Enhanced p62 expression triggers concomitant autophagy and apoptosis in a rat chronic spinal cord compression model

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Abstract. Chronic spinal cord compression is the result of mechanical pressure on the spinal cord, which in contrast to traumatic spinal cord injury, leads to slowly progressing nerve degeneration. These two types of spinal cord injuries may trigger similar mechanisms, including motoric nerve cell apoptosis and autophagy, however, depending on differences in the underlying injury severity, nerve reactions may predominantly involve the conservation of function or the initiation of functions for the removal of irreversibly damaged cells. p62 is a multidomain adapter protein, which is involved in apoptosis and cell survival as well as autophagy, and is a common component of protein aggregations in neurodegenerative diseases. In the present study, a rat chronic spinal cord compression model was used, in which the spinal cord was progressively compressed for six weeks and then constantly compressed for another 10 weeks. As a result Basso, Beattie and Bresnahan locomotor scaling revealed a gradual score decrease until the 6th week followed by constant recovery until the 16th week after spinal cord compression was initiated. During the first eight weeks of the experiment, p62 and nuclear factor- κB (NF- κB) were increasingly expressed up to a constant plateau at 12-16 weeks, whereas caspase 3 exhibited a marginally enhanced expression at 8 weeks, however, reached a constant maximum peak 12-16 weeks after the beginning of spinal cord compression. It was hypothesized that, in the initial phase of spinal cord compression, enhanced p62 expression triggered NF-κB activity, directing the cell responses

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mainly to cell survival and autophagy, whereas following eight weeks of spinal cord compression, caspase 3 was additionally activated indicating cumulative elimination of irreversibly damaged nerve cells with highly activated autophagy.

Introduction

Chronic spinal cord injury, including cervical spondylotic myelopathy (CSM), is the most common spinal cord disorder in elderly individuals and is caused by degenerative alterations in the spine, including degeneration of joints, intervertebral discs, ligaments and connective tissue of the cervical vertebrae with concomitant spinal canal stenosis (1). Canal dimensions were reported to be determinants of symptom production and neurological compromises (2,3). The gray matter in the compressed spine segments becomes atrophic with thin myelinated fibers, while denuded axons occur in the damaged white matter (4). As a result of compression, the affected area of the spinal cord may become ischemic (5,6) or hypoxic (7)and, besides mechanical observations, other studies have focused on chronic spinal cord compression-associated neural apoptosis (8-10), in particular of oligodendrocytes (11,12), which is considered to be the result of compression-associated ischemia. As an alternative to apoptosis, autophagy serves as a repair mechanism for damaged cell organelles as well as for the clearance of encapsulated proteins and has an important physiological cytoprotective function against neurodegenerative diseases (13-17). Following spinal cord injury, the major mechanism triggered is apoptosis (18), which has been verified by Fas death receptor pathway inactivation experiments (19,20) showing reduced apoptosis of neuron cells following spinal cord injuries. Other studies have demonstrated that rapamycin (an mTOR inhibitor) was also able to enhance the survival rate of neuron cells following spinal cord injury via induction of autophagy (21,22). Studies with genetically modified mice demonstrated that protein 62/sequestosome 1 (p62/SQSTM1) is important in a number of cellular functions, including bone remodeling (23), obesity (24) and cancer (25). For neurons, several studies have described p62 as a common component of protein aggregates that were identified in protein aggregation diseases affecting the brain, including Lewy bodies in Parkinson's disease, huntingtin aggregates

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in Huntington's disease and neurofibrillary tangles in Alzheimer's disease (26-28). p62 is a nucleoporin, which binds ubiquitinated proteins via its ubiquitin-associated domain and microtubule-associated protein 1A/1B light chain 3 (LC3) via its LC3-interacting region, thereby acting as an adaptor between the autophagic machinery and misfolded proteins (29). Additionally, p62 binds with polyubiquitinated caspase 8 and triggers programmed cell death with aggregation into nuclear speckles (30). The p62 protein also contains a tumor necrosis factor receptor associated factor 6 (TRAF6)-binding domain and, following binding and subsequent activation, TRAF6 induces the nuclear factor- κB (NF- κB) pathway (31,32). Since activated NF-kB leads to a reduction in apoptosis, the p62 nuclear speckles may be signaling hubs that determine whether cells survive, by triggering the NF-KB pathway, or die, by activating downstream effector caspases in spinal cord injuries (33). The present study investigated the role of p62 in chronic spinal cord injury using a rat model of chronic spinal cord compression.

Materials and methods

Chronic spinal cord injury rat model. A chronic spinal cord compression rat model was employed (34) with slight modifications. A total of 48 female Sprague-Dawley rats, weighing 170-200 g with an average weight of 185 g (Charles River Laboratories, Inc., Wilmington, MA, USA) were divided into two groups: the sham group (no compression; n=6) and the compression group (compression with a screw advanced by 0.3 mm per week; one turn; n=42) as indicated (Figs. 1 and 2). All the rats were intraperitoneally anesthetized with 10 mg/kg xylazine (Rompun; Bayer, Istanbul, Turkey) and 50 mg/kg ketamine hydrochloride (Ketalar; Pfizer, Istanbul, Turkey). Progressive cord compression was achieved at the T10 level by sequential turning of a threaded screw (diameter 2 mm; one turn=0.3 mm). The compression was performed precisely using a microscope at an oblique angle (World Precision Instruments, Inc., Sarasota, FL, USA). The sham-surgery animals underwent identical surgical procedures as above with the exception of cord compression. Acute post-surgical care included rehydration with 5 ml of sterile saline (Baxter, Mississauga, Ontario, Canada), increasing body temperature with a focused heat lamp, warm towels and subcutaneous injection of buprenorphine hydrochloride (0.05 mg/kg) immediately following surgery in order to minimize post-surgical pain. Post-surgical care also included individually housing the animals in a room with ambient temperature and controlled light conditions. All animals were sacrificed at different weeks following surgery. All experimental procedures were performed in compliance with the Guidelines for the Care and Use of Animals described in the Guidelines established by the Institute of Laboratory Animal Sciences, Faculty of Medicine of the Second Military Medical University (Shanghai, China). The present study was approved by the ethics committee of Changhai Hospital (Shanghai, China).

Basso, Beattie and Bresnahan locomotor (BBB) score and assessment. Behavioral functional deficits due to chronic compression were characterized weekly using a variety of neurobehavioral tests, including the BBB locomotor scale rating until 16 weeks post-surgery. Observers blinded to the compression group evaluated all the scores. Locomotor deficits were assessed by two independent observers using the BBB locomotor rating scale (35). The 22-point (0-21) scale included monitoring of hindlimb movements, trunk position and stability, stepping, co-ordination, paw placement, toe clearance and tail position, while the rats were placed individually in an open field with a non-slippery surface. Each rat was observed for a period of 4 min at each testing session and the scores were averaged across the right and left limbs to derive a final motor recovery score for each week of testing. A score of 21 indicated unimpaired locomotion as observed in the control rats.

Tissue preparation. For immunohistochemical analyses, the rat spines were perfused with normal saline under intraperitoneal anesthesia and then fixed with 4% paraformaldehyde (Cell Chip Biotechnology Co., Ltd., Beijing, China). Then, the spinal cords were embedded in paraffin and 4- μ m transverse slides were prepared from areas, which were located 2 mm caudal or cranial of the T10 screw compression site. For western blot analyses, the 10-cm rat cervical spinal cords (n=3 per group) were dissected and homogenized in tissue protein extraction reagent (Pierce Protein Research Products, Rockford, IL, USA).

Histological analysis. The sections prepared from treated and non-treated rats were fixed with 4% paraformaldehyde (Maya-Reagent Co., Ltd., Jiaxing, Zhejiang, China) and stained with hematoxylin and eosin (Beyotime Institute of Biotechnology Co., Ltd., Shanghai, China).

Western blot analyses. For western blot analyses, ~30 μ g protein was loaded onto 8% NuPAGE precast gels (NP0322; Invitrogen Life Technologies, Carlsbad, CA, USA) and transferred onto nitrocellulose membranes. The membranes were inhibited in Tris-buffered saline/Tween 20 buffer pH 7.5, containing 0.15 M NaCl (313-20; Beijing Biotechnology Co., Ltd., Beijing, China) and 0.05% Tween 20 (93773; Sigma-Aldrich, Shanghai, China) containing 3% milk powder for 1 h at room temperature. The membrane was incubated with the primary antibody overnight at 4°C, followed by secondary horseradish peroxidase (HRP)-labeled antibody incubation for 1 h at room temperature. Detection was performed using the ECL detection system (Millipore, Darmstadt, Germany) and protein bands were visualized using an ImageQuant digital imaging system (ChemiQ 4600, Shanghai, China).

Immunohistochemistry. For immunohistochemistry, the sections were treated with xylene for 10 min, alcohol hydration for 15 min and methanol containing 3% hydrogen peroxide for 10 min, and were incubated at a high temperature and pressure for 2 min. The sections were then washed in phosphate-buffered saline containing 0.1% Triton X-100 (Bio Basic Inc., Toronto, Canada) for 5 min, blocked with 5% bovine serum albumin for 1 h at room temperature and then incubated with the primary antibody overnight at 4°C. The following primary antibodies were used: rabbit anti-p62 (ab91526; Abcam, Cambridge, UK), mouse anti-NF- κ B protein (4764s; Santa Cruz, Dallas, TX, USA), mouse



Figure 1. Images of the screw insertion for establishing progressive cord compression (diameter 2 mm).



Figure 2. X-ray images of the rat spine with advanced screw compression of the spinal cords. (A and B) 1.8 mm depth until week 6 post-surgery.

anti-caspase-3 (9662s; Santa Cruz) and rabbit β-actin (4967; Cell Signaling Technology, Boston, MA, USA). The following secondary antibodies were used: anti-rabbit HRP-linked antibody (7074; Cell Signaling Technology) and anti-rat HRP-linked antibody (7077; Cell Signaling Technology). Finally, the sections were incubated with peroxidase-labeled polymer-HRP conjugated antibodies (HRP conjugated anti-rabbit IgG (Cell Signaling Technology) and HRP conjugated anti-rat IgG (Cell Signaling Technology) for 1 h at room temperature and then visualized with 0.02% diaminobenzidine (ZSGB-Bio Co., Ltd., Beijing, China). The sections were examined with a microscope (Leica TCS SP2; Leica, Solms, Germany) and the images were captured using an image analysis system Lumina Vision (Mitani Corp., Fukui, Japan).

Quantitative PCR (qPCR). Total RNA was extracted from spinal cord tissues using TRIzol® reagent (Invitrogen Life Technologies, Grand Island, NY, USA) and concentrations were evaluated using a Shimadzu UV-1700 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at A260 nm. From each mRNA, 1 μ g was reverse transcribed into first-strand cDNA by SuperScript[™] III Reverse Transcriptase (Invitrogen Life Technologies) in a total reaction volume of 20 μ l. qPCR amplifications were performed in triplicate using 2X FastStart Universal SYBR-Green Master mixtures (Roche Applied Science, Mannheim, Germany) with 2 μ l of cDNA samples and specific primers. The primers used were as follows: p62, forward 5'-TCCCTGTCAAGCAGTATCC-3' and reverse 5'-TCCTCCTTGGCTTTGTCTC-3'; NF-кB, forward 5'-GCACCAAGACCGAAGCAAT-3' and reverse 5'-CTCCCGTAACCGCGTAGTC-3'; caspase-3, forward 5'-CAAGGGACGGGTCATGGTTC-3' and reverse 5'-AAGTGGCGTCCAGGGAGAAG-3'; LC3, forward 5'-GACTTCCGGAAAGCTCTGCT-3' and reverse 5'-ACCAGCATCGTAGAGGGGTCT-3'; GAPDH, forward 5'-AATGCATCCTGCACCACCAA-3' and reverse 5'-GATGGCATGGACTGTGGTCA-3'. For each quantification, 35 PCR cycles were performed at 95°C for 15 sec and 60°C for 1 min and monitored using an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA, USA).

Statistical analysis. The data are presented as the mean \pm standard error of the mean. Statistical analyses were performed using SigmaStat software (SPSS, Inc., Chicago, IL, USA). Analyses of neuron quantification and neurobehavioral studies (BBB tests) were performed using two-way analysis of variance (ANOVA) followed by post-hoc pair-wise multiple comparisons. A two-way ANOVA was performed to identify the differences between groups (sham versus compressed) and time. The Bonferroni test was used for post-hoc analysis to correct for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Establishment of a chronic progressive cervical spinal cord compression rat model. In the present study, chronic spinal cord compression was established using a threaded screw. X-ray imaging demonstrated that the compression was 0% prior to screwing (Fig. 2A) and was increasingly induced by advancing the screw 0.3 mm each weak until the 6th week after initial screw implantation (Fig. 2B).



Figure 3. Basso, Beattie and Bresnahan locomotor scores of treated and control rats. \diamond indicates the scores of the control and \Box indicates the scores of the treated animals during progressive (1st-6th week) and constant (7th-16th week) spine compression.

BBB scores 1 week after surgery were unaltered with values of 18 ± 3 . At 2 and 3 weeks until 6 weeks (second, third, up to sixth compression) all animals demonstrated progressive decreases in BBB scores. From 6-16 weeks, the animals showed progressive improvements in nerve functionality scores. At 16 weeks post-surgery, compression animals had scores of 15.8 ± 0.34 (Fig. 3).

Alterations in motor neuron morphology upon compression. In the compression group, particularly in the posterior horn cell layer, large motor neurons characterized by a spindle-shape became smaller, (arrows Fig. 4B and C) and certain neurons exhibited an elongated spindle-shape (arrows Fig. 4D and E). However, the shape of the neurons returned to normal following 16 weeks (arrows Fig. 4F).

Expression of p62, *NF*-κ*B and caspase 3 in the compressed spines*. Via immunohistochemical examination, it was revealed that the expression of p62 increased in the spinal gray matter of the caudal and epicenter area (Fig. 5). In the white matter, p62 spots were observed, which spread to the epicenter and to the caudal site of the compressed area (data not shown). In addition, NF-κB staining was enhanced in the compressed spine group at 4 and 12 weeks after surgery whereas caspase 3 staining was more apparent at 12 weeks (Fig. 5).

The expression of p62, NF- κ B and caspase 3 was also quantitatively analyzed in the compressed spinal cord via western blot analysis and qRT-PCR analyses. The expression of p62 gradually increased from week 1 to week 12 with a constant high level from week 12 to week 16 after surgery. NF- κ B followed this trend with gradual increases from week 1 to week 16, whereas caspase 3 started to increase at week 8 with constant high peaks at weeks 12 and 16 (Fig. 6). Chronic spinal cord injury led to p62 and NF- κ B accumulation at 12-16 weeks, which was accompanied by enhanced caspase 3 expression from 8 weeks after surgery.

Discussion

Although the precise molecular mechanisms underlying the progressive loss of neuron cells, oligodendrocytes as well as demyelination in the spinal cord of chronic spinal cord compression remains uncertain, it is assumed that oxidative



Figure 4. Hematoxylin and eosin staining of cervical spine slides from rats subjected to spine compression. (A) Control shows a normal cord with normal shaped motor neurons (red arrows). (B-F) Subdural and intramedullary hemorrhages as well as edema led to spindle-shaped motor neurons starting at 1 week after treatment, which recovered until 16 weeks after surgery (red arrows).

stress leads to the induction of apoptosis in injured spinal cord tissues. p62 was reported to be consistently overexpressed in the pathological neurodegenerative changes of CSM and is a common component of protein aggregations in neurodegenerative diseases causing neuronal loss as well as axon degeneration in the brain (26,36). Accumulation of p62 without autophagy leads to liver damage due to activation of the caspase pathway (29,30). However, accumulation of p62 can result in cell survival and cell death mechanisms, since NF-κB is also a p62 upregulated protein (31). NF-κB proteins comprise a family of structurally associated 'rapid-acting' transcription factors. Stress-induced activation of NF-KB leads to the secretion of inflammatory cytokines in addition to increased expression of genes that regulate cell survival and growth as well as arrested proliferation, depending on the nature of the insult and extent of the damage (37). In the present study, p62 levels continually increased with spinal cord compression rate and time, which was accompanied by a similar increase in the expression of NF- κ B. These data suggested that the initial response to spinal cord compression was autophagy, as NF-KB is considered to be an anti-apoptotic gene (38). Additionally, NF-KB may have directed the cell activity from potentially unconditional apoptosis to autophagy (33), thereby preventing a profuse loss of neuron cells. In the later stage of spinal cord compression without further pressure enhancement, in addition to activated autophagy, caspase 3 expression was promoted in



Figure 5. p62, NF- κ B and caspase 3 immunostaining of rat cervical spondylotic myelopathy model spinal cord sections. The blue color indicates staining of the nuclei, whereas the brown color indicates p62, NF- κ B and caspase 3 staining. The left images indicate control spine sections, in which p62, NF- κ B and caspase 3 exhibit a low expression level. p62 and NF- κ B gradually accumulated in the nuclei 4 and 12 weeks after screw insertion, whereas caspase 3 staining increased 12 weeks after surgery. NF- κ B, nuclear factor- κ B.



Figure 6. p62, NF- κ B and caspase 3 expression in rat cervical spondylotic myelopathy model spines at the indicated time points. (A) Quantitative PCR values; (B) western blot analysis; (C) quantitative analyses of western blot data. All experiments were performed in triplicate. *P<0.05, compared with the control group. NF- κ B, nuclear factor- κ B; Con, control.

order to eliminate irreversibly damaged neuron cells. It was hypothesized that the combination of stopping screw insertion and the activation of NF- κ B from six weeks after initial surgery led to the gradual improvements of BBB scores, and this was due to repaired cell functionality via enhanced triggered autophagy, whereas the clearance of irreparable cells was enhanced from 8 weeks after initial surgery.

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