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# Exploration of Spinal Cord Aging–Related Proteins Using a Proteomics Approach

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Koshiro Kamiya<sup>1</sup>, Takeo Furuya<sup>1</sup>, Masayuki Hashimoto<sup>1</sup>,  
Chikato Mannoji<sup>2</sup>, Taigo Inada<sup>1</sup>, Mitsutoshi Ota<sup>1</sup>, Satoshi Maki<sup>1</sup>,  
Yasushi Ijima<sup>1</sup>, Junya Saito<sup>1</sup>, Mitsuhiro Kitamura<sup>1</sup>, Seiji Ohtori<sup>1</sup>,  
Sumihisa Orita<sup>1</sup>, Kazuhide Inage<sup>1</sup>, Masashi Yamazaki<sup>3</sup> and Masao Koda<sup>1</sup>

<sup>1</sup>Department of Orthopedic Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan. <sup>2</sup>Department of Orthopedic Surgery, Teikyo University Chiba Medical Center, Ichihara, Japan. <sup>3</sup>Department of Orthopedic Surgery, University of Tsukuba, Tsukuba, Japan.

**ABSTRACT:** How aging affects the spinal cord at a molecular level is unclear. The aim of this study was to explore spinal cord aging–related proteins that may be involved in pathological mechanisms of age-related changes in the spinal cord. Spinal cords of 2-year-old and 8-week-old female Sprague-Dawley rats were dissected from the animals. Protein samples were subjected to 2-dimensional polyacrylamide gel electrophoresis followed by mass spectrometry. Screened proteins were further investigated with immunohistochemistry and Western blotting. Among the screened proteins, we selected  $\alpha$ -crystallin B-subunit ( $\alpha$ B-crystallin) and peripherin for further investigation because these proteins were previously reported to be related to central nervous system pathologies. Immunohistochemistry and Western blotting revealed significant upregulation of  $\alpha$ B-crystallin and peripherin expression in aged rat spinal cord. Further exploration is needed to elucidate the precise mechanism and potential role of these upregulated proteins in spinal cord aging processes.

**KEYWORDS:** Aging, proteomics, spinal cord,  $\alpha$ B-crystallin, peripherin

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**CORRESPONDING AUTHOR:** Masao Koda, Department of Orthopedic Surgery, Graduate School of Medicine, Chiba University, 1-8-1, Inohana, Chuo-Ku, Chiba 260-8670, Japan. Email: masaokod@gmail.com

## Introduction

As the population of older individuals grows in developed countries, there is increasing need for surgical treatment of age-related compressive spinal cord lesions and spinal cord injuries. However, the way that aging affects the spinal cord at a molecular level is unclear, as evidenced by several contradictory reports describing the influence of aging on spinal cord lesions. For example, several studies reported that there was no significant difference between the neurological recovery of older and younger patients after surgical decompression for cervical myelopathy.<sup>1,2</sup> In contrast, other reports described a worse outcome after decompression surgery for cervical myelopathy in elderly patients.<sup>3</sup> Moreover, neurological outcomes of spinal cord injury (SCI) in aged patients are reported to be worse than those of younger patients.<sup>4</sup> Thus, to elucidate the influences of aging on the spinal cord, an in-depth understanding of the pathological mechanisms of spinal cord aging is essential.

The aim of this study was to explore spinal cord aging–related proteins that may be involved in pathological mechanisms of age-related changes in the spinal cord. Previous report showed the results of proteomic analyses of aging-related changes in the brain of animals and humans.<sup>5</sup> For the first time, we show the results of proteomic comparison between aged and young rat spinal cord.

## Materials and Methods

### Animals

All animals were treated and cared for in accordance with the Chiba University School of Medicine guidelines that pertain to the treatment of experimental animals. This study was approved by the Animal Care and Use Committee of Chiba University Graduate School of Medicine (Approval Number 27-91).

Two-year old and 8-week-old female Sprague-Dawley (SD) rats (Japan SLC, Inc. Hamamatsu, Japan) were used as models of aged and young animals, respectively. Animals were euthanized with pentobarbital overdose.

### Sample preparation

Thoracic spinal cords (1 cm length) were dissected from the animals and after snap-freezing in liquid nitrogen were preserved at  $-80^{\circ}\text{C}$  until use. Samples were thoroughly ground and dissolved with Dissolve buffer (8 M urea, 4% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate [CHAPS], 60 mM dithiothreitol [DTT], 10 mM Tris-HCl [pH 7.4]). Dissolved samples were cleaned with a 2-dimensional Clean Up kit (Bio-Rad, Hercules, CA), redissolved in sample buffer (7 M urea, 2% CHAPS, 20 mM DTT, 2 mM Tris-(2-cyanoethyl) phosphine, 0.2% BioLyte 3-10 [Bio-Rad]), and



the protein concentration was measured by the Bradford method.

### *Two-dimensional polyacrylamide gel electrophoresis*

Protein samples (30  $\mu$ g) from each rat were applied to an immobilized pH gradient (IPG) gel (IPG ReadyStrip 7 cm, pH 3–10; Bio-Rad) for isoelectric focusing. Next, equilibration was performed with equilibration buffer (50 mM Tris-HCl [pH 8.5], 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate [SDS], 1% DTT, 0.005% bromophenol blue) for 15 minutes and then re-equilibrated with a second equilibration buffer (50 mM Tris-HCl [pH 8.5], 6 M urea, 30% glycerol, 2% SDS, 4.5% iodoacetamide, 0.005% bromophenol blue) for 15 minutes. After equilibration, IPG gels were subjected to 12.5% SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions.

Protein spots were stained with SYPRO Ruby (Thermo Fisher Scientific, Waltham, MA) and the gel images were captured with Molecular Imager FX (Bio-Rad).

### *Mass spectrometry*

Protein spots showing a 2-fold or greater difference between aged and young rats were isolated and identified using mass spectrometry (MS). Identified spots on the gels were automatically detected by Melanie 4.02 software, excised with a Proteineer spII spot picker (Bruker Daltonics, Bremen, Germany), and dried in a speed vacuum concentrator (MaxiDry Plus, Heto, Allered, Denmark). Each dried gel piece was rehydrated with 5  $\mu$ L of 1 mM ammonium bicarbonate containing 50 ng trypsin (Roche Diagnostics, Basel, Switzerland) and incubated in the dark overnight at room temperature. Then, 20  $\mu$ L of 50% acetonitrile containing 0.3% trifluoroacetic acid was added to each gel and incubated for 15 minutes with constant shaking. The peptide mixture (1.5  $\mu$ L) was simultaneously applied to 1  $\mu$ L of matrix solution consisting of 0.025%  $\alpha$ -cyano-4-hydroxycinnamic acid (Merck Millipore, Billerica, MA), standard peptides des-Arg-bradykinin (Merck Millipore, 904.4681 Da), and adrenocorticotrophic hormone fragment 18 to 39 (Merck Millipore, 2465.1989 Da) in 65% ethanol, 35% acetonitrile, and 0.03% trifluoroacetic acid. Samples were analyzed for peptide mass fingerprinting with matrix-assisted laser desorption/ionization (MALDI)-MS in a time-of-flight mass spectrometer (Ultraflex II; Bruker Daltonics). Matching peptide and protein searches were performed automatically. Each spectrum was interpreted using Mascot Software (Matrix Sciences Ltd., London, UK). For peptide identification, monoisotopic masses were used and a mass tolerance of 0.0025% (25 ppm) was allowed. Unmatched peptides or peptides with up to 1 missed cleavage site were excluded from further consideration. The peptide masses were compared with the theoretical peptide masses of all available proteins from all species using SWISS-PROT, international protein index (IPI), and Mass Spectrometry Sequence Database (MSDB) databases.

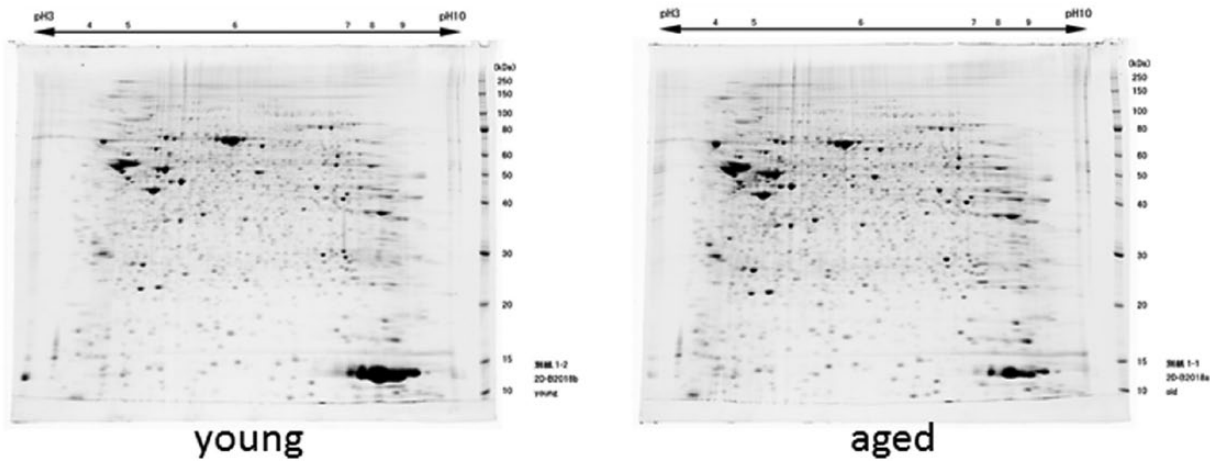
### *Western blot analysis*

Frozen spinal cord samples were homogenized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100 (homogenization buffer) containing a protease inhibitor cocktail (cOmplete; Roche Diagnostics). The homogenates were centrifuged at 10 000g for 10 minutes at 4°C to remove cellular debris. Protein concentrations of the supernatants were measured using the Bradford method and adjusted to 1 mg/mL by dilution with homogenization buffer. Protein samples were mixed with an equal volume of concentrated (2 $\times$ ) sample buffer: 250 mM Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 10%  $\beta$ -mercaptoethanol. After boiling for 5 minutes, equal volumes of samples were subjected to 10% SDS-PAGE under reducing conditions, and the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Merck Millipore). After blocking, the membrane was reacted with an anti- $\alpha$ B-crystallin (1: 1000; Merck Millipore), anti-peripherin (1: 1000; Novus Biologicals, Littleton, CO), and an anti- $\beta$ -actin antibody as a loading control (1: 1000; Santa Cruz Biotechnology, Santa Cruz, CA). For detection, a horseradish peroxidase-conjugated secondary antibody (1: 2500; Cell Signaling Technology, Danvers, MA) and an ECL chemiluminescence system (GE Healthcare, Piscataway, NJ) were used. Western blot analysis was performed in triplicate for each sample. Protein bands were quantified using ImageJ (National Institutes of Health) software and digital quantification was performed, and densitometry results calculated as mean expression level in old rats were expressed as the ratio compared with that in young rats.

### *Immunohistochemistry*

Tissues from a subset of rats ( $n = 4$ /group) were prepared for histological evaluation. Animals were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). Tissue blocks of the thoracic spinal cord were removed, post-fixed overnight in 4% paraformaldehyde, and stored at 4°C in 20% sucrose in PBS before embedding in optimum cutting temperature compound (Sakura Finetechnical, Tokyo, Japan). The cryoprotected samples were frozen and were cut into serial 20  $\mu$ m transverse sections.

For immunofluorescent labeling, sections were permeated with 0.3% Triton X in PBS and treated for 1 hour in blocking solution containing 1% bovine serum albumin. Sections were then incubated with the following primary antibodies: rabbit polyclonal anti- $\alpha$ B-crystallin antibody (1:400), mouse monoclonal peripherin antibody (1: 400), and anti-neurofilament 200 rabbit polyclonal antibody (NF200, 1: 400; Merck Millipore) for nerve fibers and anti-adenomatous polyposis coli (APC) mouse monoclonal antibody (1: 400; Abcam plc, Cambridge, UK) for oligodendrocytes. The sections were incubated with primary antibodies overnight at 4°C and then washed in PBS before incubation for 1 hour at room temperature with the secondary antibodies: Alexa 488- or Alexa



**Figure 1.** Result of 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Protein samples obtained from young (left panel) and aged (right panel) rat spinal cords were subjected to 2D-PAGE. Protein spots showing upregulation in old rats were then subjected to mass spectrometry to detect protein.

594-labeled anti-mouse, anti-rabbit IgG (1:800; Fisher Scientific, Inc.). Finally, the sections were washed twice in PBS and protected with coverslips. Positive labeling was observed using fluorescence microscopy (ECLIPSE E600; Nikon, Tokyo, Japan). To determine the specificity of staining, procedures were performed on control sections with the omission of primary or secondary antibodies.

*Statistical analyses*

Results of Western blot analyses were subjected to Student *t* test to compare the difference between the 2 groups.

**Results**

Through the screening by 2-dimensional PAGE followed by MS, several proteins were detected as upregulated proteins in aged rat spinal cord (Figure 1; Table 1). Among the screened proteins, we selected  $\alpha$ -crystallin B-subunit ( $\alpha$ B-crystallin) and peripherin for further investigation because these proteins were previously reported to be related to central nervous system (CNS) pathologies.

Immunohistochemistry revealed that peripherin expression was detected in posterior horn neurons and nerve fibers in the posterior root (Figure 2A to F) and  $\alpha$ B-crystallin expression was observed in APC-positive oligodendrocytes in spinal cord white mater (Figure 2G to L). The spatial expression pattern of  $\alpha$ B-crystallin and peripherin revealed by immunohistochemistry was similar between aged and young rats, although the degree of expression was weaker in young rat spinal cord compared with that in old rat spinal cord (Figure 2C, F, I, and L). Western blotting revealed significant upregulation of peripherin and  $\alpha$ B-crystallin expression in aged rat spinal cord (Figure 2M and N).

**Discussion**

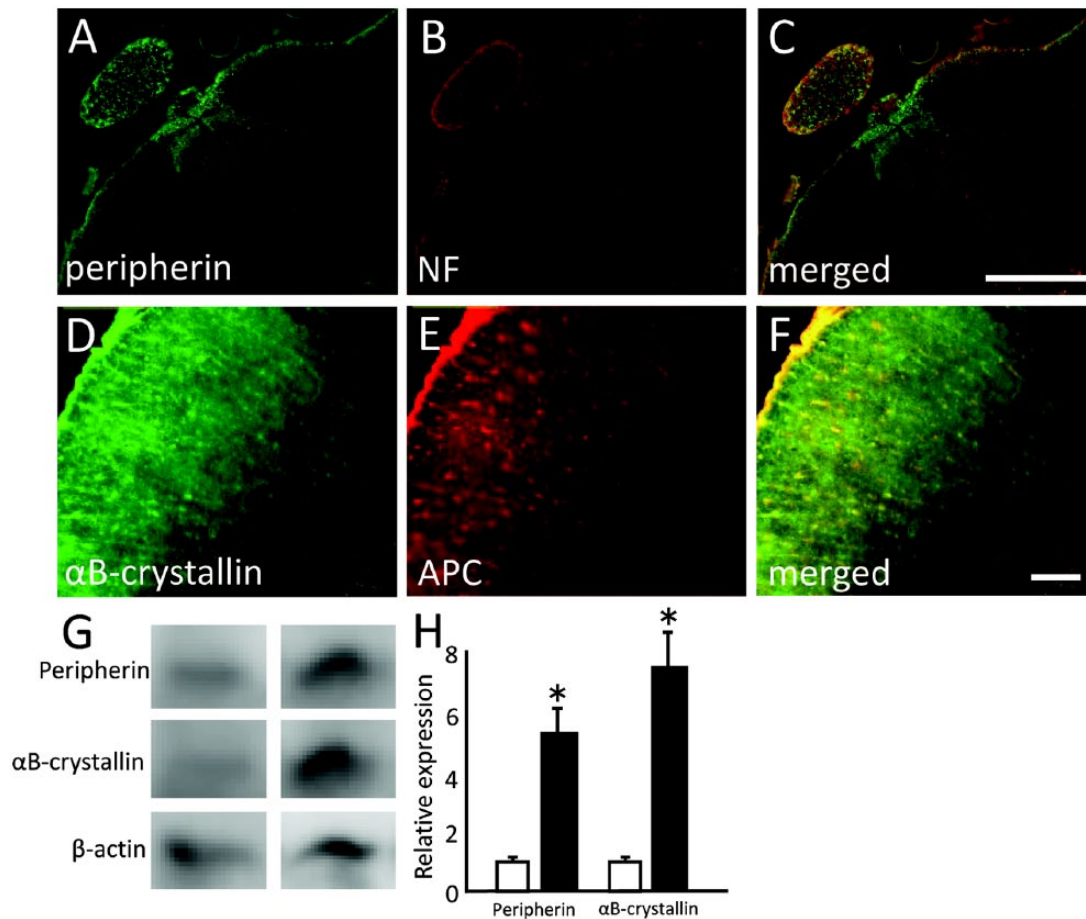
$\alpha$ -crystallin B-subunit is a small heat shock protein that acts as a molecular chaperone. The expression and chaperone activity of  $\alpha$ B-crystallin in the lens of the eye likely contribute to the

**Table 1.** List of detected proteins by mass spectrometry (*P* < .05).

PROTEIN	TOTAL SCORE
<b>Upregulated in old rat spinal cord</b>	
Glial fibrillary acidic protein	450
Glial fibrillary acidic protein	442
Neurofilament medium	439
Peripherin	436
$\alpha$ -crystallin B-subunit	434
<b>Downregulated in old rat spinal cord</b>	
Major beta-hemoglobin	432
Hemoglobin subunit alpha-1/2	392

long-term maintenance of lens clarity.<sup>6</sup> In other tissues,  $\alpha$ B-crystallin exerts molecular chaperone function and has an antiapoptotic function.<sup>7</sup> In terms of CNS pathologies,  $\alpha$ B-crystallin activity has a protective effect during brain aging.<sup>8</sup> Indeed, Klopstein et al<sup>9</sup> reported a beneficial effect of  $\alpha$ B-crystallin for a mouse model of SCI. They also provide evidence that recombinant human  $\alpha$ -crystallin modulates the inflammatory response in the injured spinal cord. Inflammation is known to be related to the aging process in various organs, tissues, and systems, including the CNS<sup>10</sup> where dysregulation of microglia, the resident immune cells in the CNS, is reportedly associated with age-related pathologies. In the same context, upregulation of inflammatory cytokines is known to occur in spinal cord tissue from aged dogs.<sup>11</sup> Moreover, aged rats showed poor recovery from SCI due to increased numbers of activated microglia.<sup>12</sup> Together, these lines of evidence suggest that the pathological changes in the aged spinal cord might restrict neurological recovery. Thus, upregulation of  $\alpha$ B-crystallin expression in aged spinal cord might counteract inflammatory reaction-related aging processes by modulating inflammation.<sup>13</sup>





**Figure 2.** Immunohistochemistry and Western blotting for  $\alpha$ -crystallin B-subunit ( $\alpha$ B-crystallin) and peripherin. Immunohistochemistry in aged rat spinal cord sample revealed that (A-C) peripherin expression was detected in neurofilament (NF)-positive neurons and nerve fibers and (D-F)  $\alpha$ B-crystallin expression was observed mainly in adenomatous polyposis coli (APC)-positive oligodendrocytes in white mater. Western blotting revealed significant upregulation of  $\alpha$ B-crystallin and peripherin expression in aged rat spinal cord (G, H, closed column) compared with that in the young rats (G, H, open column).  $\alpha$ B-crystallin indicates  $\alpha$ -crystallin B-subunit; APC, adenomatous polyposis coli.

Peripherin is a type III intermediate filament protein that is mainly expressed in neurons of the peripheral nervous system.<sup>14</sup> Although peripherin is thought to play a role in neurite elongation during development and axonal regeneration after injury, its exact function is unknown. Peripherin is also associated with some of the major neuropathologies that characterize amyotrophic lateral sclerosis. Aggregates of peripherin have been shown to induce neuronal death<sup>15</sup> and that cells can be rescued from peripherin-induced cell death by overexpression of the neurofilament heavy chain.<sup>16</sup> These lines of evidence suggest that peripherin can potentially act as a toxic agent in the spinal cord and that the peripherin upregulation seen in aged rat spinal cord in this study might reflect aging-related deposition of toxic products.<sup>17</sup>

The precise mechanisms underlying the upregulation of  $\alpha$ B-crystallin and peripherin protein expression in spinal cord of aged animals are still unclear. Although further exploration is needed to elucidate the precise mechanism and potential role of these upregulated proteins in spinal cord aging processes, inflammatory reactions and deposition of toxic products might be factors in inducing aging-related spinal cord tissue damage and vulnerability to injuries caused by external force.

Precise understanding of molecular basis of spinal cord aging is essential for clarification of aging-related spinal cord vulnerability. Although further exploration is needed, this study is the very first step toward explication of spinal cord aging.

### Author Contributions

MY and MK conceived and designed the experiments. KK, TF, MH, CM, TI, MO, and SM conducted data acquisition. YI, JS, and MK analyzed the data. KK wrote the first draft of the manuscript. SeO and SuO contributed to the writing of the manuscript. All the authors agree with manuscript results and conclusions. SeO, SuO, and KI jointly developed the structure and arguments for the paper. MY and MK made critical revisions and approved the final version. All authors reviewed and approved of the final manuscript.

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