Ependymal Cell Reactions in Spinal Cord Segments after Compression Injury in Adult Rat

MASAKI TAKAHASHI, MD, YASUHISA ARAI, MD, HISASHI KUROSAWA, MD, NORIYOSHI SUEYOSHI, PhD, AND SHUNICHI SHIRAI, MD

Abstract. Recently, it has been suggested that neural stem cells and neural progenitor cells exist in the ependyma that forms the central canal of the spinal cord. In this study, we produced various degrees of thoracic cord injury in adult rats using an NYU-weight-drop device, assessed the degree of recovery of lower limb motor function based on a locomotor rating scale, and analyzed the kinetics of ependymal cell proliferation and differentiation by proliferating cell nuclear antigen (PCNA), nestin, glial fibrillary acidic protein (GFAP), or GAP-43 immunostaining. The results showed that the time course of the ependymal cell proliferation and differentiation reactions differed according to the severity of injury, and that the responses occurred not only in the neighborhood of the injury but in the entire spinal cord. An increase in the locomotor rating score was related to an increase in the number of PCNA-positive cells, and the differentiation of ependymal cells into reactive astrocytes was involved in injury repair. No apoptotic cells in the ependyma were detectable by the TUNEL method. These results indicate that the ependymal cells of the spinal central canal are themselves multipotent, can divide and proliferate according to the severity of injury, and differentiate into reactive astrocytes within the ependyma without undergoing apoptosis or cell death.

Key Words: Apoptosis; Ependymal cell; Glial fibrillary acidic protein (GFAP); Neural progenitor; Proliferating cell nuclear antigen (PCNA); Proliferation; Spinal cord injury.

INTRODUCTION

Ependymal cells are remnants of the primitive neuroepithelium from which neural progenitor cells originate during pre- and perinatal development (1). The proliferative activity of ependyma has already been described in adult spinal cord (2, 3). It is well known that in adult mammals, ependymal cells proliferate under certain pathological or traumatic conditions (4). In previous studies this proliferation after spinal cord injury has been interpreted as a mechanical displacement of the ependymal cells at the time of the lesion (5), or as a response to increased pressure within the lumen of the ependymal canal (6, 7).

Because ependymal cells proliferate adjacent to an injury site and migrate there, it has recently been recognized that ependymal cells are progenitor cells capable of proliferation and differentiation (8, 9). Progenitor cells capable of differentiating into neurons and glial cells occur in the adult spinal cord, and at least some of them have the same properties as stem cells (10–12). Johansson et al (10) reported that the ependymal cells lining the central canal of the spinal cord correspond to stem cells. The differentiation of endogenous precursors into mature neurons and reconstruction of nerve tracts were observed in the adult brain, and these precursors were shown to originate from neural stem cells around the cerebral ventricles (13). Multipotent progenitor cells are found in the dentate gyrus, where neurogenesis occurs (14). A study reported that only the cells in the subependymal layer around the lateral ventricles, and not the ependymal cells, are neural stem cells (15).

In recent studies, these cells have been isolated from lower vertebrates and human fetal and adult brains and have been cultured (16, 17). Subsequently, attempts were made to transplant them into the injured central nervous system (18, 19). However, neural stem cells often differentiate into glial cells (20–23). Neural progenitor cells that have proliferated in the damaged spinal cord fail to differentiate into neurons, presumably because the environment of spinal cord tissue itself limits the differentiation.

In addition to transplantation, spinal cord regeneration requires the maintenance of the intraspinal environment and the activation of endogenous neural stem cells. To that end, it is necessary to grasp the reaction of the entire spinal cord to injury and its time course. Ependymal reaction to injury is thought to be a local reaction of the damaged spinal cord rather than triggered by systemic reaction to injury-induced stress or a signal from the central nervous system (9). However, we anticipated that if injury-induced signals were conducted to the ependymal-lined central canal, not only at the local site of injury but also the ependymal cells would respond in some way. Many studies have investigated the injury site and the adjacent area after spinal cord injury, but none have reported the ependymal cell reaction throughout the spinal cord. Moreover, there are few or no reports of variations in the ependymal cell reaction according to the severity of injury. In this study, we experimentally produced 3 different severities of spinal cord injury in adult rats, and
immunohistologically investigated the ependymal cell reaction throughout the spinal cord, particularly changes outside the local site of injury.

MATERIALS AND METHODS

Rats

Eight-week-old adult female Wistar rats weighing 205 to 230 g were purchased from Sankyo Labo Service Co, Inc. (Tokyo, Japan). The animals were housed in individual cages in a temperature-controlled room (24 ± 1°C) with a 14/10 hour light/dark cycle. Spinal cord-injured rats were given manual bladder evacuation 2 times per day and allowed free access to food and water. Anesthesia was performed with 3% to 4% isoflurane in oxygen and maintained with 0.5% to 1.5% isoflurane derived from water. Anesthesia was performed with 3% to 4% isoflurane in oxygen and maintained with 0.5% to 1.5% isoflurane derived through a face mask. To protect from infection, a subcutaneous injection of cefmetazole sodium (10 mg/kg) was administered for 3 days after surgery. The animal experimentation procedures described in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Juntendo University.

Procedures for Spinal Cord Injury

Under anesthesia a laminectomy was made at the T7–8 vertebral level under aseptic conditions. The spinal cord was then contused with an NYU-weight-drop device and different injury groups were produced. A 10-g stainless rod with a tip diameter of 2.5 mm was dropped from a height of either 6.25 mm (group I, n = 40), 25 mm (group II, n = 40), or 50 mm (group III, n = 40) onto the exposed intact dura overlying the dorsal spinal cord. Immediately after the spinal cord injury (SCI), the impact was observed on the exposed intact dura overlying the dorsal spinal cord. To assess injury, a laminectomy was performed at the T7–8 vertebral level under aseptic conditions. The spinal cord was then contused with an NYU-weight-drop device and different injury groups were produced. A 10-g stainless rod with a tip diameter of 2.5 mm was dropped from a height of either 6.25 mm (group I, n = 40), 25 mm (group II, n = 40), or 50 mm (group III, n = 40) onto the exposed intact dura overlying the dorsal spinal cord. Immediately after the spinal cord injury (SCI), the impact was removed and the wounds irrigated with saline, and the muscle and skin openings were sutured. Six sham-operated rats given only laminectomy and 5 untreated rats were used as a control. Of 142 rats thus treated, 11 rats were excluded from the experiments because of surgical complications and undefined postsurgical complications.

Behavioral Assessment

The rats were trained preoperatively to move around an open field, consisting of a molded-plastic circular enclosure with a smooth, nonslip floor (100-cm diameter, 20-cm wall height), for 4 min to ensure that all rats consistently obtained a maximum score of 21, based on the Basso, Beattie, Bresnahan (BBB) locomotor rating scale (24). Postoperative open field testing was done from day 1 to 8 weeks for all rats and videotaped for subsequent BBB locomotor scoring by blinded observers.

Immunohistochemistry

Animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (50–70 mg/kg) and then transectedally perfused with saline, followed by 10% neutral buffered formalin at the following intervals after injury: 1 and 3 days, 1, 2, 3, 4, 6 and 8 weeks. Following perfusion, the spinal cord was removed along with spinal nerve roots and further fixed in 20% formalin for 48 hours at room temperature. Using spinal nerve roots as landmarks, the 26 spinal cord segments were then carefully blocked in the transverse plane from C1 to L6 (cervical 7, thoracic 13, lumbar 6). Consecutive levels were placed side by side into sliced liver obtained from the cadaver, embedded in paraffin, and cut transversely into 3 to 4-µm sections. Every section was stained with hematoxylin and eosin (H&E) to count the number of ependymal cells per spinal cord segment. The remaining sections were processed for subsequent immunohistochemistry. After deparaffinization and blocking of endogenous peroxidase with 3% methanol peroxidase, tissues were incubated in phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) and 10% normal goat serum for 30 min and then reacted with mouse monoclonal anti-proliferating cell nuclear antigen antibody (EPO anti-PCNA/HRP, clone PC10; DAKO, Copenhagen, Denmark), rabbit polyclonal anti-glial fibrillary acidic protein antibody (GFAP, 1:50 dilution; DAKO), and mouse monoclonal anti-nestin antibody (clone Rat-401, 1:10 dilution; PharMingen, San Diego, CA) for 1 hour at room temperature (RT; 21°C), followed by biotinylated antimouse or rabbit secondary antibody and streptavidin-HRP (LSAB2 system, DAKO, Carpenteria, CA). Diaminobenzidine was applied as the chromogen. Pretreatment was not necessary to enhance immunoreactivity for the PCNA staining. The slides for all staining were lightly counter-stained with hematoxylin. The primary antibodies were omitted in the negative controls. The spinal cords of group II rats were also stained with growth-associated phosphoprotein 43 antibody (GAP-43, clone IG7, 1:2 dilution; YLEM, Rome, Italy) following pretreatment with 0.1% trypsin in PBS at 37°C for 40 min. To assess apoptosis, DNA fragmentation of tissue cells was examined by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL), using a commercially available kit (ApopDETTEK Cell Death Assay System, Enzo Diagnostics, Farmingdale, NY).

Quantitative Analysis of Ependymal Cell Proliferation

For all sections of the spinal cord segments except the lesion epicenter, the number of ependymal cells lining the wall of the central canal was counted using H&E staining. Cells touching the ependymal layer with more than 50% of their circumference in contact with cells in the ependyma were regarded as ependymal cells. In this study, the numbers of ependymal cells and positive cells in the ependyma per section per spinal cord segment were measured for 26 spinal cord segments (7 cervical, 13 thoracic, and 6 lumbar segments), and their sums were defined as the total numbers of ependymal cells and positive cells, respectively, in 1 spinal cord. A total of 16,776 cross-sections of the spinal cord were obtained: 15,585 sections after injury (5,136 sections from group I, 5,359 sections from group II, and 5,090 sections from group III), 582 sections from the normal control group, and 609 sections from the sham operated group.

Double Label Immunofluorescence

Dual color tissue immunofluorescence for GAP-43 and GFAP antibodies was carried out using FITC-conjugated anti-mouse immunoglobulins (1:20 dilution; DAKO) for the former, and biotinylated anti-rabbit goat serum (1:100 dilution; Vector, Burlingame, CA) and streptavidin–Alexa 594 (1:500 dilution; Molecular Probes, Eugene, OR) for the latter. To combine antibodies to nestin and GFAP, Alexa 568-conjugated anti-mouse IgG (1:200 dilution) and FITC-conjugated anti-rabbit IgG (1:
improvement between 3 days and 4 weeks postoperation. The rats in group II (25-mm group) had little or no hind limb movement until 1 week postoperation and then demonstrated progressive recovery over the next 2 wk, and after that showed gradual recovery until 8 wk. The rats in the 50-mm group (group III) demonstrated flaccid paralysis, which persisted for 1 wk postoperation. Behavioral recovery occurred between 2 and 6 wk postoperation when the rats were able to move the 3 joints slightly but not to walk, and the average BBB score was 5.6 ± 1.1. No further recovery was observed over the remaining 2 wk. Thus, even after 8 wk training, the 50-mm group rats (group III) did not progress beyond the behavioral performance level attained by the 6.25-mm rats (group I) by 1 day postoperation. All the rats in the sham operated group maintained the maximum BBB score until they were killed.

**Histopathology and Immunohistochemistry in the Normal Ependyma**

The morphology of the central canals formed by ependymal cells in the cervical and thoracic regions was round, but oval-shaped at the lumbar level. The ependyma was composed of some chromatin-abundant and faint cells in the H&E stain. To assess proliferation activity, the PCNA-labeled cells in the ependyma in each segment were counted. The labeled cells were preferentially located in the ependyma, and very few labeled cells were observed in the white and gray matter except the ependyma in 1 section. Nestin immunoreactivity was examined to assess the neural stem/progenitor cell qualities, although very few positive cells were detected in the normal ependyma. GAP-43 was used to detect neuronal cells during the different stages of cellular development, from proliferation and migration to differentiation. GAP-43 is known to play an important role in neural development, axonal regeneration, and the modulation of synaptic function (25, 26). GAP-43 immunoreactivity was observed in all of the neuronal cells in the gray matter, and almost all the ependymal cells were positive. One to 2 TUNEL-positive cells were observed in the gray and white matter in some sections, but no positive cells were seen in the ependyma of any sections.

**RESULTS**

**Behavioral Recovery**

The BBB scores differed significantly between the 3 groups in all postoperative testing sessions (1 day: F = 210.62, p < 0.001; 3 days: F = 157.79, p < 0.001; 1 wk: F = 644.93, p < 0.001; 2 wk: F = 337.56, p < 0.001; 3 wk: F = 116.67, p < 0.001; 4 wk: F = 201.24, p < 0.001; 6 wk: F = 83.25, p < 0.001; and 8 wk: F = 76.78, p < 0.001). These increasingly severe contusion injuries produced concomitant degradations in behavioral performance as measured by the BBB scores (Fig. 1). Group I (6.25-mm group) had the greatest locomotor recovery. Most rats in this group demonstrated behavioral recovery. Most rats in this group demonstrated behavioral recovery. Most rats in this group demonstrated behavioral recovery. Most rats in this group demonstrated behavioral recovery.

**Time-Dependent Changes in the Number of Positive Cells in Ependyma after Spinal Cord Injury**

The total number of ependymal cells increased in all groups in a time-dependent manner, and the more severe the injury, the greater the number of cells (Fig. 2). There were significant group differences in the maximal number of PCNA-, nestin-, and GFAP-positive cells, with F = 11.9 (p < 0.05), F = 5.36 (p < 0.05), F = 9.4 (p < 0.05), respectively, indicating different changes depending on the severity of injury. Comparisons among the 3 groups showed significant differences in PCNA and

**Statistical Analysis**

The BBB scores and the numbers of cells under investigation in each experimental and control group were averaged and compared using a 1-way analysis of variance (ANOVA). Pairwise post hoc comparisons were made using Bonferroni corrected t-tests. Differences of the rates of labeled cells between normal control and experimental groups were examined by Student t-tests.

![](http://jnen.oxfordjournals.org/)

**Fig. 1.** The kinetics (time course) of locomotor recovery, as measured by BBB open field scores for 3 groups. Following spinal cord injury (SCI), group I demonstrated the greatest recovery and group III the least, while group II attained intermediate levels. There was significant difference between the 3 groups in the final BBB score (p < 0.001, ANOVA). Data represent means ± SD.
Kinetics of changes in the number of ependymal cells except in lesion sites, as determined by tissue sections stained with H&E and labeled PCNA, nestin, and GFAP following graded spinal cord injury. Group I, 6.25-mm SCI (A); Group II, 25-mm SCI (B); Group III, 50-mm SCI (C). The number of cells is presented as the mean ± SD. Data of 5 rats for each time point. In groups I and II (A, B), asterisk indicates significant difference from the normal control (p < 0.05, t-test). Abbreviations: N, normal control; SCI, spinal cord injury.

GFAP (p < 0.05), and in nestin (p < 0.05), except group I vs group II (p = 0.67).

In all groups, the number of PCNA-positive cells fell to a minimum after injury, indicating a transient decrease in proliferative activity. The more severe the injury, the longer the interval from the time of injury to the time the minimum number was reached, and the later the subsequent upward turn. The more severe the injury, the greater the maximum number, that is, the higher the proliferative activity.

The number of nestin-positive cells increased in the early stage after injury, and the more severe the injury, the greater the maximum number, that is, the higher the nestin reactivity. The number of nestin-positive cells significantly increased after the early post-traumatic increase in groups I and II (p < 0.05), but not in group III.

The number of GFAP-positive cells increased after injury and the more severe the injury, the greater the maximum number. The post-traumatic increases in the numbers of GFAP-positive cells and nestin-positive cells occurred almost in parallel in all groups, but not in group III 2 weeks or later after injury.

BBB-based behavioral assessment showed that the more severe the injury, the later the lower limb motor function tended to recover. In all groups, the recovery of lower limb motor function coincided with a rapid increase in the number of PCNA-labeled cells subsequent to the early postoperative decrease; however, in group III, the recovery of lower limb motor function was insufficient despite a marked increase in PCNA-positive cells.

Ependymal cell reaction in adult rats was not restricted to the neighborhood of injury, and systemic reaction occurred throughout the spinal cord, varying according to the severity of the injury.

Two rats each in the sham-operated group were killed 1 day, 3 days, and 1 wk after surgery. The numbers of cells and PCNA-, nestin-, and GFAP-positive cells did not significantly differ from those in the normal control (H&E, p = 0.74; PCNA, p = 0.82; nestin, p = 0.89; and GFAP, p = 0.78).

Differences in Reactivity by Spinal Cord Region

Although Figure 2 shows time-dependent changes in measured values, these are expressed in Figure 3 as a
percentage to indicate differences in the reactivity of ependymal cells in each region within the spinal cord irrespective of the timing of increases and decreases for comparison with the normal control (data not shown for groups I and III). The percentage of ependymal cells in each region after injury to those in the entire spinal cord did not differ from that in the normal control, remaining almost constant until 8 weeks after injury. This indicates that an increase in ependymal cells occurs throughout the spinal cord, and not primarily in the thoracic region (Fig. 3A). The percentage of PCNA-positive cells significantly increased in the cervical region in all stages after injury in the 3 groups (p < 0.05), except 8 wk after injury in group II (Fig. 3B). The percentage of nestin-positive cells in the cervical region significantly increased on day 1 in all groups (p < 0.001). In group II the percentage of nestin-positive cells increased in the cervical region 1 and 4 weeks after injury (p < 0.05), but in the other 2 groups tended to increase in the thoracic region on day 3 and thereafter. The percentage of nestin-positive cells in the lumbar region decreased in all the 3 groups in all stages except day 1 and 2 wk after injury in group I (Fig. 3C).

The percentage of GFAP-positive cells after injury decreased in the cervical region and increased in the thoracic and lumbar regions in all 3 groups (Fig. 3D). The Table shows the number of cells in each region in group II.

After injury, the cervical region had the highest proliferative activity and rapidly expressed nestin, but few cells within the ependyma became GFAP-positive. The thoracic region expressed nestin and many cells within the ependyma became GFAP-positive. The lumbar region hardly expressed nestin and many cells rapidly became GFAP-positive.

In all sections except the injury site, no TUNEL-positive cells were observed within the ependyma (Fig. 4A); however, many TUNEL-positive cells were observed in the injured epicenter and the adjacent gray and white matter (Fig. 4B). These results indicate that, except in injury sites, no apoptosis or death of ependymal cells occurs in response to injury regardless of severity.

In this study, ependymal cell division was observed in the injury groups but not in the normal control or sham group (Fig. 5): symmetric cell division in 14 of 15,585 sections, and asymmetric cell division in 18 of 15,585 sections. Johansson et al reported that the majority of ependymal cells underwent asymmetric cell division (10), but in this study no significant difference was noted in the frequencies of symmetric and asymmetric cell division.

Immunohistochemical analysis using double labeling showed the presence of cells that were positively double-stained by GAP43 and GFAP, leading to the notion that some of the ependymal cells combine the properties of neurons and glial cells. Nestin- and GFAP-double-stained

---

### Table

<table>
<thead>
<tr>
<th></th>
<th>H&amp;E</th>
<th>Nestin</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thoracic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumbar</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1 day</th>
<th>3 days</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
<th>6 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>472</td>
<td>294</td>
<td>5987</td>
<td>6171</td>
<td>6515</td>
<td>6693</td>
<td>6953</td>
<td>7015</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>74.4</td>
<td>74.4</td>
<td>45.9</td>
<td>45.9</td>
<td>45.9</td>
<td>45.9</td>
<td>45.9</td>
<td>45.9</td>
</tr>
<tr>
<td>Nestin</td>
<td>75.2</td>
<td>75.2</td>
<td>37.7</td>
<td>37.7</td>
<td>37.7</td>
<td>37.7</td>
<td>37.7</td>
<td>37.7</td>
</tr>
<tr>
<td>GFAP</td>
<td>75.7</td>
<td>75.7</td>
<td>39.4</td>
<td>39.4</td>
<td>39.4</td>
<td>39.4</td>
<td>39.4</td>
<td>39.4</td>
</tr>
</tbody>
</table>

---

Note: Kinetics of changes in the number of ependymal cells stained with H&E or with labeled PCNA, nestin, or GFAP in cervical, thoracic, and lumbar segments in group II. N: normal control. Data represent means ± SD.
cells were observed, indicating that ependymal cells can express nestin during proliferation. PCNA- and GFAP-double-stained cells were observed, indicating that ependymal cells can change to GFAP-positive cells during proliferation. Nestin- and GFAP-double-stained cells were observed and were considered to represent reactive astrocytes (Fig. 6).

**DISCUSSION**

The ependymal cells in the ventricular zone/subventricular zone and the spinal cord have been reported to have proliferative activity. Recent studies have shown that multipotent stem cells occur in these regions (10, 15, 27) and in the spinal cord substance other than the ependyma (28). We examined the reaction of ependymal cells throughout the spinal cord. In the past, the proliferation of ependymal cells was thought to result from the expansion of the central canal due to hemorrhage from the injury site (7), but this study showed that it is not the only cause of ependymal cell proliferation. Although it has been reported that after injury to the spinal cord ependymal mitotic activity adjacent to the injury site reaches a maximum level on day 2 after injury in young rats and then declines to a normal control level on day 4 (29), we confirmed in this study that symmetric and asymmetric cell division persisted on and after day 4, indicating that ependymal cell proliferation continues during injury repair. Past studies showed that ependymal proliferation differed in the brain and spinal cord (3, 29), whereas this study showed that ependymal proliferation also differed according to the region of the spinal cord and was enhanced in the cervical spinal cord.

Ki-67, for which cells in the G1, S, G2, and M phases of the cell cycle are positive, and BrdU, for which cells in S phase are positive (30, 31), are generally used to assess proliferative activity. Although studies have reported that the Ki-67 and BrdU labeling indices in T1 in the normal adult rat are $1.5\% \pm 0.8\%$ (9) and $1.7\% \pm 1.3\%$ (32), respectively, the PCNA labeling index in this study was slightly higher at $2.6\% \pm 1.0\%$ than the previously reported values. Since a study on brain tumors reported that PCNA could be used instead of BrdU (33), and because it is easier to handle, it was used in this study. PCNA is one of the non-histone acid nuclear proteins that are expressed in proliferating cells in the G1 and S phases of the cell cycle, and is an auxiliary protein (36 kDa) of DNA polymerase δ (34). In this study, proliferative activity was evaluated by PCNA and its changes were related to lower limb motor-function recovery.

Injury stimulated the progression of the cell cycle in the ependyma that had been in a static state. This event involves various signals. Cyclin E, which completes the transition from the G1 to S phase, and the related cyclin-dependent kinase (CDK2) are expressed in proliferating neuroepithelial cells (35, 36). Presumably, these factors were working in the ependymal cells before injury, or some negative or antimitotic growth signal acted on these factors, halting the cell cycle; injury stimuli made the cell cycle progress to the M phase through the action of CDK1, cyclin A, and cyclin B (37). It has been reported that Notch 1 is expressed in the ependymal cell nucleus after spinal cord injury (38), and that Notch activity maintains cells in a nondifferentiated state (39). The notch signal is involved in the regulation of the cell cycle time of ependymal cells.

Proliferation and apoptosis are involved in the development of the nervous system in the fetal period (40, 41): numerous apoptotic cells can be demonstrated in the spinal cord of chick embryos by the TUNEL method, but they decrease with the growth of the embryos (42). However, in rats, on postnatal days 14 to 16, 60% of cell death is observed in the subventricular zone (43). Morshed et al (31) reported that the fate of proliferating cells in the subependymal layer of the adult mouse brain was death.
In this study, no TUNEL-positive cells were observed in spinal cord ependymal cells except in lesion sites, suggesting that, in ependymal cells, a signal is transduced only in the direction of proliferation, inhibiting apoptosis. This indicates that ependymal cells receiving injury stimuli resemble fetal cells in their proliferation but differ from them in apoptosis, and suggests that ependymal cells proliferate to the extent necessary for injury repair without undergoing cell death or apoptosis.

In this study, nestin was expressed in ependymal cells not only adjacent to an injury of any severity but throughout the spinal cord. Nestin re-expression means the return of cells to a less mature state, that is, the fetal period (44). Nestin is also known as a radial glia marker (45). In this study, nestin-positive cells having long processes and morphologically resembling radial glia occurred within the ependyma. A previous study (46) reported that radial glia transformed into astrocytes postnatally; however, a recent study has indicated that radial glia are neuronal precursor/stem cells (47). Cells expressing activated Notch 1 become radial glia during embryogenesis and, after birth, these cells become dispersed astrocytes and GFAP-positive perivascular cells (48), which are regarded as neural stem cells in adults (15).

Although we were not able to confirm cell division in the gray or white matter, we observed some PCNA-positive cells. They may correspond to neural precursor cells that were shown to exist in areas other than the ependyma by Yamamoto (28). In this study, we were not able to determine whether these cells existed in these regions or had migrated from the ependyma. Based on the presence of BrdU-positive, nestin-positive cells, Kojima reported that dividing progenitor cells had migrated from the ependyma to gray matter (32). This possibility was also suggested by the observation of the asymmetrical cell division of PCNA-positive cells in this study. Although the failure to demonstrate glial or neuronal markers in ependymal cells after injury suggests that neuronal precursor cells do not differentiate within the ependyma (49), this study demonstrated nestin and GFAP in ependymal cells, indicating that neural precursor cells differentiate within the ependyma. These observations indicate that some ependymal cells differentiate within the ependyma, while others migrate into the gray matter without differentiation.

The findings that almost all ependymal cells in the normal control were positive for GAP43 show that the ependyma contains some neuron components. In vitro, embryonic neural stem cells differentiate first into neurons alone through symmetric division. Beyond the switch point in the course of time, embryonic stem cells undergo asymmetric division to change into mature stem cells that produce glial cells (50). In the CNS, neurons develop earlier than glial cells, but neural stem cells have a strong tendency toward differentiation into glial cells, with 11% to 18% differentiating into neurons and 30% to 64% into glial cells (20–22).
Fig. 6. Dual color tissue immunofluorescence of ependymal cells after spinal cord injury (SCI) showing the immunoreactivity with neuronal and glial markers. GAP43- (green) and GFAP-positive (red) cells in the ependyma of the C4 segment 2 weeks after 6.25-mm SCI (A). The insets in (A) show GAP43 immunoreactivity in ependyma under optic microscopy. Nestin- (red) and GFAP-positive (green) cells in the ependyma of the L1 segment 1 week after 25-mm SCI (B). Double-positive cells (yellow) are shown by arrows. Nestin- (red) and PCNA-positive (green) cells in the ependyma of the T4 segment 1 week after 50-mm SCI (C). PCNA- (red) and GFAP-positive (green) cells in the ependyma of the T4 segment 1 week after 25-mm SCI (D). Double-positive cells are shown by arrows. Scale bars: A, B: 20 μm; C, D: insets in A: 50 μm.

Neural stem cells differentiate into glia through the Notch pathway (48, 51) and the STAT/Smad pathway (52, 53). Notch signaling in the local site of spinal cord injury and the adjacent area reportedly suppresses the new formation of neurons and selectively stimulates astrogensis (38). In this study we showed that nestin was expressed in the ependymal cells not only immediately after injury but also several times thereafter. The expression varied with the severity of the injury, and the more frequent the expression, the better the recovery of lower limb motor function. In group III, no reincrease in the number of nestin-positive cells was observed and the recovery of lower limb motor function was insufficient. In other words, repeated expression of nestin after spinal cord injury appeared important for injury repair. Reactive astrocytes are known to express both nestin and GFAP (8, 44, 54). In this study, nestin-positive, GFAP-positive reactive astrocytes and nestin-negative, GFAP-positive nonreactive astrocytes were present in the ependyma. We found that when the number of nonreactive astrocytes increased as in group III, the recovery of lower limb motor function halted and no further repair of injury occurred. Thus, the presence of reactive astrocytes in the ependyma was needed for the continuance of injury repair. Glial cells are known to have injury-repairing ability and release nitric oxide (55), various cytokines (56), and neurotrophins (57, 58).

In other words, it is likely that ependymal cells differentiate into reactive astrocytes, which produce cytokines available for the repair of the local site of injury. After injury to the spinal cord, gliogenesis occurs in preference to neurogenesis. In controlling neural stem cells intrinsic to the spinal cord, it seems important to consider how to make reactive astrocytes persist in the injured spinal cord, in addition to promoting neurogenesis.

ACKNOWLEDGMENTS

The authors thank the Central Laboratory of Medical Sciences, the Division of Pathology staff for exceptional technical assistance, and the Division of Biomedical Research Resources staff for strict animal care. We also thank Katumi Miyahara for support with confocal microscopy.

REFERENCES


5. Matthews MA, St Onge MF, Faciane CL. An electron microscopic analysis of abnormal ependymal cell proliferation and envelopment of sprouting axons following spinal cord transection in the rat. Acta Neuropathol (Berl) 1979;45:27–36


23. Cao QL, Zhang YP, Howard RM, Walters WM, Tsoufas P, Whittemore SR. Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. Exp Neurol 2001;167:48–58


41. Cao QL, Zhang YP, Howard RM, Walters WM, Tsoufas P, Whittemore SR. Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. Exp Neurol 2001;167:48–58

42. Wako KC, Park IS, Chi JG, Lee MS. Cytokine pattern in the thoracic spinal cord of chick embryos (incubation day 5–13) using...

Received July 1, 2002
Revision received October 3, 2002
Accepted October 22, 2002