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Original article

Degenerative and regenerative changes in the dorsal funiculus of the cryoinjured spinal cord of rats

—— electron microscopic study ——

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The morphological changes were examined in the dorsal funiculus after cryoinjury to the spinal cord at Th10 in the rat. Cryoinjury was performed by contacting a liquid nitrogen-frozen metal rod with the dorsal surface of the spinal cord. The frozen spinal cord was thawed spontaneously. This freeze-thawing treatment was repeated three times. The histological changes were examined by light and electron microscopy from 2 to 60 days after cryoinjury. The present study focused on the electron microscopic findings of the degenerative and regenerative changes of nerve fibers and glial cells following injury.

In typical Waller degeneration, myelin sheaths of degenerated axons were separated from oligodendrocytes, and phagocytozed by macrophages. Within the lesion, while glial cells including oligodendrocytes were degraded, some axons were rescued from the damage, surviving as demyelinated axons after the degradation of associated oligodendrocytes. Such demyelinated axons were later remyelinated by oligodendrocytes or Schwann cells. This might be a major factor contributing to the locomotive recovery of the animal. Growth cones were formed even after a long period following cryoinjury at the proximal stump of the injured nerves. This suggests that nerve fibers have a strong ability to regenerate in the spinal cord dorsal funiculus. A cavity was usually formed in the epicenter to rostral part of the lesion. Cavity formation is a critical barrier to spinal cord regeneration.

The main strategies for spinal cord regeneration might be to rescue and restore neural tissues from degeneration, and prevent cavity formation by providing a sufficient blood supply to ensure tissue survival and axonal outgrowth.

Key word: spinal cord cryoinjury, regeneration, electon microscopy, oligodendrocyte, Schwann cell, myelination

Introduction

Spinal cord regeneration has been extensively studied by employing various techniques including transplantation of Schwann cells (Oudega and Xu, 2006), olfactory ensheathing cells, (Plant et al, 2003), foetus spinal cord (Iwashita et al., 1994), foetus-derived neural stem cells (Wu et al., 2002; Bai et al., 2003), macrophages (Rapalino et al., 1998), choroid plexus (Ide et al., 2001; Matsumoto et al., 2003) and bone marrow stromal cells (Chopp et al., 2000; Hofstetter et al., 2002; Lee et al., 2003; Ohta et al, 2004; Someya et al, 2008; Shichinohe et al, 2008), and suppression of inhibitory molecules such as Nogo A (Freund et al., 2007). On the other hand, adult brain-derived neural stem cells (Mligiliche et al., 2005) were shown to survive after transplantation. Similarly, neural stem cells obtained from adult brain of dead rats (Xu et al., 2003) were demonstrated to be available as transplants. The first clinical case of treatment of spinal cord injury by bone marrow stromal cell transplantation was reported (Saito et al., 2008).

In such spinal cord regeneration studies, precise histological findings are important to evaluate nerve regeneration within the injured site. Immunohistochemistry has been used as the main tool to identify cells and reveal interactions between different cell types in the same section in recent spinal cord regeneration studies. Over the long history of spinal cord regeneration studies, electron microscopy remained the most powerful technique to demonstrate the precise changes of cells in the injured spinal cord tissue up until 90s. The electron microscopic findings obtained at that time are still very informative for understanding the changes in nerve fibers and glial cells following injury to the spinal cord. However, probably owing to its complicated technical procedures, electron microscopy has been decreasingly used in studies of spinal cord regeneration. Young researchers in this field have had few opportunities to look at electron micrographs in the laboratory or in papers of this field.

With this background, we think that it is important to demonstrate the degeneration and regeneration of nerve fibers and glial cells by electron microscopy. The present study focused on the electron microscopic findings, effectively demonstrating how the nerve fibers and accompanying glial cells degenerate and regenerate following cryoinjury to the white matter (dorsal funiculus) of the spinal cord.

Materials and Methods

Surgery

Adult male rats, weighing 200–250 g, were used. Rats were anesthetized initially by the inhalation of ether, and maintained under anesthesia by inhalation of halothane. The spinal cord covered by the dura mater was exposed at the Th8–9 level. For cryoinjury, a copper rod cooled with liquid nitrogen was contacted with the dorsal part of the spinal cord (Fig. 1). The spinal cord was frozen immediately. After 3–5 seconds, the copper rod was detached from the spinal cord, and it thawed spontaneously within 10 seconds. Such freezing-thawing was repeated 3–5 times (Kitada et al., 1999). The wound was sutured layer by layer. Rats were maintained up to 2 months after surgery before sacrifice. Animals were sacrificed at 2, 4, 7, 11, 16, 22, 35, and 60 days after surgery. Three to 5 rats were used at each time point.

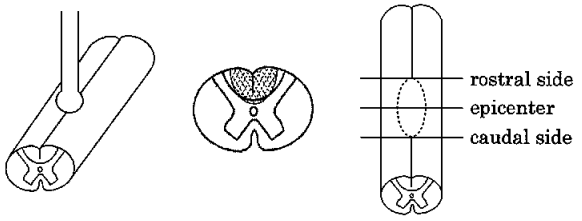

Fig. 1 This picture shows how the spinal cord was cryoinjured, and the tissues were prepared
A copper rod cooled with liquid nitrogen was contacted with the dorsal surface of the spinal cord at Th10. The frozen dorsal column thawed spontaneously. This treatment was repeated three times. The cryolesion spread over the area including the dorsal funiculus. After various time periods, the spinal cord was fixed, and transverse tissue sections were obtained from rostral, middle (epicenter), and caudal parts of the lesion.

Tissue preparation

Rats were anesthetized by the intraperitoneal injection of Nembutal (pentobarbiturate sodium, 50 mg/kg body weight), and fixed by perfusion through the heart with 50 ml Ringer's solution followed by 150 ml fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate

buffer (pH 7.4). The cryoinjured segment was excised, and stored for 1 day in the same fixative. The segment was transversely cut into thin slices at three points as shown in Fig. 1, and then post-fixed for 2 hours in 1% osmium tetroxide solution (pH 7.4) at an ice-cold temperature. After post-fixation, specimens were dehydrated through the graded series of ethanol, and embedded in Epon 812. Thin sections for electron microscopy were cut with a diamond knife and observed using a Hitachi 700 electron microscope after double staining with lead citrate and uranyl acetate.

Results

Histology

The dorsal white column has the shape of an inverted triangle with the base at the dorsal surface of the spinal cord. The height and the base of the triangle are approximately 1 and 1.5–2 mm, respectively. Axons in the dorsal column are 0.5–2.5 μm in diameter, with the myelin sheaths 0.5–0.05 μm thick. The ventral region of the dorsal white column is occupied by thin myelinated fibers of the cortico-spinal tract.

Cryoinjury was caused on the mid-line of the dorsal column. The extension of the cryoinjury was variable from 1/3 to 1/2 of the depth, and from 1/2 to 2/3 of the width at the dorsal surface (Fig. 1).

Two-four days after cryoinjury

Axons were degraded by Waller degeneration. Myelin sheaths of Waller-degenerated axons were separated from oligodendrocytes, and subsequently phagocytosed by macrophages (Fig. 2). In the lesion, axons and glial cells including astrocytes and oligodendrocytes were degraded, and myelin sheaths were completely disrupted into fragments (Fig. 3a,b). Blood vessels were also damaged. No extracellular matrix remained after the degeneration of cell components. The basal laminae of blood vessels disappeared. This feature is a finding unique to the central nervous system. In peripheral nerve injury, collagen fibrils and Schwann cell basal laminae in the endoneurium remained following the degradation of axons and Schwann cells.

In some cases, demyelinated axons were found (Fig. 3c). Probably, oligodendrocytes had been injured, whereas axons remained intact in such cases. Subsequent to oligodendrocyte degradation, myelin sheaths were disintegrated, and phagocytosed by macrophages. As a result, axons became naked.

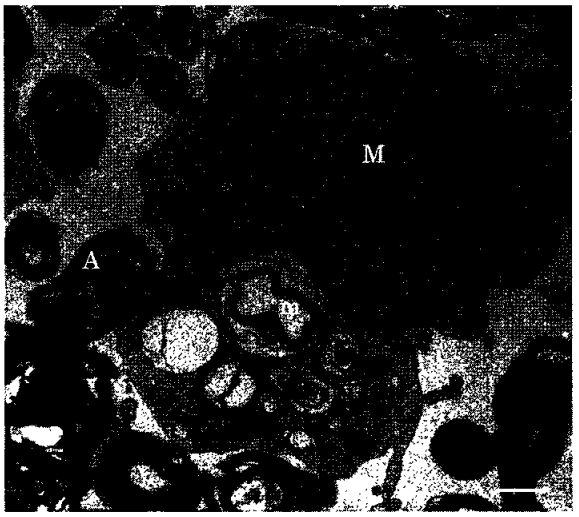

Fig. 2 Two days after cryoinjury. Waller degeneration in the rostral part
This electron micrograph shows the Waller degeneration in the rostral part of the cryoinjured spinal cord. Axons (A) are all degenerated, and myelin sheaths are degraded. A macrophage (M) can be seen to phagocytose myelin sheath debris (m). No glial cells were found. Scale bar: 1μm

In the periphery of the lesion, while many axons were degraded, some apparently intact myelinated axons with an oligodendrocyte remained (Fig. 3d). There were vacant spaces, probably due to edema, between these small-diameter nerve fibers. There were no astrocyte processes associated with these surviving myelinated nerve fibers. This suggests that astrocytes might have been more extensively degraded or retracted from the lesion than oligodendrocytes.

In the caudal part of the lesion, noticeable changes occurred in nerve fibers. As the axons ran in the caudo-rostral direction in the dorsal column, the caudal site corresponded to the proximal stump of the severed axons. The myelinated axons were swollen, and contained numerous organelles including mitochondria and membranous organelles (Fig. 4a,b). These features resemble those seen in the proximal stump of the injured peripheral nerve axons. This is

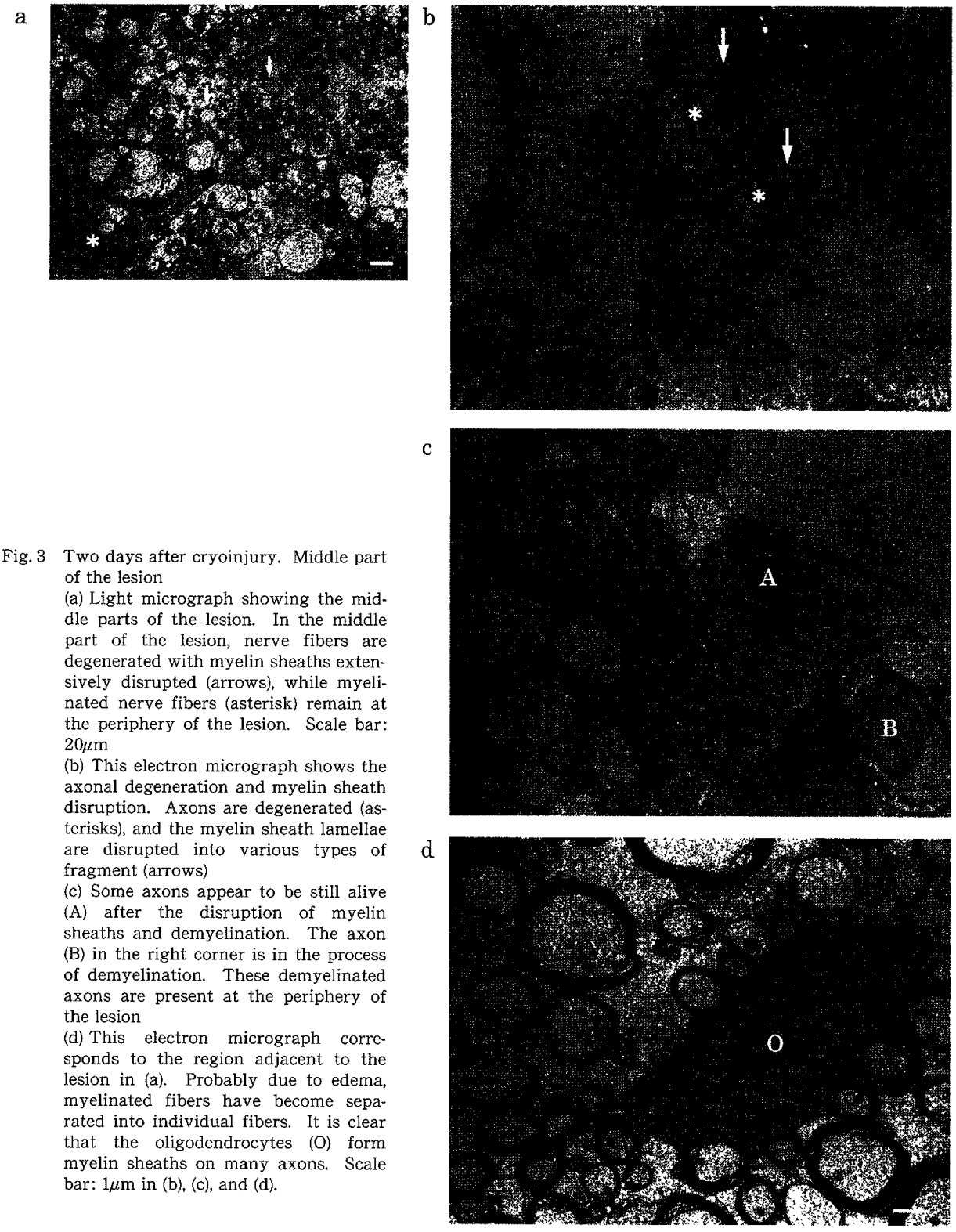

Fig. 3 Two days after cryoinjury. Middle part of the lesion
(a) Light micrograph showing the middle parts of the lesion. In the middle part of the lesion, nerve fibers are degenerated with myelin sheaths extensively disrupted (arrows), while myelinated nerve fibers (asterisk) remain at the periphery of the lesion. Scale bar: 20μm
(b) This electron micrograph shows the axonal degeneration and myelin sheath disruption. Axons are degenerated (asterisks), and the myelin sheath lamellae are disrupted into various types of fragment (arrows)
(c) Some axons appear to be still alive (A) after the disruption of myelin sheaths and demyelination. The axon (B) in the right corner is in the process of demyelination. These demyelinated axons are present at the periphery of the lesion
(d) This electron micrograph corresponds to the region adjacent to the lesion in (a). Probably due to edema, myelinated fibers have become separated into individual fibers. It is clear that the oligodendrocytes (O) form myelin sheaths on many axons. Scale bar: 1μm in (b), (c), and (d).

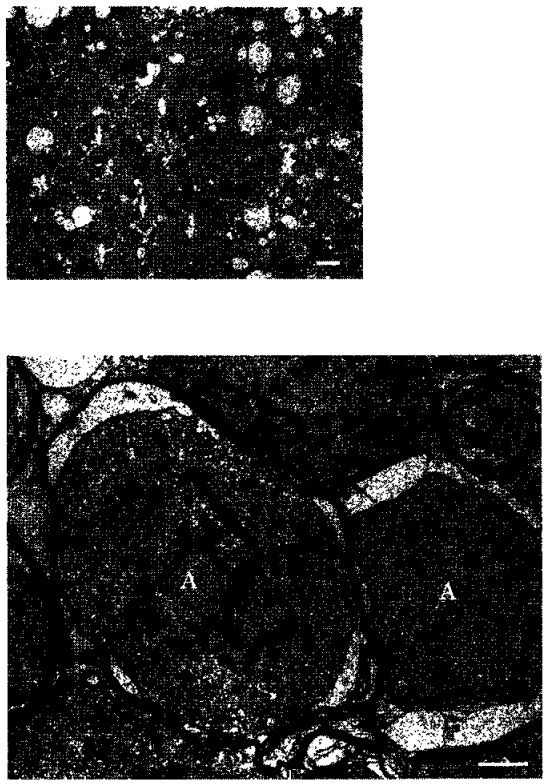

Fig. 4 Two days after cryoinjury. Caudal part of the lesion
(a) Light micrograph showing the most caudal part of the lesion. Affected axons are almost demyelinated and swollen (asterisks). Considering that nerve fibers run in a caudo-rostral direction in the dorsal column, this feature indicates changes of injured axons at the proximal stump in the spinal cord. Thin myelin sheaths (arrows) remain. Scale bar: 20μm
(b) This electron micrograph was taken from the same level as (a). Axons (A) are swollen with thin myelin sheaths, and contain many mitochondria and membranous organelles. Scale bar: 1μm

probably due to the interruption of axonal flow at the injured axonal stumps.

Although the basal laminae of blood vessels disappeared following the degradation of endothelial cells, the basal laminae faci ng the pia mater remained intact. Regenerating axons, 0.5–3.0 μm in diameter, were found beneath such basal laminae (Fig. 5). They were in direct contact with the basal laminae or associated with glial cell processes. Though it was unknown whether these axons came from dorsal roots or from the intrinsic axons within the spinal cord, it was clear that the basal laminae served as scaffold for the growth of regenerating axons.

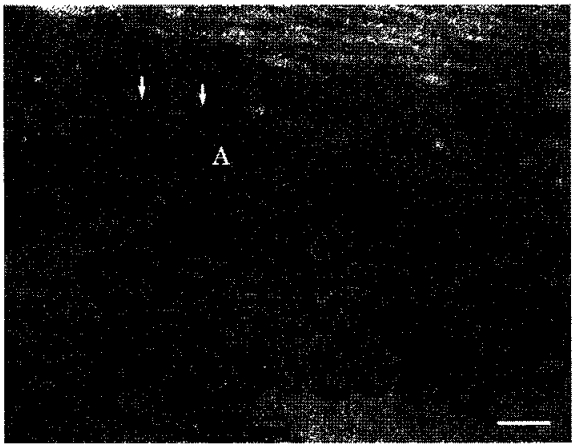

Fig. 5 Two days after cryoinjury
This electron micrograph shows regenerating axons extending along the basal lamina (arrows) of the spinal cord surface on the pia mater. Axons (A) contain numerous membranous organelles, indicating the feature of growth cones.
Scale bar: 1μm

One-three weeks after cryoinjury

Numerous macrophages were found in the space formed in the severely damaged region. No cellular reactions were noted except for the invasion of macrophages into such spaces. No cellular repair occurred in these spaces, resulting in cavity formation (Figs. 6a, 7a,b).

Axons with ordinary axoplasmic structures containing microtubules and occasional mitochondria were considered as those that had survived, and become naked due to demyelination. There were various types of remyelination on such naked axons by oligodendrocytes (Fig. 6b). Oligodendrocyte processes containing bundles of microtubules in the dark cytoplasm surrounded such naked axons, and occasionally formed thin myelin sheaths on them.

In the caudal part of the lesion, there were many axons with a diameter of 0.5–3.0 μm that contained numerous mitochondria and membranous organelles including vesicles. These features resembled those of growth cones of regenerating axons seen in the peripheral nerve (Fig. 6c). These regenerating axons were usually accompanied by the thin processes of oligodendrocytes. Oligodendrocytes characteristically showed relatively dark cytoplasm, and contained an abundance of ribosomes and short and straight rER. Thin oligodendrocyte processes with dark cytoplasm were attached to

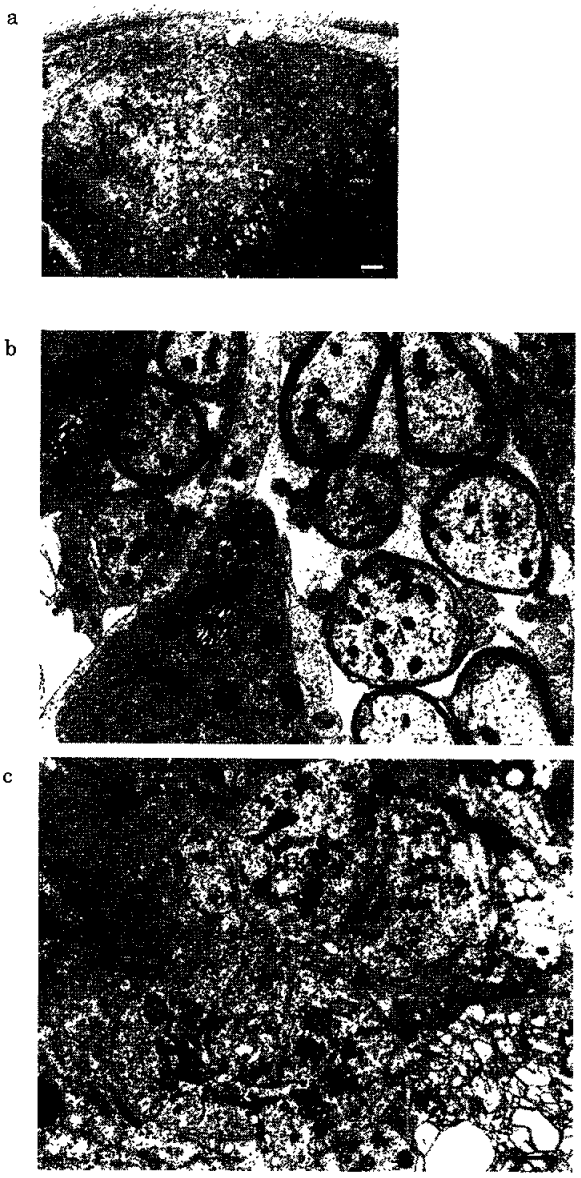

Fig. 6 One to three weeks after cryoinjury
(a) This light micrograph shows the caudal part of the lesion 11 days after cryoinjury. Almost all the dorsal column is affected by cryoinjury. The left part of the dorsal column became a cavity (asterisk) containing macrophages, while in the right part (star), the affected tissue remains. The cavity occupied the dorsal column in the rostral and middle part. Scale bar: 10μm
(b) Remyelination of the axons occurred in the tissue-retaining region as shown in (a). These thinly myelinated axons (A) are considered to be those that had survived after demyelination by myelin sheath disruption. Remyelination was carried out by oligodendrocytes (O)
(c) Growth cones (G) are noted in the caudal part of the lesion. These axons are enlarged, and contain numerous mitochondria and membranous organelles in the axoplasm, the features of growth cones. Growth cone presence means that the spinal cord axons retain the ability to regenerate even in the later stage of injury
Scale bar: 1μm in (b) and (c)

growth cones.

A large cavity was formed in the rostral part (Fig. 7a). In the middle part, many myelinated nerve fibers were found beneath the surface of the spinal cord (Fig. 7 b,c). These nerve fibers were myelinated by oligodendrocytes or Schwann cells (Fig. 7e). In addition, there were many unmyelinated axons that were associated by Schwann cells (Fig. 7d) in the area near the spinal cord surface. This feature indicated processes of myelination of axons by Schwann cells. Presumable arachnoidal cells invaded into the lesion and surrounded myelinated fibers (Fig. 7c)

Astrocytes were scanty. It appeared that astrocytes were more vulnerable to injury, and were easily lost from the lesion. There were no findings indicating astrocyte proliferation leading to glial scar in the border of the lesion.

Four-eight weeks after cryoinjury

Cavities of various sizes were formed in the epicenter to the rostral part of the lesion (Fig. 8a). The cavity wall was composed of oligodendrocytes and occasional astrocytic processes. The so-called astrocytic scar was not necessarily formed in the wall of the cavity. Axons were also found in the cavity wall.

There was no cavity in the caudal part. Repairing tissues reacted to cryoinjury were found in the caudal part (Fig. 8b).

Electron microscopy demonstrated that such repairing tissues contained astrocytes and some oligodendrocyte-myelinated nerve fibers (Fig. 8c). Occasionally, some growth cones were found in such tissues (Fig. 8c). This suggested that spinal cord nerves retained their regenerative ability even after a long period following injury.

Remyelination on the surviving or regenerating axons appeared to have been completed. Thick myelin sheaths were formed on such axons by oligodendrocytes or Schwann cells. Axons myelinated by Schwann cells were scattered among oligodendrocyte-myelinated axons (Fig. 8d). Myelin sheaths by Schwann cells were much thicker than those by oligodendrocytes. Collagen fibril deposition was not conspicuous around Schwann cells.

In some places, astrocyte processes proliferated to form local astrocytic scars. It

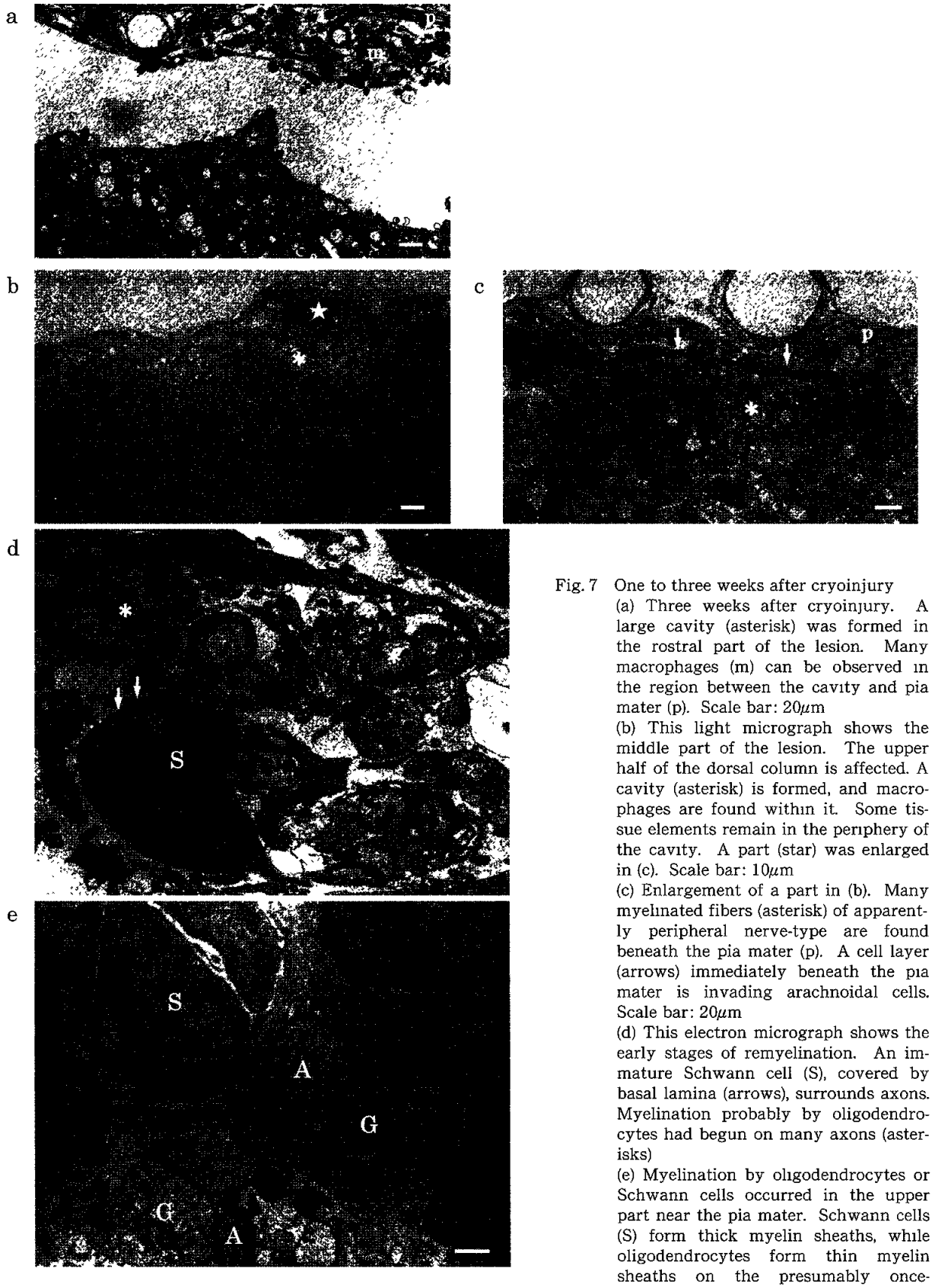

Fig. 7 One to three weeks after cryoinjury
(a) Three weeks after cryoinjury. A large cavity (asterisk) was formed in the rostral part of the lesion. Many macrophages (m) can be observed in the region between the cavity and pia mater (p). Scale bar: 20μm
(b) This light micrograph shows the middle part of the lesion. The upper half of the dorsal column is affected. A cavity (asterisk) is formed, and macrophages are found within it. Some tissue elements remain in the periphery of the cavity. A part (star) was enlarged in (c). Scale bar: 10μm
(c) Enlargement of a part in (b). Many myelinated fibers (asterisk) of apparently peripheral nerve-type are found beneath the pia mater (p). A cell layer (arrows) immediately beneath the pia mater is invading arachnoidal cells. Scale bar: 20μm
(d) This electron micrograph shows the early stages of remyelination. An immature Schwann cell (S), covered by basal lamina (arrows), surrounds axons. Myelination probably by oligodendrocytes had begun on many axons (asterisks)
(e) Myelination by oligodendrocytes or Schwann cells occurred in the upper part near the pia mater. Schwann cells (S) form thick myelin sheaths, while oligodendrocytes form thin myelin sheaths on the presumably once-demyelinated axons (A). Growth cone-like axons (G) are found among these myelinated fibers.
Scale bar: 1 μm in (d), and (e)

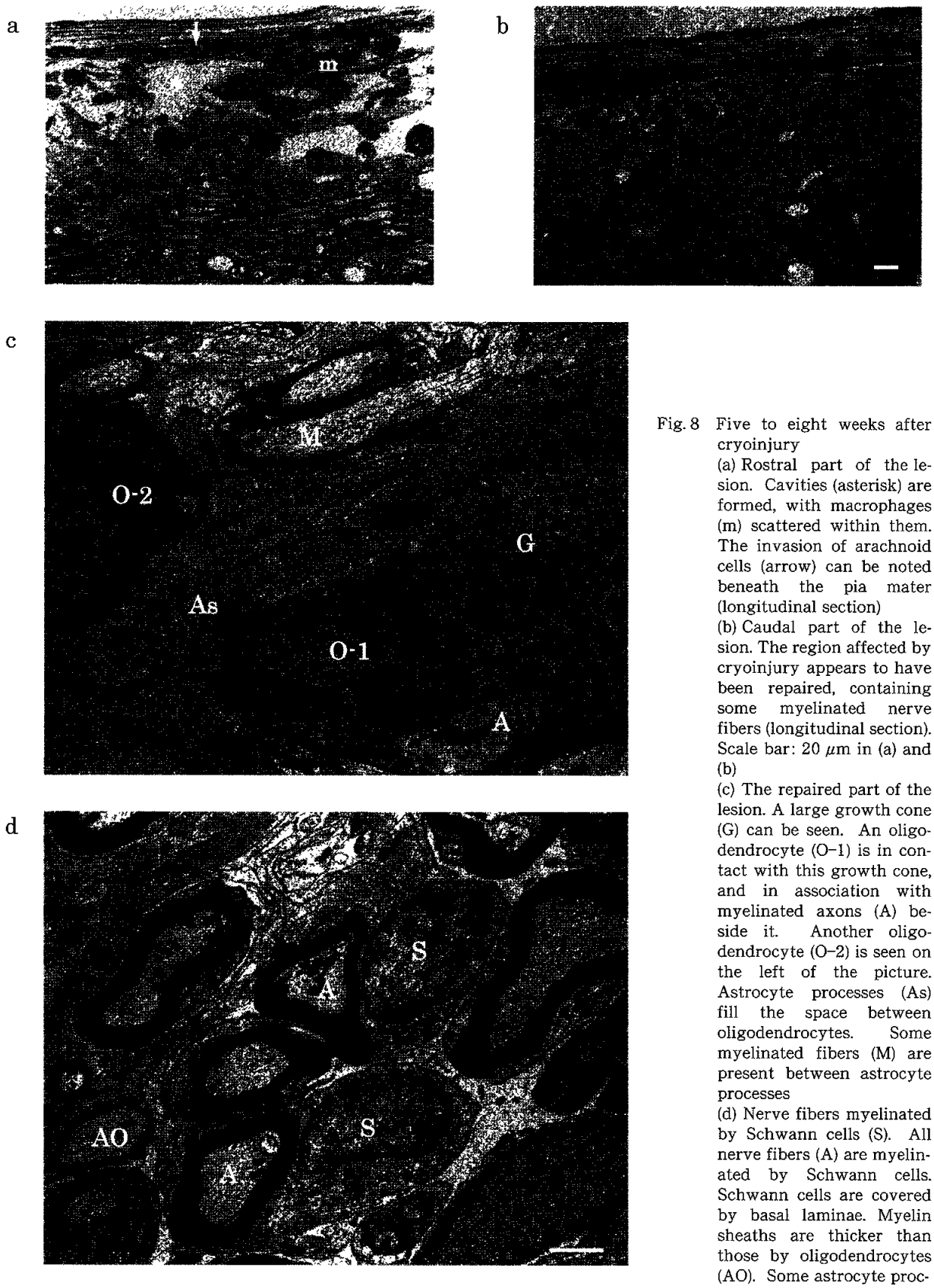

Fig. 8 Five to eight weeks after cryoinjury
(a) Rostral part of the lesion. Cavities (asterisk) are formed, with macrophages (m) scattered within them. The invasion of arachnoid cells (arrow) can be noted beneath the pia mater (longitudinal section)
(b) Caudal part of the lesion. The region affected by cryoinjury appears to have been repaired, containing some myelinated nerve fibers (longitudinal section). Scale bar: 20 μm in (a) and (b)
(c) The repaired part of the lesion. A large growth cone (G) can be seen. An oligodendrocyte (O-1) is in contact with this growth cone, and in association with myelinated axons (A) beside it. Another oligodendrocyte (O-2) is seen on the left of the picture. Astrocyte processes (As) fill the space between oligodendrocytes. Some myelinated fibers (M) are present between astrocyte processes
(d) Nerve fibers myelinated by Schwann cells (S). All nerve fibers (A) are myelinated by Schwann cells. Schwann cells are covered by basal laminae. Myelin sheaths are thicker than those by oligodendrocytes (AO). Some astrocyte processes have extended between these Schwann cell-myelinated fibers.
Scale bar: 1μm in (c) and (d)

was not clear where such scars would be formed in the tissue around the cavity.

Discussion

The present study demonstrated that rigorous regenerating reactions occurred in the caudal part of the dorsal funiculus in the cryoinjured spinal cord of the rat. In contrast, the tissues at the epicenter of the lesion were degraded, degenerated, and became necrotic, resulting in cavities of various sizes. Regenerating axons and glial cells cannot invade the damaged tissue at the epicenter, resulting in the formation of a cavity as a necrotic space. The cavity formation prohibited the extension of regenerating axons up to the rostral part of the lesion.

Axonal changes

Many characteristic changes were observed in myelinated fibers following cryoinjury in the caudal part of the lesion. When they were completely destroyed by cryoinjury, axons underwent Waller degeneration, and myelin sheaths were subsequently removed by macrophages. If axons survived while their associated oligodendrocytes were damaged, demyelination occurred. These demyelinated axons probably loose their function. It is conceivable that such demyelinated axons were remyelinated by oligodendrocytes, or occasionally by Schwann cells (Sasaki et al., 1989; Sato et al., 1997). Axons can probably recover their original physiological function as impulse conductors when remyelinated. This phenomenon will greatly contribute to the functional recovery of animals after spinal cord injury.

Growth cones were formed in the caudal part of the injured spinal cord (Kitada et al., 1999). Such growth cones were found even 30 days after injury, suggesting that spinal cord nerves retain the ability to regenerate a long time after injury. It was not clear whether growth cones, once formed, could extend for a certain distance in the lesion. It is probable that the growth cones could not extend over a long distance. This will be one of the most critical points to be addressed in future studies on spinal cord regeneration. The finding that growth cones extended along the pia mater basal lamina of the spinal cord surface is interesting. It was not determined whether growth cones were derived from root fibers or from intrinsic spinal cord fibers. However, this suggests that even in the spinal cord, axons can extend over a long distance if they are provided with a suitable scaffold to grow along.

Myelin sheath and glial cells

In the typical Waller degeneration, if axons were degenerated, oligodendrocytes separated myelin sheaths from the cytoplasm by some unknown mechanisms. Isolated myelin sheaths appeared to be degraded by autolysis or phagocytosed by macrophages.

If both axons and oligodendrocytes were destroyed by injury, myelin sheaths were disrupted into various forms of fragments, which were also finally removed by macrophages. In the case in which axons survived and the associated oligodendrocytes were degraded, myelin sheaths were disintegrated into varying types of lamellae, and removed by macrophages, resulting in the demyelination of axons. These axons were usually remyelinated by oligodendrocytes. Although it is not known whether new oligodendrocytes are generated by mitosis within the lesion, at least oligodendrocytes surviving after injury might contribute to the myelin formation on demyelinated axons.

It is notable that astrocytes disappeared from the lesion. Astrocytes might be more vulnerable to cryoinjury than oligodendrocytes. It has been commonly believed that astrocytes remain to form networks after axons and oligodendrocytes are damaged. This is not the case. The lesion is usually devoid of astrocytes, containing surviving axons and associated oligodendrocytes. As far as we examined, astrocytic scar formation was not common on the cavity wall. In the later stage, astrocytes extended their cytoplasmic processes, to fill in the spaces between nerve fibers and/or oligodendrocytes.

The invasion of arachnoid tissue into the spinal cord, though restricted in its extension, might be an important problem. If the spinal cord receives an open injury such as transection, arachnoid cells invade extensively, and form a barrier to the growth of axons. The reaction of the spi-

nal cord to the open injury is fundamentally different from closed injury. In closed injury, like the cryoinjury in the present study, external cell invasion including fibroblasts and arachnoidal cells is minimal. Invasion by fibroblasts or arachnoidal cells from the outside leads to astrocytic scar formation. Astrocytes proliferate and form a barrier of basal laminae at the border facing non-spinal cord tissues such as fibroblasts or arachnoidal cells. Accordingly, the condition for regeneration is markedly different between the open- and close-injured spinal cord. In the present cryoinjury, albeit the injury was a closed one, a small amount of arachnoid cells invaded through locally disrupted basal laminae, or through the root entry space after the root had been degenerated.

Spinal cord regeneration

From the standpoint of spinal cord regeneration, the survival of axons after demyelination is regarded as a structural basis for the functional recovery of animals following spinal cord injury. As stated above, axons that had been demyelinated seem to be again myelinated by oligodendrocytes or Schwann cells. It is conceivable that remyelinated axons can recover their impulse conduction function. In this respect, the conservation of axons and glial cells in the early injury stages might be critical for achieving functional recovery in spinal cord regeneration. Axons and glial cells should be rescued from degeneration, e. g., by growth factors (Ohta et al., 2004; Scharma, 2007; Wright et al., 2007; Ohtaki et al., 2008), in the early stage of injury, to achieve the functional recovery of the animal.

The presence of growth cones is a positive sign of nerve regeneration. The fact that growth cones are formed even 30 days after injury suggests that spinal cord nerve fibers retain their regenerative ability for a long time after injury. This leads to the consideration that spinal cord regeneration might be realized even in the chronic condition if some appropriate techniques can be applied for the extension of growth cones. Growth cones need scaffolds to grow along, as in the case of peripheral nerve regeneration (Ide et al., 1983). If some suitable scaffolds as basal laminae in the peripheral nerve can be provided, nerve regeneration is more likely in the spinal cord.

The biggest hindrance to spinal cord regeneration might be cavity formation. No tissue components invade or survive in such cavities. Many attempts have so far been made to fill in these cavities with transplant materials effective for nerve outgrowth, including various kinds of cells or artificial substances (Kataoka et al., 2004). We think that the blood supply is the most critical factor in cavity formation. Blood vessels are destroyed by injury. Apparently no blood vessel formation occurs in the spinal cord lesion. This is a fundamental difference from peripheral nerve injury. In the peripheral nerves, blood vessels regenerate extensively through the endoneurial connective tissue. On the other hand, there is no connective tissue component such as collagen fibrils and basal laminae in the spinal cord. Unlike in the case of peripheral nerves, basal laminae of blood vessels did not survive, but disappeared when blood vessels were destroyed in the spinal cord. This means that there is no scaffold for blood vessel formation in the spinal cord. This fact might be a major factor in cavity formation in the spinal cord.

We should keep in mind that simple cell transplantation might not be effective for spinal cord regeneration. An appropriate environment should be provided to keep the transplants alive and/or promote blood vessel invasion into the lesion. In this respect, any kind of scaffold or supporting material can be studied along with the cell transplantation. The fate of transplanted cells and histological changes should be examined precisely.

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