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Upregulation of S100A4 after spinal cord transection in adult rats¹

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KEY WORDS spinal cord injury; S100A4; gene expression

ABSTRACT

AIM: To investigate whether spinal cord transection induces changes of gene expression of S100A4 protein.

METHODS: In a spinal cord transection model, S100A4 expression and cellular localization were examined using cDNA microarray, Northern blot, immunohistochemistry, and immunofluorescence double-labeling methods.

RESULTS: There was very limited S100A4 mRNA expression in the control spinal cord. However, S100A4 mRNA expression was increased significantly in both the rostral and caudal spinal cord segments adjacent to the injury site. Specifically, S100A4 gene expression was substantially increased at d 2, peaked at d 7 and d 14, and remained high up to 28 d post-injury. During its peak expression, S100A4 protein was localized in astrocytes of the spinal cord within 5 mm from the site of spinal transection. **CONCLUSION:** Spinal cord transection induces prolonged S100A4 expression at both mRNA and protein levels in areas close to the injury site. Increased expression of S100A4 in astrocytes after spinal cord transection may indicate that this molecule may play a role in astrocytic responses to injury.

INTRODUCTION

Members of the S100 family of calcium-binding proteins are involved in numerous intracellular processes which include regulation of enzyme activity, cell proliferation and differentiation, and cytoskeletal dynamics^[1]. The precise role of these proteins in the normal and diseased nervous system remains elusive.

One member of the S100 family is the S100A4

protein (also referred to as mts1, 18A2/mts1, pEL-98, p9Ka, or metastasin) which is now known to be capable of regulating cell cycle progression and modulating intercellular adhesion and invasive and metastatic properties of cancer cells^[2-4]. The expression level of S100A4 has been found to be closely related to metastatic activity of some malignant tumors^[5]. S100A4 expression in astrocytes in the region of the dorsal funiculus can be induced following sciatic nerve or dorsal root injury suggesting that S100A4 plays a role in the response of astrocytes to degeneration of myelinated axons^[6,7]. Moreover, recent *in vitro* study demonstrated that S100A4 protein strongly promoted neurite outgrowth and induced differentiation of primary hippocampal neurons, an effect associated with oligomeric forms of S100A4^[8].

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Traumatic spinal cord injury (SCI) has a devastating impact on patients resulting from complete or partial loss of motor and sensory function below the level of lesion. It is likely that both intrinsic neuronal and extrinsic environmental factors existed in the lesion site contribute to the lack of axonal regeneration after SCI. In the present study, we investigated whether S100A4 expressed following SCI and, if so, which cell type contributed to its expression. Understanding S100A4 expression following SCI may allow us to further study its function in nerve injury and regeneration after SCI.

MATERIALS AND METHODS

Spinal cord transection For spinal cord transection, adult Sprague-Dawley female rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and a laminectomy was performed at the 10th vertebral level to expose the dorsal surface of T11. The cord was then completely transected at this level using a pair of surgical microscissors. Sham-operated rats receiving no spinal cord transection served as controls. Post-operative care for spinal cord transected rats followed previously reported protocols^[9,10]. All surgical interventions and postoperative animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996, USA) and were approved by the Chinese National Committee to the Use of Experimental Animals for Medical Purposes, Shanghai Branch. The rats were allowed to survive for 2 (6 rats), 7 (6 rats), 14 (6 rats), or 28 (6 rats) d post-injury, then were sacrificed with deep anesthesia and two 5-mm pieces of cord segments immediately rostral and caudal to the transection or equivalent segments of control spinal cords were taken and frozen immediately in liquid nitrogen. Total RNA was extracted with TRIzol reagent (Life Technologies, Rockville, MD, USA). The mRNAs were purified with an Oligotex mRNA kit (Qiagen, Hilden, Germany).

cDNA microarray Our homemade rat 6.5 k Array containing 6509 genes and expressed sequence tags (ESTs) was used as previously reported^[11]. Hybridization was performed as described previously^[11], then bound probe was detected with the PhosphorImager (Molecular Dynamics, Uppsala, Sweden) and images were analyzed with ARRAYVISION 5.1 (Imaging Research, Ontario, Canada).

Northern blotting For Northern blotting, 15 μ g

of total RNA (control: $n=5$) was separated and blotted to the nylon membrane. The probes were labeled at the 3' end by d[³²P]ATP (NEN) using terminal deoxynucleotidyl transferase (Amersham Pharmacia Biotech, Piscataway, NJ) and purified. The filters were hybridized at 42 °C for 16-18 h and washed 2 times, and the hybridization signal was scanned with the PhosphorImager. The grey scale of each hybridization band was calculated by using Scion Image software for image processing with GAPDH as internal control. The oligonucleotide probes used in this study were: S100A4: 5'gcatcatcatggcaatgcaggacaggaagacac3'; GAPDH: 5'gcaggatgcattgctgacaatcttgaggaggattgtcatat3'.

Production and purification of recombinant S100A4 The cDNA of S100A4 was obtained by RT-PCR (data not shown) and the PCR product of S100A4 was inserted into the *E coli* expression vector pGEX-KG. The plasmid DNA was then transformed into bacteria host BL21. Induction of expression of S100A4 inserts and purification of the recombinant proteins named GST-S100A4 were done according to the manufacturer's manual (GST gene fusion system, Amersham pharmacia biotech). Briefly, bacteria growing to A₆₀₀=0.4 were induced by 0.05 mmol/L isopropyl-1-thio- β -D-galactopyranoside at 37 °C overnight. Cells were harvested and lysed by sonication. After centrifugation to remove debris, the supernatant was mixed with a 50 % slurry of glutathione sepharose 4B (Amersham Pharmacia Biotech), which bound to the glutathione S-transferase (GST) at the amino terminus of the recombinant protein. The slurry was then loaded onto a column and eluted with 5 bed volumes of elution buffer (10 mmol/L reduced glutathione in 50 mmol/L Tris-HCl, pH 8.0). Fractions of 500 mL were collected, and samples from the column were analyzed in 15 % SDS-polyacrylamide gel electrophoresis. Fractions containing the recombinant protein GST-S100A4 were stored at -70 °C. A sample of each eluate was analyzed by electrophoresis on a 15 % SDS-polyacrylamide gel to verify the purity of elution protein.

Preparation of polyclonal antibody against S100A4 New zealand rabbits were injected with 1 mg recombinant fusion protein GST-S100A4 in Freund's complete adjuvant. Three weeks later, they were injected with 0.5 mg GST-S100A4 in Freund's incomplete adjuvant, which was repeated four times at 2-week intervals. After a sample serum was collected from the rabbit's ear and was proven positive by a double agar diffusion test, the rabbit's sera were taken from its ear

and stored in 0.1 % sodium azide at -20°C .

Immunohistochemistry and immunofluorescence double-labeling For immunohistochemistry, rats (transection: $n=4$; control: $n=4$) were deeply anaesthetized and perfused via the ascending aorta with 50 mL warm (37°C) saline followed by 50 mL warm fixative composed of 4 % paraformaldehyde and 0.2 % picric acid in 0.16 mol/L phosphate buffer at pH 6.9. This was immediately followed by 200 mL of the same, but ice-cold fixative. After perfusion, the spinal cord was dissected out, post-fixed in the same fixative at 4°C for 90 min, and immersed in 10 % sucrose in 0.01 mol/L phosphate buffered saline (PBS) overnight. Immunohistochemical labeling was used to localize S100A4. Briefly, 20 mm-thick horizontal sections of the spinal cord were washed in 0.01 mol/L PBS, rinsed in 1 % H_2O_2 , and incubated overnight at 4°C with rabbit anti-S100A4 (1:2000) in PBS containing 1 % BSA. The sections were then incubated with a biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA), and subsequently incubated with an avidin-biotin-peroxidase complex (1:100; Vector). Immunolabeling was visualized with PBS containing 0.05 % 3,3'-diaminobenzidine and 0.3 % H_2O_2 . The sections were then dehydrated through graded ethanol and xylene and coverslipped.

Immunofluorescence double-labeling was used for cellular colocalization of S100A4. The sections were incubated with a mixture of rabbit anti-S100A4 antibody (1:2000) and a monoclonal antibody as the cell-specific marker overnight at 4°C . The cell-specific monoclonal antibodies included mouse anti-gial fibrillary acidic protein antibody (GFAP, 1:200; Sigma) to identify astrocytes, mouse anti-RIP antibody (1:50, a gift from Dr Scott R WHITTEMORE, University of Louisville) to identify oligodendrocytes and mouse anti- β tubulin III antibody (1:400; Sigma) to recognize neuron. On the following day, the sections were incubated with fluorescein-conjugated goat anti-rabbit (FITC, 1:100; Jackson ImmunoResearch Lab, West Grove, PA) and rhodamine-conjugated goat anti-mouse (TRITC, 1:100; Jackson ImmunoResearch Lab) antibodies. Sections were washed, coverslipped, and examined using a Leica SP2 confocal microscope.

RESULTS

S100A4 mRNA was upregulated in injured spinal cord after SCI In sham-operated control rats, there was little S100A4 mRNA expression (Fig 1A). SCI induced significant increase in S100A4 mRNA

expression in spinal segments immediately rostral and caudal to the transection at 14 d post-injury (Fig 1A). Northern blot analysis further demonstrated the time course of S100A4 gene expression: it was significantly increased at 2 d post-injury, peaked at 7 and 14 d, and decreased but still remained at a high level at 28 d post-

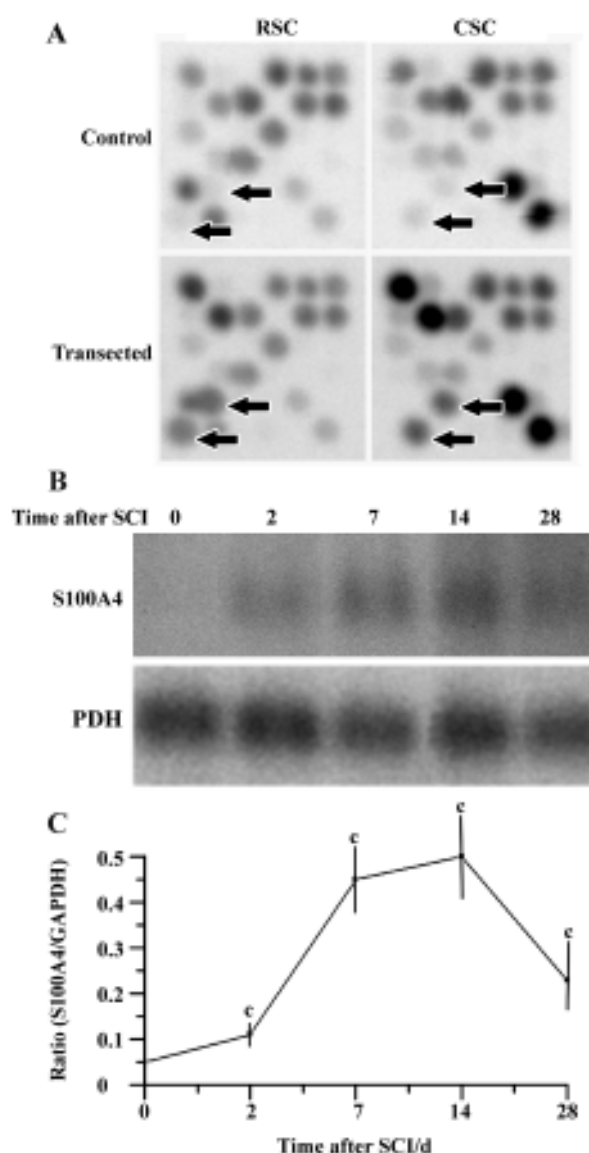


Fig 1. S100A4 mRNA was upregulated in the spinal cord after SCI. A) S100A4 mRNA was upregulated in the spinal cord at 14 d post-injury (arrows). B) The time course of S100A4 mRNA expression in both the control and spinal cord-transected rats. The same blot nitrocellulose membrane was re-hybridized with the probe of GAPDH for an internal control. C) S100A4 gene expression was significantly increased at 2 d, peaked at 7 d and 14 d, and decreased at 28 d post-injury. The mean and standard error among 3 repeated experiments were calculated. $n=5$. Mean \pm SEM. ($P<0.01$ vs with the control. RSC - rostral spinal cord; CSC - caudal spinal cord).

injury (Fig 1B, 1C). As expected, S100A4 mRNA expression was very low in the control spinal cords (Fig 1B, 1C).

S100A4 expressed in astrocytes after injury In order to investigate cellular localization of S100A4 after SCI, we prepared an antibody against the rat S100A4 protein. At first, the *E coli* expression vector pGEX-KG/S100A4 was constructed and confirmed by DNA sequencing. Then recombinant GST-S100A4 fusion protein was expressed and purified (Fig 2A). After purification, only one band around 35 kD was seen, indicating that GST-S100A4 was highly purified (Fig 2A). Further, the polyclonal antibody against rat S100A4 was obtained from two immunized New Zealand rabbits. The quantity of antiserum was tested by double agar diffusion with the titer greater than 1:16 (Fig 2B). Theoretically, GST gene of pGEX-KG vector was derived from *Schistosoma japonicum* and the antibody against GST-S100A4 could be directly used to immunolabel rat tissues without any cross-staining with rat GST. This was proven by immunostaining of rat tissues with the GST-preabsorbed antibody against GST-S100A4 (Fig 3C).

The immunohistochemical results revealed that there was a baseline S100A4 protein expression in sham-operated control rats (Fig 3A). Spinal cord injury substantially increased S100A4 expression which were seen

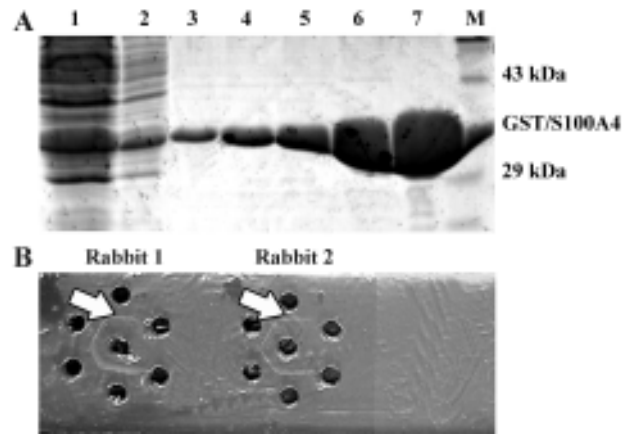


Fig 2. Purification of recombinant S100A4 and preparation of polyclonal antibody against recombinant rat S100A4. A) Lane 1: supernatant of BL21 transformed with pGEX-KG/S100A4 containing recombinant protein GST-S100A4 (about 35 kD); Lane 2-7: serial wash solution eluted from purification column; M: protein molecular weight markers. B) Double agar diffusion test showed the quantity of the polyclonal antibody against rat S100A4 with the titer greater than 1:16 (arrows).

mainly in glial cells close to the site of injury (7 d post-injury; Fig 3B). Similar immunostaining pattern was seen in the spinal cord 14 d post-injury (data not shown). In control sections, pre-immune serum was used to confirm the specificity of the antibodies (Fig 3D). To

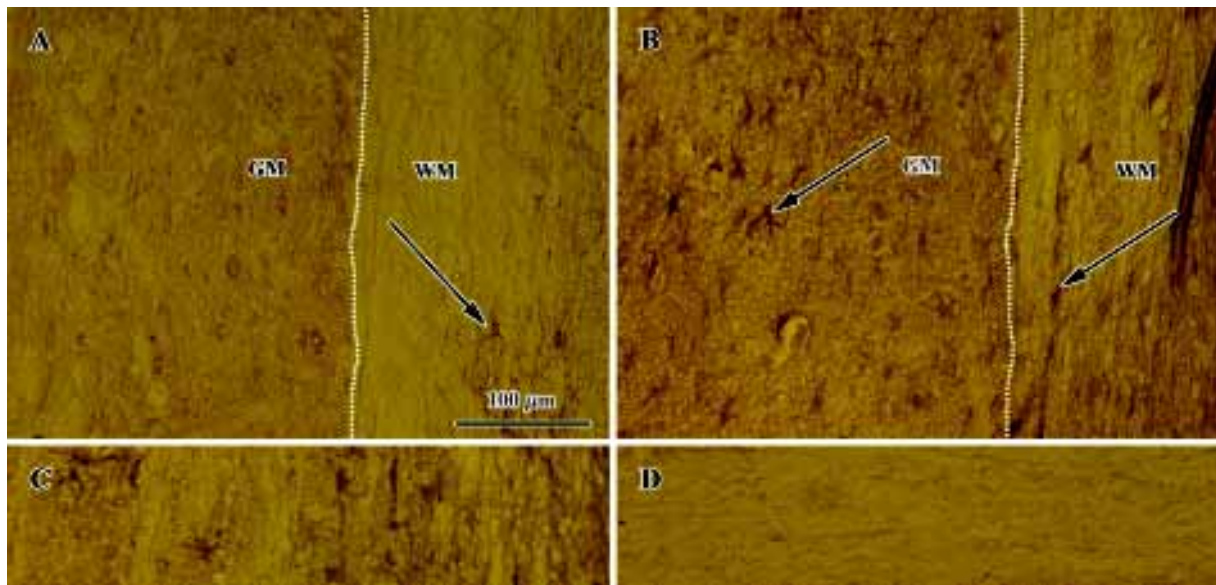


Fig 3. The immunohistochemical results revealed that there was a baseline S100A4 protein expression in sham-operated control rats (A). Spinal cord injury substantially increased S100A4 expression which were seen mainly in glial cells close to the site of injury (7 d post-injury; B). Immunostaining of rat spinal cord with the GST-preabsorbed antibody against GST-S100A4 confirmed the specificity of the antibody (C). Additionally, pre-immune serum was also used to confirm the specificity of the antibodies (D). GM, grey matter; WM, white matter.

determine specific cell types that express S100A4, an immunofluorescence double-labeling experiment was performed. Colocalization of S100A4 was found mainly in astrocytes (GFAP-IR, Fig 4A-4C). Colocalization of S100A4 protein was found neither in oligodendrocytes (Rip-IR; Fig 4D-4F) nor in neuron (β -tublin III-IR; Fig 4G-4I).

DISCUSSION

In the present study, we demonstrated that S100A4 expressed at a very low level in the sham-operated control spinal cord. Such an expression was significantly increased in the spinal cord after injury. Interestingly, S100A4 protein was localized specifically

in astrocytes close to the injury site and was not localized in oligodendrocytes nor in neurons. To our knowledge, the observation that SCI induced increased expression of S100A4 and that its expression was localized in astrocytes has not been reported previously.

It has been reported that the expression of S100A4 was up-regulated ipsilaterally after a sciatic nerve or dorsal root injury^[6,7]. Both sciatic nerve and dorsal root injury induced a marked and prolonged up-regulation of S100A4-IR in astrocytes in the region of dorsal funiculus containing the central processes of the injured primary sensory neurons, suggesting that S100A4 played a unique physiological role in the response of astrocytes to degeneration of myelinated axons. Thus, the significant up-regulation of S100A4 in astrocytes

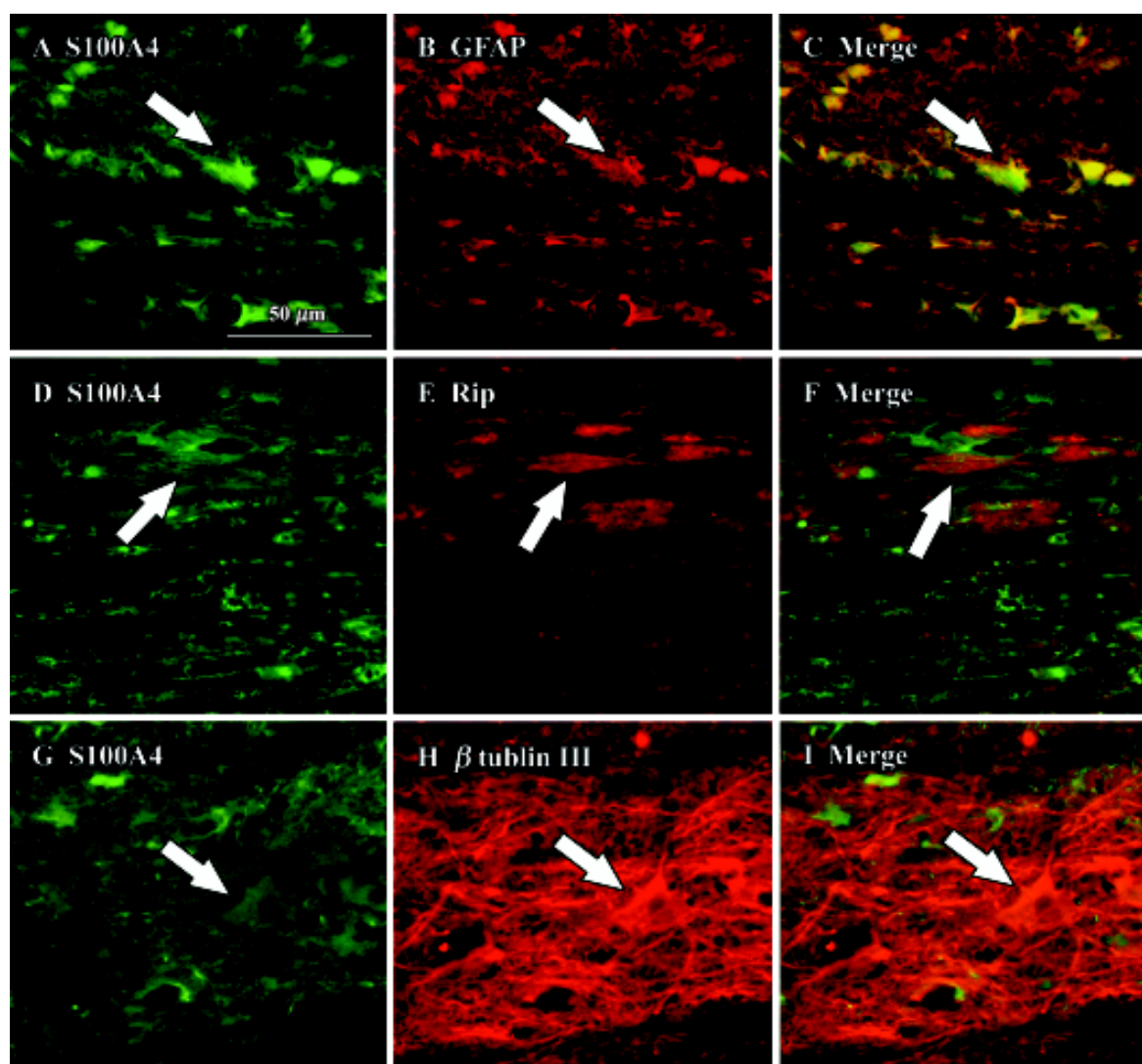


Fig 4. S100A4 expression 14 d post-injury was found mainly in astrocytes (GFAP-IR, A-C) which can be appreciated in the merge of the double exposure. Colocalization of S100A4 protein was found neither in oligodendrocytes (Rip-IR; D-F) nor in neuron (β -tublin III-IR; G-I).

close to the injury site in the present study might play a role in the degeneration or demyelination of nerve fibres after SCI.

S100A4 is a secreted protein released by some types of cells^[12,13] which can function as an efficient neuritogenic factor^[8]. After injury, S100A4 may be released from astrocytes into the lesion site which may in turn promote neurite outgrowth of damaged axons. However, this S100A4-induced neurite outgrowth effect may not be sufficient since there has been no evidence showing that spontaneous regeneration can occur in the spinal cord after injury unless additional repair strategies are applied.

Reactive gliosis, which is characterized by the proliferation of reactive astrocytes and activated microglia, is a common response to the central nervous system injury. A major cause of the failure of axon regeneration after SCI is the inhibitory nature of this glial environment. Due to the fact that S100A4 had the capability of regulating cell cycle progression, modulating intercellular adhesion and invasion, and exhibiting metastatic properties in cancer cells^[2-4], increased expression of S100A4 molecule in reactive astrocytes after SCI may be responsible for astrocyte proliferation and migration to the injury site and, therefore, involved in the formation of glia scar.

Taken together, spinal cord transection induces S100A4 expression at both mRNA and protein levels in areas close to the injury site. This expression is mainly localized in reactive astrocytes. Increased expression of S100A4 after SCI may be involved in neuronal and astrocytic responses to the injury.

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