



Analysis of gene expression following spinal cord injury in rat using complementary DNA microarray

Toshiya Tachibana^{a,b,*}, Koichi Noguchi^b, M.A. Ruda^a

^aCellular Neuroscience Section, Pain and Neurosensory Mechanisms Branch NIDCR, NIH Bethesda, MD 20892, USA

^bDepartment of Anatomy and Neuroscience, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan

Received 6 March 2002; received in revised form 3 April 2002; accepted 9 April 2002

Abstract

To identify genes that were altered by spinal cord injury (SCI), we used complementary DNA microarray consisting 1176 rat genes. Rats were subjected to contusive injury of the thoracic spinal cord. Sham animals received only laminectomy. Twenty-four hours later, spinal cord was dissected out, a ³²P labeled probe was prepared and hybridized to the microarray. We identified three genes that showed a greater than 2-fold increase in SCI tissue, heat shock 27-kDa protein, tissue inhibitor of metalloproteinase-1 and epidermal fatty acid-binding protein. Seven genes, lecithin:cholesterol acyltransferase, dipeptidyl aminopeptidase related protein, phospholipase C delta 4, plasma membrane Ca²⁺-ATPase isoform 2, G-protein G(O) alpha subunit, GABA transporter 3, and neuroendocrine protein 7B2 were down-regulated greater than 50% in SCI tissue. Changes in expression of these genes were confirmed by reverse transcription-polymerase chain reaction. These genes may play a role in the response to tissue damage or repair following SCI. © 2002 Published by Elsevier Science Ireland Ltd.

Keywords: Microarray; Spinal cord Injury; Reverse transcription-polymerase chain reaction; Heat shock protein; Tissue inhibitor of metalloproteinase; Fatty acid-binding protein; Lecithin:cholesterol acyltransferase; Plasma membrane Ca²⁺-ATPase

The response to spinal cord injury (SCI) is known to occur in two phases. The first phase, the primary injury, is the mechanical trauma initially sustained. The second phase, termed secondary injury, is posttraumatic tissue damage. The neurological deficits related to secondary injury occur within minutes of the injury and can continue for days, weeks or longer [2]. This autodestructive process includes physiological, biological and metabolic changes that result in axonal damage and cell loss. In addition, it has become increasingly recognized that the nervous system also initiates reactive process in response to trauma that are neuroprotective and regenerative [1,14]. All these responses to SCI are, in part, reflected in changes in gene expression. New technologies to identify the nervous system's response at the molecular level have increased in the last decade. Microarray analysis is a powerful new tool to quickly examine expression of thousands of genes [2,10]. In the present study, we used complementary DNA (cDNA) microarray technology to identify genes that were altered after SCI. The changes in gene expression identified on microarrays

after SCI were confirmed using reverse transcription-polymerase chain reaction (RT-PCR).

Male Sprague–Dawley rats (7 weeks of age) were subjected to contusive injury of the thoracic spinal cord using the NYU impactor following anesthesia with sodium pentobarbital (50 mg/kg i.p.) [4]. After a laminectomy at the tenth thoracic vertebra to expose the T12 spinal segment, a 10 g weight was dropped 2.5 cm onto the dura mater ($n = 5$ for microarray; $n = 3$ for RT-PCR). Sham animals received only the laminectomy ($n = 5$ for microarray; $n = 3$ for RT-PCR). Twenty-four hours later, the animals were anesthetized and 15 mm of spinal cord surrounding the epicenter of injury or the same area in the sham animals was dissected out and homogenized. The experimental protocol was approved by the Animal Care and Use Committee at NIDCR, NIH and the International Association for the Study of Pain ethical guidelines were adhered to in these experiments [20]. Samples from the injured or sham animals were pooled for cDNA microarray. ³²P labeled probes were prepared using the Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA). Poly(A)⁺ RNA was isolated from total RNA (50 μg) and treated with DNase I. Poly(A)⁺ RNA was transcribed by MMLV reverse tran-

* Corresponding author. Tel.: +81-798-45-6416; fax: +81-798-45-6417.

E-mail address: tachi@hyo-med.ac.jp (T. Tachibana).

scriptase in the presence of [α - 32 P]dATP and specific primers of the genes represented on the Atlas Rat 1.2 Array, a cDNA microarray containing 1176 known genes (Clontech). The radiolabelled cDNA probes were hybridized to the microarray membranes at 68°C overnight. All probes were 1×10^6 cpm. After a series of washes, the membranes were analyzed by autoradiography and quantified using a PhosphorImager system and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Gene expression from the SCI tissue was compared to that from sham animals. Expression levels of individual genes were represented in arbitrary units after subtracting the background and normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A greater than 2-fold increase or a greater than 50% decrease in expression were used as criteria for a meaningful change in gene expression between the SCI and sham groups. RT-PCR was performed with total RNA from individual animals in the SCI and sham groups and was distinct from that used for the microarray analysis. cDNA was synthesized with oligo (dT) primer and MMLV reverse transcriptase (Advantage RT-for-PCR kit, Clontech)

and amplified by PCR during 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min (Taq PCR Master Mix Kit, Qiagen, Valencia, CA). PCR products were analyzed by electrophoresis in 2% agarose gels. Images of ethidium-bromide-stained bands were obtained with a CCD camera system (Atto, Tokyo, Japan) and quantified using ImageQuant software. The messenger RNA (mRNA) level of each sample for each gene was normalized to GAPDH. The specific primers used for amplification are listed on Table 1 and were designed by Clontech. Data are expressed as means \pm SD. The RT-PCR data were analyzed using the Student's *t*-test.

Several differentially expressed genes were observed on the microarray between control spinal cord (Fig. 1A) and injured spinal cord (Fig. 1B). For example, in the SCI array, the expression of heat shock 27-kDa protein (HSP27) was increased (compare Figs. 1C,D, arrow) while the expression of lecithin:cholesterol acyltransferase (LCAT) (compare Figs. 1E,F, arrowhead) was decreased. Alterations in gene expression in injured spinal cord compared to control spinal cord could be differentiated in ten genes by scatter plot

Table 1
Specific primers used for amplification^a

| Gene | Microarray SCI/control | RT-PCR | | GenBank # | Primers |
|--|------------------------|---------|-------|-----------|--|
| | | Control | SCI | | |
| Heat shock 27-kDa protein (HSP27) | 4.22 | 0.90 | 1.91* | M86389 | 5'-ACGTC AACCACTTCGCTCCTGAGG-3' 5'-CTTGGCTCCAGACTGTTCCGACTC-3' |
| Tissue inhibitor of metalloproteinase-1 (TIMP-1) | 2.81 | 0.23 | 0.94* | L31883 | 5'-CTGCAACTCGGACCTGGTTATAAGG-3' 5'-AACGGCCCGCGATGAGAACTCC-3' |
| Epidermal fatty acid-binding protein (E-FABP) | 2.08 | 0.48 | 0.78* | U13253 | 5'-GGCCAAACCAGACTGCATCATTACC-3' 5'-TCTCATAGACCCGAGTACAGATGGC-3' |
| Lecithin:cholesterol acyltransferase (LCAT) | 0.34 | 0.34 | 0.12* | U62803 | 5'-CAGAAGCTGGCAGGACTGGTAGAG-3' 5'-TGATCGGGATGCCCTGGTTGTAC-3' |
| Dipeptidyl aminopeptidase related protein (DPP6) | 0.44 | 0.27 | 0.09* | M76426 | 5'-CCGTGTTCTCCAAGGATGGCCGG-3' 5'-GCGCTGTAGAGGTGTCGTCTTCGT-3' |
| Phospholipase C delta 4 (PLCD4) | 0.45 | 0.13 | 0.06* | D50455 | 5'-GTCCAAGGCCAAGAACCTCATCAGG-3' 5'-CTAACGGGCTTCATGGGATTGAAGG-3' |
| Plasma membrane Ca ²⁺ -ATPase isoform 2 (PMCA2) | 0.47 | 0.63 | 0.42* | J03754 | 5'-ATCCAGACACAGATCCGCGTCGTG-3' 5'-GATTTGCTCGTGTGCGTCGTCAGG-3' |
| G-protein G(O) alpha subunit (GNAO) | 0.47 | 0.24 | 0.18* | M17526 | 5'-AAACAACGGCATCGTAGAAACCCAC-3' 5'-CAGATGGAGTCAAGAGCATGAGAG-3' |
| GABA transporter 3 (GAT3) | 0.48 | 0.71 | 0.31* | M95738 | 5'-CTGGGATCTGTGCGGGCATCTTC-3' 5'-GGTACCGTCGCCTTTGACCTTGG-3' |
| Neuroendocrine protein 7B2 (7B2) | 0.48 | 0.62 | 0.45* | M63901 | 5'-AGCTCACGAGGGTCTTCAGCATCTG-3' 5'-CCACTTGCCCAACCTGGGTAGTC-3' |
| GAPDH | | | | | 5'-TGCAACGGATTTGGCCGTATTGGC-3' 5'-GAAGACGCCAGTAGACTCCACGAC-3' |

^a **P* < 0.05.

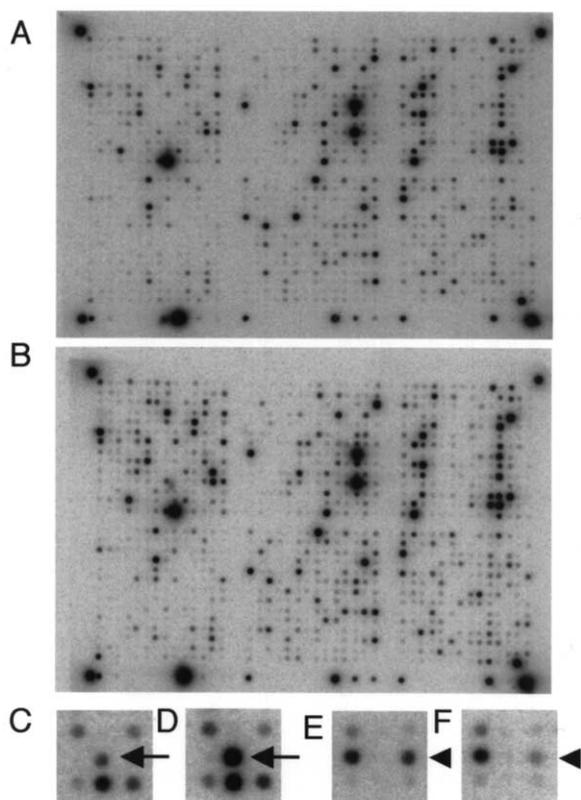


Fig. 1. PhosphorImager photographs of microarray membranes containing 1176 rat genes hybridized with a ^{32}P labeled probe made from control spinal cord mRNA (A); or injured spinal cord mRNA (B). Enlargements of corresponding areas of the microarrays comparing control (C and E); and SCI (D and F). The expression of HSP27 was increased (compare spot at arrow in C and D) while the expression of LCAT (compare spot at arrowhead in E and F) was decreased in SCI tissue.

analysis (Fig. 2). The greater than 2-fold up-regulated genes were HSP27, tissue inhibitor of metalloproteinase-1 (TIMP-1) and epidermal fatty acid-binding protein (E-FABP). The seven down-regulated genes were LCAT, dipeptidyl aminopeptidase related protein (DPP6), phospholipase C delta 4 (PLCD4), plasma membrane Ca^{2+} -ATPase isoform 2 (PMCA2), guanine nucleotide-binding protein G(O) alpha subunit (GNAO), gamma-aminobutyric acid transporter 3 (GAT3) and neuroendocrine protein 7B2 (7B2). RT-PCR was performed with total RNA from individual SCI and sham surgery animals. RT-PCR indicated that the expression of HSP27, TIMP-1 and E-FABP were up-regulated and that of LCAT, DPP6, PLCD4, PMCA2, GNAO, GAT3 and 7B2 were down-regulated in injured spinal cord (Fig. 3). Quantification of gene expression identified that all changes had a statistical significance ($P < 0.05$). These observations on RT-PCR analysis confirm the changes in gene expression found with the microarray. These data are summarized in Table 1.

Three genes, HSP27, TIMP-1, and E-FABP exhibited differential induction after SCI. HSP27 is a low molecular weight heat shock protein and is considered to be a mole-

cular chaperone [17]. In other central nervous system injury models HSP27 was increased in glial cells where it is thought to play a role in protection from cell death [8]. HSP27 may have a similar role following SCI. TIMP-1 is an inhibitor of matrix metalloproteinases (MMPs). The induction of TIMP-1 has previously been observed in activated astrocytes circumscribing the lesions in an animal model of experimental allergic encephalomyelitis (EAE) [16]. TIMP-1 likely acts to confine the extent of tissue destruction by MMPs in the EAE model. Our data suggests that TIMP-1 may have a similar role following SCI or may be involved in shaping reconstruction. E-FABP is a fatty acid binding protein that binds hydrophobic ligands such as long chain free acids [5]. Fatty acid binding proteins are involved in the intracellular transport of free fatty acid molecules by facilitating their movement within the cell nucleus and cytoplasm. Release of free fatty acids (FFAs) is increased in SCI where it contributes to edema and inflammation related to secondary tissue damage [6]. An increase in E-FABP may be a protective response to the increase in FFA after SCI. This also suggests that cells in the injured spinal cord may mobilize FFAs to synthesize new membranes.

The present study also demonstrated differential reduction of gene expression after SCI. LACT is an enzyme made in the liver, brain and testes that catalyzes the esterification of cholesterol in high density lipoprotein, thereby playing a role in removal of excess cholesterol from the tissue [19]. In

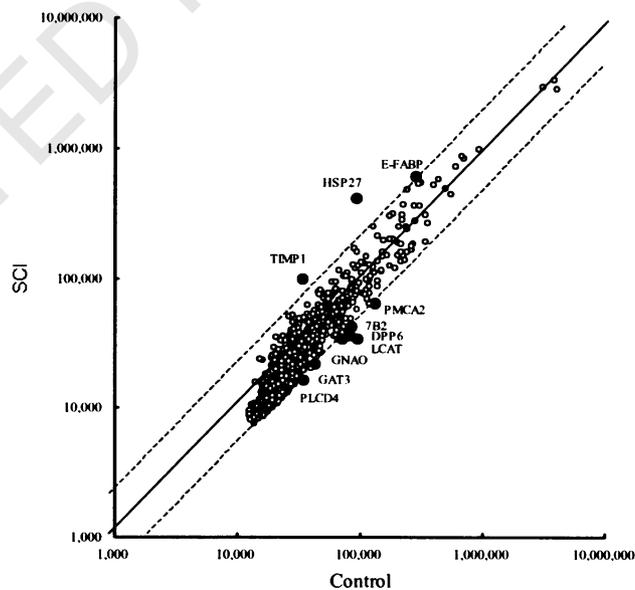


Fig. 2. Scatter plot analysis representing gene expression of the microarray analysis in arbitrary units. The position of ten genes whose expression was different in injured spinal cord and control spinal cord are marked by filled circles. Three genes, HSP27, TIMP-1 and E-FABP showed a greater than 2-fold increase in SCI tissue. Seven genes, LCAT, DPP6, PLCD4, PMCA2, GNAO, GAT3 and 7B2 were down-regulated greater than 50% in SCI tissue. The broken lines indicate 2-fold expression differences between control and SCI.

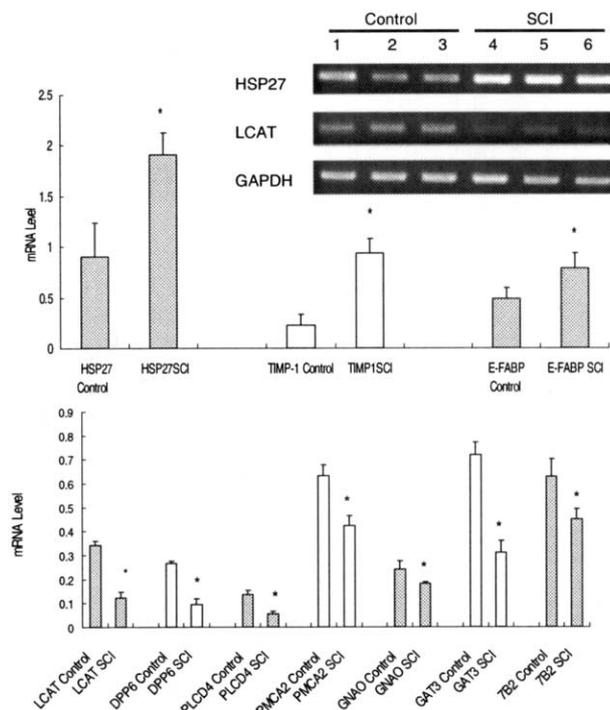


Fig. 3. RT-PCR was performed with specific primers for the ten genes whose gene expression had a greater than 2-fold expression difference between control and SCI on the microarrays. (A) Photographs represent examples of ethidium-bromide-stained bands of HSP27, LCAT and GAPDH in control (lane 1–3, $n = 3$) and SCI (lane 4–6, $n = 3$). (B) Quantification of the levels of HSP27, TIMP1 and E-FABP mRNA identified increases while that of LCAT, DPP6, PLCD4, PMCA2, GNAO, GAT3 and 7B2 mRNA were decreased significantly in injured spinal cord as compared to control ($*P < 0.05$). The mRNA levels were normalized to GAPDH and expressed as means \pm SD. These changes in gene expression were consistent with that observed on the microarrays.

the brain, LCAT has been localized to glial cells [3]. Cholesterol is a major component of plasma membranes and myelin. Down regulation of LACT may be a response to an increase in mobilization of cholesterol to synthesize new membranes and myelin or a decreased presence of cholesterol in the damaged tissue. PMCA2 is one of four isoforms of calcium pumps that is found in the nervous system [7]. Calcium pumps regulate intracellular Ca^{2+} levels by Ca^{2+} extrusion from cells. It is known that intracellular Ca^{2+} levels are increased after SCI and a significant increase in Ca^{2+} may be cytotoxic [6]. A decrease in PMCA2 expression may contribute to intracellular Ca^{2+} accumulation after SCI. Some down-regulated genes, 7B2, DPP6 and GAT3 involve in neuropeptides and neurotransmitters. 7B2 designates an acidic protein which is stored within and released from dense core secretory granules of neuronal and endocrine cells [12]. 7B2 is regarded as a peptide/hormone-associated secretory protein. 7B2 is widely present in spinal neurons, where it may be involved in the secretory mechanisms of neuropeptides [13]. DPP6 is a peptidase that regulates the biological activity of neuro-

peptides by converting precursors to active forms or active forms to inactive forms [18]. GAT3 is one of four isoforms of GABA transporters and involved in the termination of GABA transmission by rapid uptake of GABA [15]. These suggests that some neurotransmitter systems may lose their functions following SCI. PLCD4 and GNAO which have a role in the intracellular second messenger system were also down-regulated following SCI. PLCD4 is a δ -type phospholipase C that plays an important role in receptor-mediated signal function by generating two second messenger molecules, inositol 1,4,5-trisphosphate and diacylglycerol, from phosphatidylinositol 4,5-bisphosphate [11]. GNAO is an α subtype of the guanine nucleotide binding proteins (G-protein) [9]. G-proteins are a family of proteins that couple extracellularly activated membrane receptors to intracellular second messenger enzymes. The down regulation of those genes suggests that the some intracellular signaling system may be impaired selectively in injured spinal cord.

We thank Mrs E.H. Franklin for her excellent technical assistance. This work was supported by the Intramural Research Program, NIDCR, NIH and Hyogo College of Medicine.

- [1] Beattie, M.S., Bresnahan, J.M., Komon, J., Tovar, C.A., Van Meter, M., Anderson, D.K., Faden, A.I., Hsu, C.Y., Noble, L.J., Salzman, S. and Young, W., Endogenous repair after spinal cord contusion injuries in the rat, *Exp. Neurol.*, 148 (1997) 453–463.
- [2] Bowtell, D.D.L., Options available-from start to finish-for obtaining expression data by microarray, *Nat. Genet.*, 21 (1999) 25–32.
- [3] Collet, X., Francone, O., Besnard, F. and Fielding, C.J., Secretion of lecithin:cholesterol acyltransferase by brain neuroglial cell lines, *Biochem. Biophys. Res. Commun.*, 258 (1999) 73–76.
- [4] Constantini, S. and Young, W., The effects of methylprednisolone and the ganglioside GM1 on acute spinal cord injury in rats, *J. Neurosurg.*, 80 (1994) 97–111.
- [5] De León, M., Welcher, A.A., Nahin, R.H., Liu, Y., Ruda, M.A., Shooter, E.M. and Molina, C.A., Fatty acid binding protein is induced in neurons of the dorsal root ganglia after peripheral nerve injury, *J. Neurosci. Res.*, 44 (1996) 283–292.
- [6] Faden, A.I., Experimental neurobiology of central nervous system trauma, *Crit. Rev. Neurobiol.*, 7 (1993) 175–186.
- [7] Guerini, D., The significance of the isoforms of plasma membrane calcium ATPase, *Cell Tissue Res.*, 292 (1998) 191–197.
- [8] Imura, T., Shimohama, S., Sato, M., Nishikawa, H., Madono, K., Akaike, A. and Kimura, J., Differential expression of small heat shock proteins in reactive astrocytes after focal ischemia: possible role of β -adrenergic receptor, *J. Neurosci.*, 19 (1999) 9768–9779.
- [9] Jones, T.D. and Reed, R.R., Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium, *J. Biol. Chem.*, 262 (1987) 14241–14249.
- [10] Kelly, D.L. and Rizzino, A., DNA microarray analyzes of genes regulated during the differentiation of embryonic stem cells, *Mol. Reprod. Dev.*, 56 (2000) 113–123.
- [11] Liu, N., Fukami, K., Yu, H. and Takenawa, T., A new phospholipase C $\delta 4$ is induced at s-phase of cell cycle and appears in nucleus, *J. Biol. Chem.*, 271 (1996) 355–360.

- 449 [12] Marcinkiewicz, M., Benjannet, S., Seidah, N.G., Cantin, M.
450 and Chr stien, M., The pituitary polypeptide '7B2' is asso-
451 ciated with LH/FSH and TSH cells and localized within
452 secretory vesicles, *Cell Tissue Res.*, 250 (1987) 205–214.
- 453 [13] Marcinkiewicz, M., Touraine, P. and Chr stien, M., Pan-
454 neuronal mRNA expression of the secretory polypeptide
455 7B2, *Neurosci. Lett.*, 177 (1994) 91–94.
- 456 [14] Namiki, J. and Tator, C.H., Cell proliferation and nestin
457 expression in the ependyma of the adult rat spinal cord
458 after injury, *J. Neuropathol. Exp. Neurol.*, 58 (1999) 489–
459 498.
- 460 [15] Nelson, N., The family of Na⁺/Cl⁻ neurotransmitter trans-
461 porters, *J. Neurochem.*, 71 (1998) 1785–1803.
- 462 [16] Pagenstecher, A., Stalder, A.K., Kincaid, C.L., Shapiro, S.D.
463 and Campbell, I.L., Differential expression of matrix metal-
464 loproteinase and tissue inhibitor of matrix metalloprotei-
465 nase genes in the mouse central nervous system in
466 normal and inflammatory states, *Am. J. Pathol.*, 152
467 (1998) 729–741.
- 468 [17] Sharp, F.R., Massa, S.M. and Swanson, R.A., Heat-shock
469 protein protection, *Trends Neurosci.*, 22 (1999) 97–99.
- 470 [18] Wada, K., Yokotani, N., Hunter, C., Doi, K., Wenthold, R.J.
471 and Shimasaki, S., Differential expression of two distinct
472 forms of mRNA encoding members of a dipeptidyl amino-
473 peptidase family, *Proc. Natl. Acad. Sci.*, 89 (1992) 197–201.
- 474 [19] Warden, C.H., Langner, C.A., Gordon, J.I., Taylor, B.A.,
475 McLean, J.W. and Lusic, A.J., Tissue-specific expression,
476 developmental regulation, and chromosomal mapping of
477 the lecithin:cholesterol acyltransferase gene, *J. Biol.*
478 *Chem.*, 36 (1989) 21573–21581.
- 479 [20] Zimmermann, M., Ethical guidelines for investigations of
480 experimental pain in conscious animals, *Pain*, 16 (1983)
481 109–110.
- 482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504

UNCORRECTED PROOF