Accumulation of p62 promotes spinal cord neurodegeneration under chronic mechanical compression

Tanabe F., Yone K., Kawabata N., Komiya S., and Setoguchi T.
Department of Orthopaedic Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University
Department of Physical Therapy, School of Health Sciences, Faculty of Medicine, Kagoshima University
The Near-Future Locomotor Organ Medicine Creation Course (Kasunoki Kai), Graduate School of Medical and Dental Science, Kagoshima University
e-mail address: 5689@n2.kufm.kagoshima-u.ac.jp

INTRODUCTION
Cervical spondylotic myelopathy is the most common cause of spinal cord dysfunction by neurodegeneration in people over 55 years of age. The pathology of cervical myelopathy of the spinal cord consists of irreversible neurodegenerative changes, including neuronal loss, axonal degeneration, and myelin destruction. Intracellular accumulation of altered proteins, including p62 and ubiquitinated proteins, is the basis of most neurodegenerative disorders. The relationship among the accumulation of altered proteins, autophagy, and spinal cord dysfunction by cervical spondylotic myelopathy has not been clarified. We examined the expression of p62 and autophagy in the chronically compressed spinal cord of tip-toe-walking Yoshimura mice. In addition, we examined the expression of p62 and autophagy in hypoxic neuronal cells.

METHODS: Mouse model of chronic spinal cord compression. The twy mouse displays spontaneous calcified deposits posteriorly at the C1-C2 vertebral level. The deposits compress the cervical cord progressively with age, resulting in profound motor paresis 16–24 weeks after birth. This experiment was conducted on 24-week-old male twy mice, which display neurological dysfunction, and 24-week-old ICR mice as control.

Immunohistochemical examinations, western blot analysis, and electron microscopic examinations were performed to assess the expression of p62, ubiquitinated proteins, and autophagy markers. Cell lines: The immortalized mouse neural precursor cell line, ME85, and the U251MG human glioma cell line were used to examine the functions of p62 and autophagy under hypoxic stress. Hypoxic stress: We used CoCl2, a transition metal that mimics hypoxia, and AnaeroPack system, which absorbs O2 in the cell culture and generates conditions consisting of less than 0.1 % O2 concentration. For autophagy inhibition, cells were cultured in the presence of 3-MA. For autophagy induction, cells were cultured in the presence of LiCl.

RESULTS:
Accumulation of p62, ubiquitinated proteins, and LC3 in compressed spinal cord. Western blot analysis indicated that the expression levels of ubiquitinated proteins and p62 were up-regulated in the compressed spinal cord of twy mice compared to the spinal cord of ICR mice. p62 is a multifunctional protein that interacts with a central component of the autophagy machinery, autophagic marker microtubule-associated protein 1 light chain 3 (LC3), and transports altered proteins to degradation by autophagy. Western blot analysis showed that the expression of LC3-I and LC3-II was increased in the compressed spinal cord of twy mice. These findings suggest that the accumulation of p62, ubiquitinated proteins, and LC3 in the compressed spinal cord. Nissl staining and Luxol fast blue staining showed that the compressed spinal cords were significantly degenerated in the gray matter and white matter of the 2-mm rostral site, the epicenter site, and the 2-mm caudal site in the spinal cords of twy mice. Immunohistochemical examination revealed that p62 immunoreactivity was exhibited as a dot structure in the gray matter and white matter of the spinal cord of twy mice (Fig. 1). The number of p62 punctae was significantly increased in the spinal cords of twy mice.

Immunohistochemical examination showed that p62 was detected in MAP2-positive neurons, NF-H positive axons, GFAP-positive astrocytes, and olig2-positive oligodendrocytes. Electron microscopy showed the expression of autophagy markers, including autolysosomes and autophagic vesicles, in the compressed spinal cord. These findings suggest the presence of p62 and autophagy in the degenerated compressed spinal cord.

Hypoxic stress increased the expression of p62, ubiquitinated proteins, LC3-II, and autophagy flux in neuronal cells. Because hypoxia caused by ischemia of the cord affects the clinical manifestations of myelopathy resulting from mechanical compression, we examined the expression of p62 and autophagy in neuronal cells under hypoxic stress. Hypoxic stress increased the expression of polyubiquitated proteins, p62 protein, and LC3-II in neuronal cells. In order to assess the activation of autophagy, we performed LC3 turnover assay by examining the LC3-II expression level in the presence of the lysosomal inhibitor chloroquine. LC3 turnover assay showed that chloroquine treatment increased the amount of LC3-II expression under hypoxic stress. To confirm the autophagy activation, we used GFP-LC3. It has been reported that the response of GFP to lysosomal degradation is more resistant than that of LC3; therefore, free GFP has been suggested to be an indicator of functional autophagy flux. Western blot analysis showed that hypoxic stress increased the free GFP fragment in neuronal cells. Furthermore, histological examination showed the number of GFP-LC3 punctae was significantly increased in the presence of chloroquine under hypoxic conditions (Fig. 2). These findings suggest that hypoxic stress increased p62 and autophagy in neuronal cells.

Forced expression of p62 decreased neuronal cell proliferation. In order to examine the effect of p62 accumulation in neuronal cells, we performed WST assay following the forced expression of p62. The forced expression of p62 decreased the number of neuronal cells under hypoxic stress. These findings suggest that p62 accumulation under hypoxic stress promotes neuronal cell death.

Autophagy increased the number of neuronal cells under hypoxic stress. Autophagy contributes to the degradation of damaged proteins such as damaged mitochondria, microvesicles, and p62. In order to examine the function of autophagy in neuronal cells under hypoxic stress, we used 3-MA, an autophagy inhibitor, and LiCl, an autophagy inducer. Treatment with 3-MA decreased the number of neuronal cells, whereas LiCl increased the number of cells under hypoxic stress. These findings suggest that autophagy promotes neuronal cell survival under hypoxic stress.

DISCUSSION: We showed that the expression of p62 dots was increased with neuronal loss in the gray matter of the caudal and epicenter sites. In white matter, the p62 dots were observed spread to the epicenter site and to the caudal site of the compressed site, along with demyelination and degeneration. These findings suggest that the distribution of p62 was consistent with the pathologic neurodegenerative changes of cervical spondylotic myelopathy. We showed that hypoxic stress up-regulated p62 in neuronal cells. In addition, the forced expression of p62 decreased the number of neuronal cells. These findings suggest that the up-regulation of p62 contributed to neuronal loss in the compressed spinal cord. In addition, we showed that induction of autophagy participates in neuronal cell protection.

SIGNIFICANCE: Our findings suggest that pharmacological inducers of autophagy may be useful for treating cervical spondylotic myelopathy patients.