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REGULAR ARTICLE

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Regulation of clusterin expression following spinal cord injury

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Abstract We have investigated the localization and regulation of a putative extracellular chaperone, clusterin, in the rat spinal cord after lesion. In control animals, clusterin is expressed in motoneurons, in meningeal and ependymal cells, and in astrocytes mainly located beneath the pial surface. Beginning at day 2 after hemisection at segmental level C6, clusterin levels increase in GFAP-positive astrocytes within the lesioned segment. Three weeks after trauma, clusterin mRNA and protein are elevated in neurons close to the lesion site and in glial elements within scar tissue and within degenerating fiber tracts rostral and caudal to the lesion. This study provides evidence for a role of clusterin in the subacute and late phase of spinal cord injury.

Keywords Clusterin · Complement inhibitor · Spinal cord injury · Hemisection · In situ hybridization · Rat (Sprague Dawley)

Introduction

Synthesis of clusterin (also known as ApoJ or SGP-2) increases in response to the injury of various organs including the brain (Rosenberg and Silkensen 1995; May and Finch 1992). Direct contusion injury (Bellander et al. 1996), deafferentation (Lampert-Etchells et al. 1991), axotomy (Liu et al. 1999), inflammation (Wu et al. 1993), and peripheral nerve lesions (Svensson et al. 1995; Liu et al. 1995) lead to up-regulation of clusterin in the central nervous system. The function of this heterodimeric glycoprotein remains elusive. It has been suggested that clusterin plays a role in maintenance of cell-cell or cell-substratum contacts, in lipid transport, in the regulation of apoptosis, and in the inhibition of complement-mediated cell lysis (Jenne and Tschopp 1989; Rosenberg and Silkensen 1995). Recent evidence has pointed to a neuroprotective function of clusterin as a secreted chaperone, because of its similarity to heat shock proteins (Wilson and Easterbrook-Smith 2000).

Inflammation, tissue disintegration, and apoptosis are associated with acute and subacute traumatic spinal cord injury (SCI). Therefore, it would be of interest to establish a possible role of clusterin as molecule regulating the spatial and temporal extent of these pathological events. The aim of the present study is to reveal the distribution and regulation of clusterin at different time points following a hemisection lesion in the adult rat.

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Materials and methods

Animals

Female Sprague-Dawley rats weighing 250–300 g were used. Animals were fixed in a spinal unit under deep anesthesia induced by Xylazine (10 mg/100 g body weight, i.m.) and Ketamin (100 mg/100 g, i.m.). The spinal column was visualized between the 6th and 7th vertebral body, the dura mater incised for a slit opening, and the right half of the cervical cord transected by using a fine scalpel. The cutting edges of the cord were inspected under a stereoscopic microscope for completeness of the procedure. A small sterile spongue was inserted into the lesion site to prevent bleeding. Spinal muscles were sutured, and the skin was closed with metal clips. Non-operated and sham-operated animals (durotomy, 2 days and 21 days survival time) served as controls (n=4 for each group). Animals were re-anesthesized with phenobarbital (120 mg/kg, i.p.) after survival times of 12 h, 2, 7, or 21 days (n=4 for each time point) and transcardially perfused with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) for in situ hybridization or with Zamboni's fixative (2% paraformaldehyde, 15% picric acid in 0.1 M phosphate buffer, pH 7.4) for im-

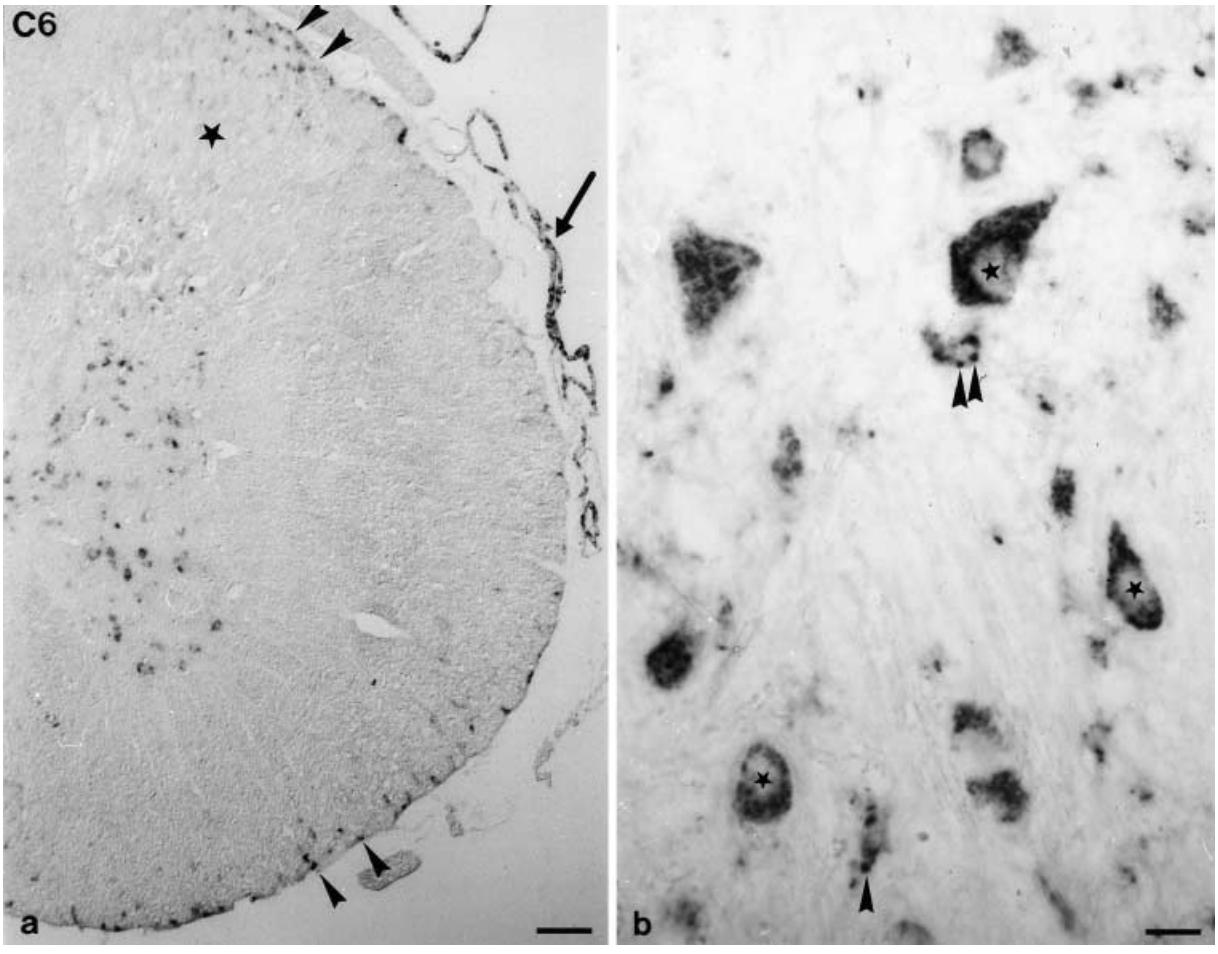


Fig. 1a, b Non-radioactive in situ hybridization for clusterin mRNA in unlesioned rats. **a** Low magnification reveals staining in neuronal cell bodies located in the ventral and intermediate but not in the dorsal zone (*star*). Few non-neuronal cells, which are oriented radially beneath the spinal surface (*arrowheads*), are detected. Note the prominent labeling of the dura mater (*arrow*). **b** Higher magnification of the ventral horn demonstrates the "patchy" distribution of formazan reaction product in neuronal cell bodies (*stars* non-reactive nucleus) and glial cells (*arrowheads*). *Bar* 20 µm (**a**), 100 µm (**b**)

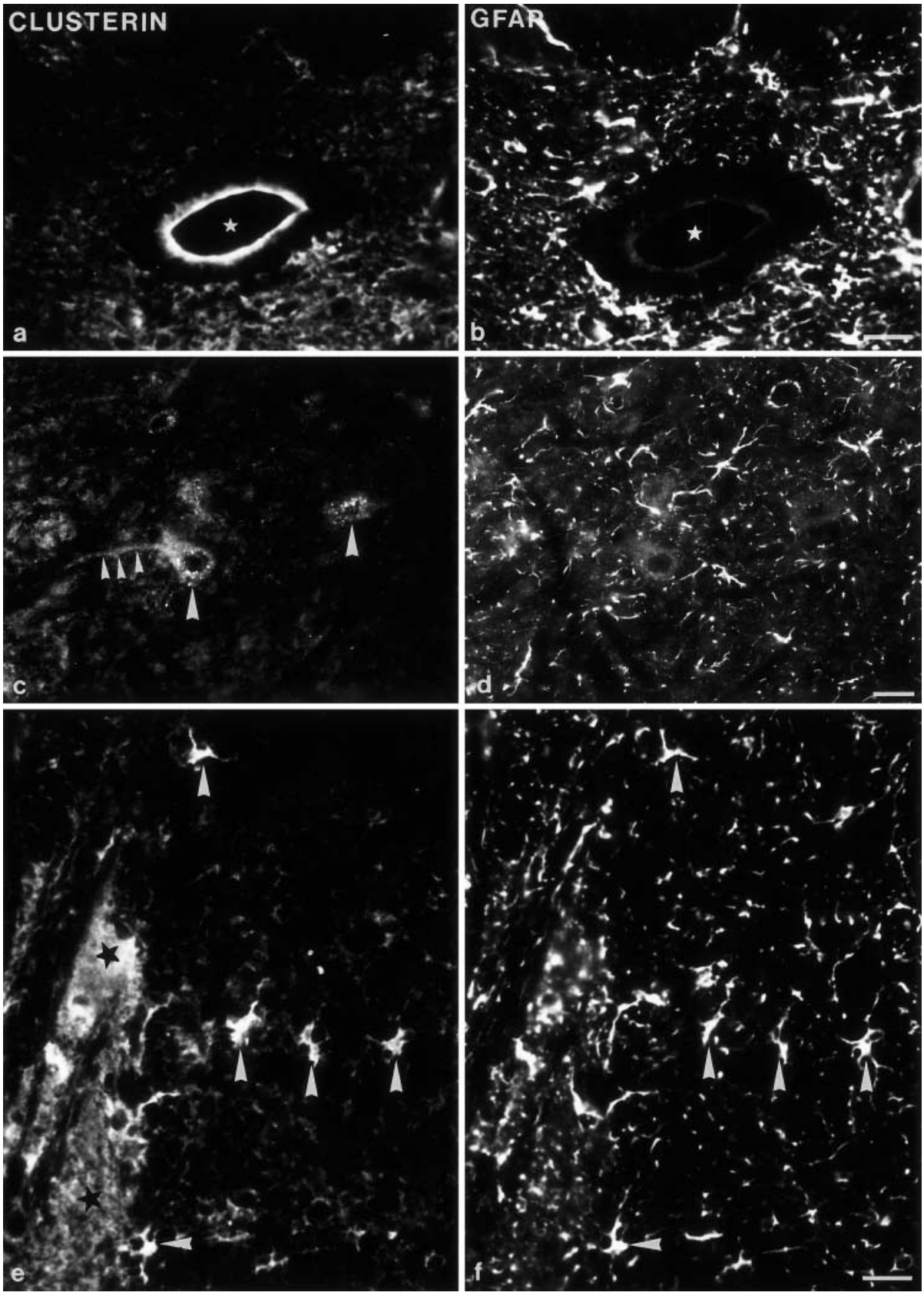
munofluorescence studies. The guidelines of the National Institute of Health concerning animal care were closely observed, and all experiments were approved by the ethical commission of the local government.

In situ hybridization

For in situ hybridization, a 1.36-kb rat clusterin cDNA fragment (kindly provided by R. Buttyan, New York; Buttyan et al. 1989) was subcloned into pBluescript-KS$^-$ vector. Antisense RNA was obtained after restriction with *Bam*HI and in vitro transcription with T7 bacteriophage RNA polymerase. For generation of the control probe, the corresponding sense RNA was obtained by restriction of the plasmid DNA with *Hin*dIII and subsequent in vitro transcription with T3 RNA polymerase. The length of the digoxigenin (DIG)-labeled riboprobes was checked on a denaturing formaldehyde agarose gel stained with ethidium bromide. The probes were fragmented by alkaline hydrolysis to an average fragment length of 200 bases. The concentration of labeled antisense or sense probes was 0.5–1 ng/µl hybridization mixture.

Cryostat sections (12 µm) from spinal cord segments C4–C8 were thawed onto silanized glass slides and hybridized as described previously (Obermüller et al. 1995; Klimaschewski et al. 1996). Briefly, overnight hybridization at 43°C was followed by several washes at 48°C in decreasing concentrations of SSC (2×SSC to 0.5×SSC) containing 50% formamide (1×SSC=50 mM NaCl, 15 mM sodium citrate, pH 7.0). Subsequently, slides were rinsed in buffer I (100 mM TRIS-HCl, 150 mM NaCl, pH 7.5) and immersed in 1% Boehringer's blocking reagent plus 0.5% bovine serum albumin dissolved in buffer I. Alkaline-phosphatase-coupled sheep polyclonal anti-DIG antibodies (F_{ab} fragments, dilution 1:500 in blocking medium; Boehringer Mannheim, Germany) were administered to the sections at room temperature for 2 h followed by a 16-h incubation at 4°C. Slides were then washed in buffer I and equilibrated for 2 min in buffer II (100 mM TRIS-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). Signal development was performed over 48 h by using NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) diluted in buffer II.

Fig. 2 Double-labeling immunofluorescence for clusterin (**a, c, e**) and GFAP (**b, d, f**) reveals the cellular localization of clusterin protein (**a–d** control tissue, **e, f** lesioned tissue). Ependymal cells lining the central canal are strongly immunoreactive for clusterin (**a**, *star*). A weaker granular signal is observed within neuronal cell bodies (**c**, *large arrowheads*). Some dendritic processes also exhibit granular clusterin immunofluorescence (**c**, *small arrowheads*). Whereas the gray matter neuropil is diffusely stained by clusterin antibodies (**a, c, e**, *black star* in **e**), co-localization with GFAP-positive cellular profiles is not detected under normal conditions (**c, d**). In contrast, 7 days after hemisection, clusterin immunoreactivity is clearly visible adjacent to the lesion in enlarged white matter astrocytes that are strongly GFAP-positive (**e, f**, *arrowheads*). *Bar* 20 µm ▶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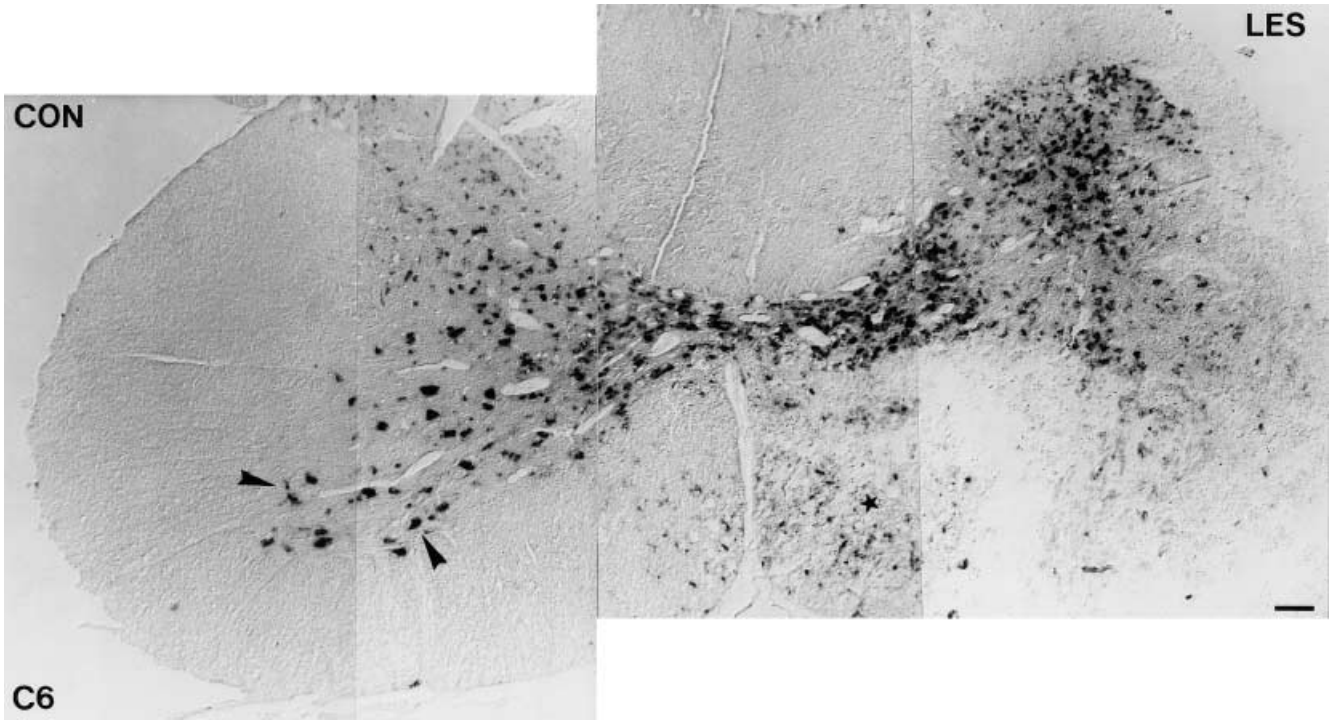

Fig. 3 Clusterin mRNA expression in segment C6 approximately 1 mm adjacent to a hemisection, 1 week after lesion. Intact neuronal cell bodies located within the ventral horn of the contralateral side (*CON*, *arrowheads*) synthesize clusterin, whereas no neurons are detected in the corresponding area of the lesioned side (*LES*). However, an increased number of clusterin-mRNA-positive non-neuronal cells (presumably astrocytes) is observed in the gray and in the degenerating white matter (ventral funiculus; *star*). *Bar* 100 µm

Double-labeling immunofluorescence

Cryostat sections were air-dried for 45 min on gelatine-coated slides and incubated for 1 h in phosphate-buffered saline (PBS), containing 10% swine serum and 0.5% Tween 20. After several washes in PBS, rabbit polyclonal antibodies against rat clusterin (diluted 1:1000; kindly provided by C.Y. Cheng, Population Council Center for Biomedical Research, New York, USA), and mouse monoclonal anti-GFAP antibodies (diluted 1:1000; Sigma) were applied overnight. Anti-mouse IgG conjugated to Cy2 (diluted 1:100; Rockland) combined with anti-rabbit IgG conjugated to Cy3 (diluted 1:100; Jackson), each applied for 1 h, served as the detection system. Slides were rinsed in PBS and mounted in carbonate-buffered glycerol at pH 8.6. Specificity tests were performed by preincubation of the diluted antiserum with 20 µg/ml of the respective peptide overnight at 4°C or by omission of the primary antisera. Slides were evaluated by confocal microscopy (Leica TCS^{4D}) or with a Polyvar microscope (Reichert Jung) equipped for epifluorescence microscopy.

Results

Localization of clusterin in the cervical spinal cord

Clusterin mRNA is strongly expressed in spinal motoneurons located in ventral horn laminae 6–10 under normal conditions, whereas neuronal perikarya in other laminae are rarely stained (Fig. 1a). The formazan reaction product indicating the presence of clusterin mRNA is distributed inhomogeneously within the cytoplasm of neuronal and non-neuronal cell bodies (Fig. 1b). Fine granular clusterin immunoreactivity is observed in neuronal perikarya that occasionally extend into the dendritic compartment of motoneurons (Fig. 2c). The diffuse labeling of the neuropil suggests that clusterin is secreted from neuronal somata or from their processes (Fig. 2). Meningeal cells of the dura mater and ependymal cells lining the central canal exhibit intense mRNA and protein signals (Figs. 1a, 2a). Clusterin-mRNA-positive glial elements are detected mainly beneath the pial surface (Fig. 1a). These radially oriented cells represent astrocytes as revealed by double-staining with GFAP antibodies and are rarely observed within the gray matter.

No obvious difference was visible between untreated and sham-operated animals. Sections incubated with the control sense probe did not reveal any staining.

Regulation of clusterin in spinal segments C4–C8 following hemisection at C6

Although the expression pattern of clusterin contralateral to the lesion side at segmental level C6 is not different from that of sham-operated animals, an increased number of clusterin mRNA positive astrocytes is observed adjacent to the lesion (Fig. 3). This up-regulation starts in white matter astrocytes located in the vicinity of the lesion after 2 days and is further increased after 1 week. Compared with sham-operated animals, the proportion of clusterin mRNA and protein containing non-neuronal cells is elevated within gray and white matter at day 7

Table 1 Staining intensity (+ weak, ++ moderate, +++ strong, *(+)* presence of very few positive cellular profiles per section) for clusterin on mRNA and protein levels after hemisection at C6, 1 week after lesion. Only single neuronal profiles are detected close to the lesion (C6)

Segment		Gray matter (neurons in laminae 6–10)		Gray matter (astrocytes)		White matter descending tracts		White matter ascending tract	
		Intact	Lesion	Intact	Lesion	Intact	Lesion	Intact	Lesion
C4	mRNA	++	++	+	+	(+)	(+)	(+)	+++
	Protein	+	+	+	+	(+)	(+)	(+)	++
C6	mRNA	++	(+)	+	+++	(+)	+++	(+)	+++
	Protein	+	(+)	+	+++	(+)	++	(+)	++
C8	mRNA	++	++	+	+	(+)	+++	(+)	(+)
	Protein	+	+	+	+	(+)	++	(+)	(+)

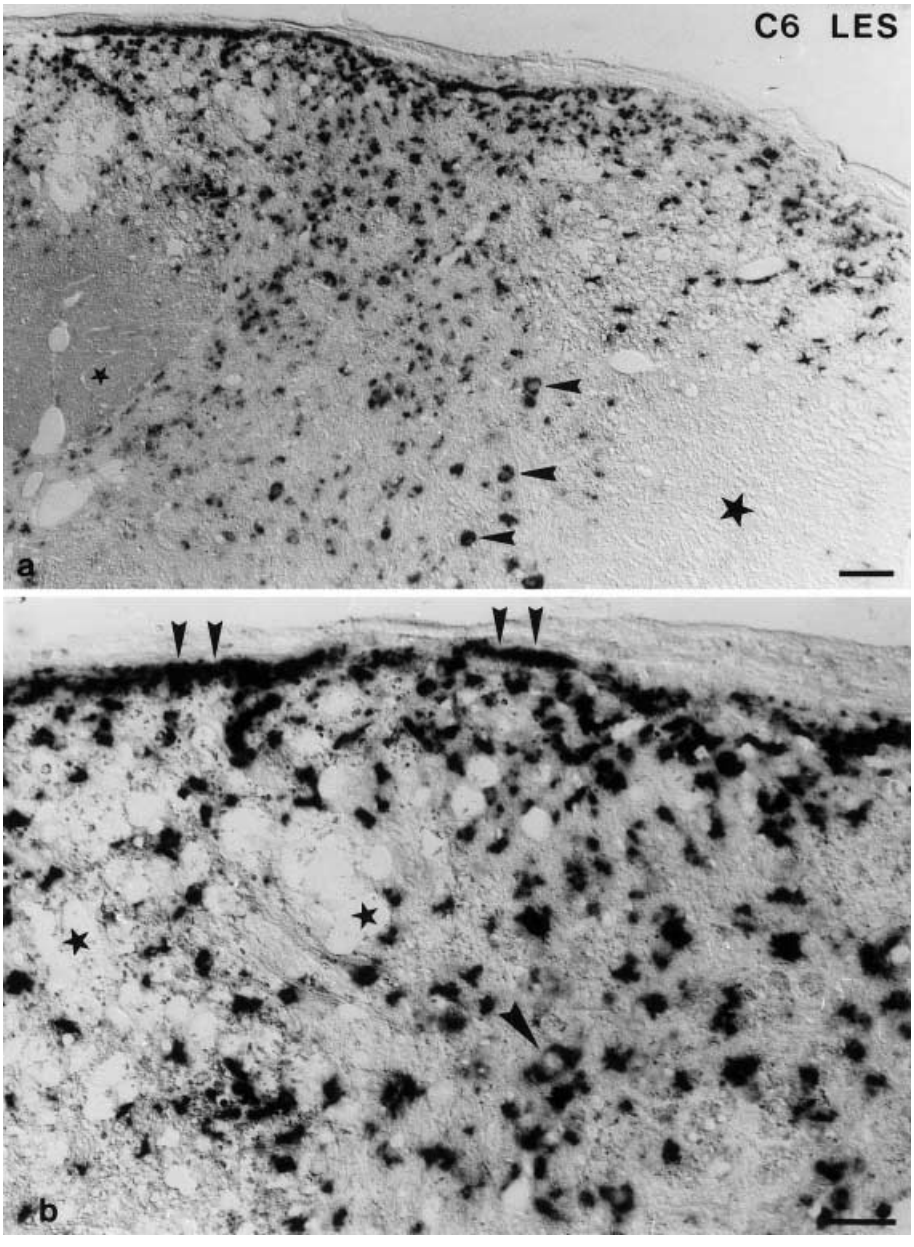

Fig. 4a, b In situ hybridization of clusterin mRNA in segment C6, 3 weeks after lesion. **a** Increased staining is detected in regions that undergo degenerative changes, whereas intact white matter areas, such as the corticospinal tract (*small star*) or the lateral funiculus (*large star*), do not contain clusterin-mRNA-positive cells. Intensely labeled neuronal perikarya are found within gray matter (*arrowheads*). **b** Higher magnification reveals prominent staining of hypertrophic cells throughout the spongiform tissue degeneration (*stars*). Note the aggregation of strongly stained cells at the glia limitans (*small arrowheads*) and a surviving neuron in the dorsal horn (*large arrowhead*). *Bar* 100 µm (**a**), 50 µm (**b**)

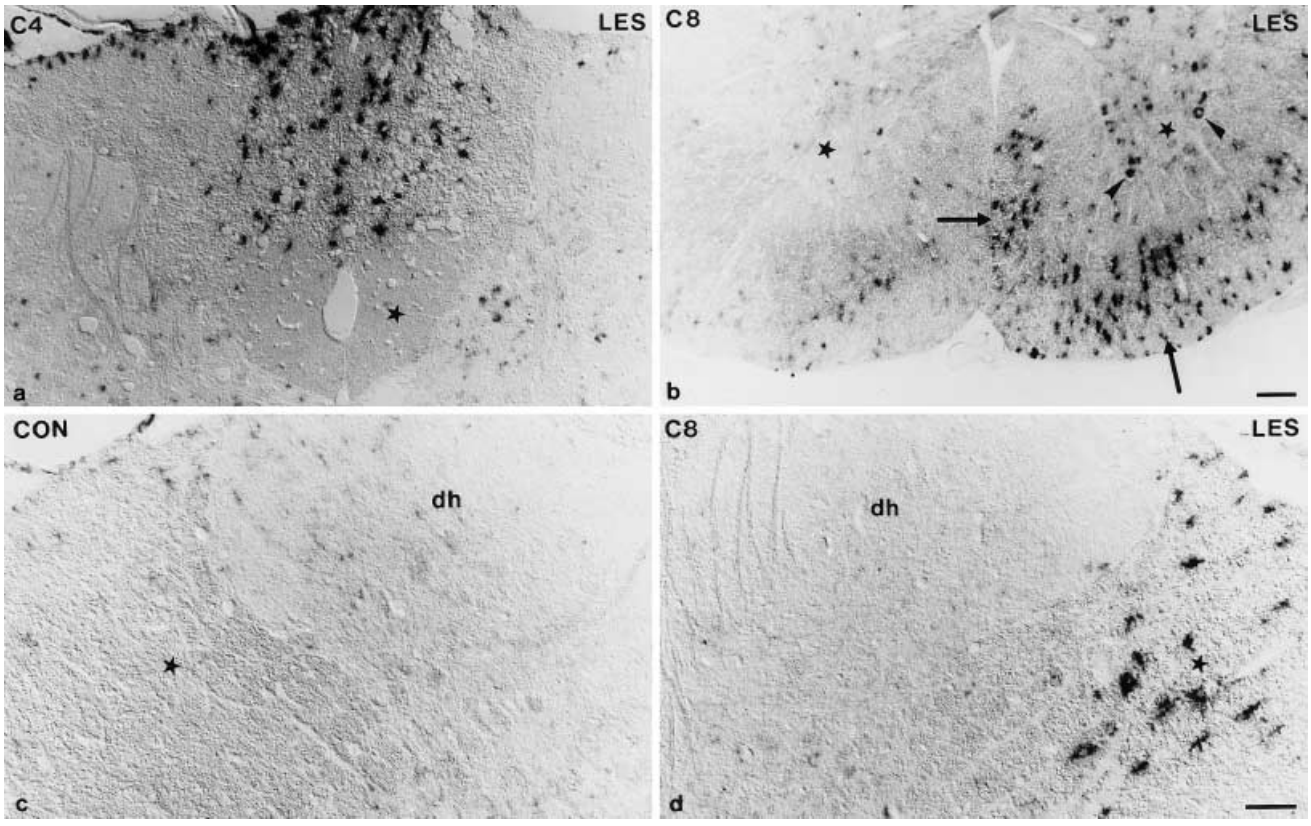

Fig. 5a–d Expression of clusterin mRNA in degenerating pathways, 21 days after hemisection, at level C6. Marked staining of hypertrophic astrocytes is detected rostral to the lesion in the dorsal column (**a**), whereas no clusterin-mRNA-positive cells are observed in the intact descending corticospinal tract (*star*). In this case, the knife-lesion crossed the midline also resulting in contralateral degeneration. Caudal to the lesion, increased labeling of astrocytes is found within the ventrolateral funiculus (**b**, *arrows*). Motoneurons (*arrowheads*) are more intensely stained within the ventral horn (*star*) of the lesioned side compared with the contralateral side. Higher magnification of segment C8 (**c, d**) reveals prominent labeling of astrocytes on the lesioned side (*LES*) within a major descending projection from the brainstem, the dorsolateral funiculus (*star*). Clusterin mRNA is not expressed within the area of the contralateral dorsolateral funiculus (*CON*; *dh* dorsal horn). *Bar* 100 µm (**a, b**), 50 µm (**c, d**)

after lesion (Figs. 2e, 3). Within gray matter, the number of mRNA-positive cellular profiles increases dramatically, whereas white matter fiber tracts rarely exhibit positive non-neuronal cells. Semiquantitative estimations of clusterin mRNA and protein staining intensities are provided in Table 1.

Three weeks following lesion, clusterin-mRNA-positive scar-forming astrocytes are considerably enlarged and aggregate below the pial surface at the interface between the wound and intact spinal cord (Fig. 4). Close to the lesion, the number of clusterin-expressing neurons is reduced because of tissue destruction (Fig. 3). However, in those sections that contain intact neuronal cell bodies, the neuronal clusterin mRNA signal is stronger on the lesioned side than on the contralateral side (Fig. 5b). Compared with sham-operated animals, clusterin-mRNA-expressing cells and granular reaction product are found in segments C4 and C8 in areas of degenerating axonal tracts (Fig. 5). These include ascending pathways (ventrolateral funiculus and dorsal columns) in segments located rostral to the lesion (C4) and descending pathways (corticospinal, ventral, and dorsolateral funiculus) in segments located caudal to the lesion (C8).

In general, the changes visible after immunohistochemical staining were less pronounced than those observed in sections hybridized for clusterin mRNA. Although double-labeling with GFAP antibodies indicated that most of the clusterin immunoreactive profiles represented astrocytes, we could not exclude de-novo synthesis of clusterin in oligodendrocytes or macrophages.

Discussion

Clusterin expression in the spinal cord

In agreement with previous observations (Pasinetti and Finch 1991; Liu et al. 1995; Garden et al. 1991; Danik et al. 1993), prominent expression of clusterin protein and mRNA is observed in spinal motoneurons of the untreated rat. Furthermore, ependymal cells and astrocytes close to the pial surface are clusterin-immunoreactive as revealed by co-staining with antibodies against GFAP.

The novel findings presented in this study include the up-regulation of clusterin synthesis in neurons and non-neuronal cells at the level of a spinal cord hemisection and in degenerating fiber tracts at least two segments

away from the lesion. A recent study provided evidence for an increase of clusterin mRNA and protein at level C3 in response to transection of the lateral funiculus (Liu et al. 1999). However, the application of radioactive in situ-hybridization did not allow the identification of specific cell types newly synthesizing clusterin in the spinal cord. Here, we describe clusterin synthesis in neurons that are present in the lesioned segment and that are probably either deafferented or axotomized or both. Moreover, double-immunolabeling with antibodies against GFAP reveals astrocytes as another source of clusterin after lesion, whereas oligodendrocytes may also be involved in degenerating fiber tracts (Liu et al. 1999). Changes in clusterin synthesis are observed within a few days after lesion, but the strongest clusterin mRNA signals are detected in the later stage of injury beginning after 1 week. This late induction resembles the time course observed in other lesion paradigms, e.g., in the rat hippocampus after entorhinal cortex lesioning (Lampert-Etchells et al. 1991) or in the dorsal horn following sciatic nerve lesion (Liu et al. 1995).

In embryonic spinal cord tissue cultures, clusterin is expressed in neurons and non-neuronal cells in response to glutamate excitotoxicity (Messmer-Joudrier et al. 1996). The latter study has revealed that induction of clusterin mRNA requires the presence of neurons, since clusterin synthesis is not elevated in non-neuronal cell cultures. The authors conclude that neuronal stress attributable to the activation of *N*-methyl-D-aspartate (NMDA) receptors may "prime" non-neuronal cells to produce and release clusterin. Since pharmacological studies suggest that NMDA receptors are involved in the pathogenesis of spinal cord injury (Faden and Salzman 1992), they may also play a role in clusterin induction in vivo. It is possible that injured neurons or their degenerating axons release factors that induce clusterin expression in glial cells. This hypothesis could also explain the strong induction of clusterin in non-neuronal cells located within degenerating white matter regions. Further support for this assumption is provided by studies showing clusterin up-regulation in astrocytes that surround axotomized hypoglossal motoneurons (Svensson et al. 1995) or degenerating central terminals of dorsal root ganglion neurons after peripheral nerve lesion (Liu et al. 1995).

Putative functions of clusterin following spinal cord injury

The increase of clusterin mRNA in some non-neuronal tissues undergoing programmed cell death has led to the suggestion that this protein is directly involved in apoptosis. However, a developmental study by Garden et al. (1991) has revealed that clusterin expression does not correlate with areas of apoptosis. In contrast, they report that clusterin induction coincides with neuronal differentiation. In the developing spinal cord, clusterin mRNA levels increase during and after the period of developmental cell death and continue to increase postnatally. In line with this observation, clusterin mRNA is rapidly induced in pheochromocytoma (PC12) cells upon treatment with differentiating agents, e.g., nerve growth factor or vasoactive intestinal peptide (Lee et al. 1995; own unpublished observations). In the spinal cord, apoptosis as revealed by TUNEL labeling is associated with Wallerian degeneration of long spinal tracts (Crowe et al. 1997), andwe have detected increased clusterin expression in regions known to exhibit apoptotic nuclei.

It has been suggested that clusterin prevents the disintegration of injured tissue in response to trauma. Indeed, clusterin has been named after its pronounced ability to promote cell aggregation (Rosenberg and Silkensen 1995). In vitro studies (Tung et al. 1992) have demonstrated that, under conditions that inhibit interactions between cellular and extracellular proteins, clusterin exerts its strongest effect on aggregation. It is therefore conceivable that astrocytes and neurons that lose their adhesive properties in response to lesion up-regulate clusterin in order to preserve cell proximity.

Some evidence suggests a role of clusterin as complement inhibitor. Complement factors mediate a variety of inflammatory processes and promote phagocytosis and cell lysis. Increased levels of complement have been found in microglial cells after direct injury to the central nervous system (Pasinetti et al. 1992), in the vicinity of axotomized central neurons, or near degenerating central terminals of axotomized peripheral neurons (Tornqvist et al. 1996). However, complement activation is not observed during Wallerian degeneration in spinal white matter, and recent studies argue against an involvement of clusterin in complement inhibition (Hochgrebe et al. 1999). Finally, because of its similarity to heat shock proteins and its ability to inhibit stress-induced protein precipitation, clusterin probably acts as a chaperone that binds toxic or damaged molecules in the extracellular environment. These complexes may be subsequently removed through receptor-mediated endocytosis (Wilson and Easterbrook-Smith 2000).

Note added in proof A pro-apoptotic role of clusterin is suggested by a recent paper published in *Nat. Med.* (7: 338–343, 2001). Clusterin-deficient mice exhibit 50% less brain injury following neonatal hypoxic-ischemic injury and exogenous clusterin-exacerbated neuronal cell death in vitro.

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