Increased immunoreactivity of c-Fos in the spinal cord of the aged mouse and dog

JI HYEON AHN1*, MYOUNG CHUL SHIN2*, JOON HA PARK1, IN HYE KIM1, JAE-CHUL LEE1, BING CHUN YAN3, IN KOO HWANG4, SEUNG MYUNG MOON5, JI YUN AHN2,6, TAEK GEUN OHK2,7, TAE HUN LEE8, JUN HWI CHO2, HYUNG-CHEUL SHIN9 and MOO-HO WON1

Departments of 1Neurobiology, and 2Emergency Medicine, School of Medicine, Kangwon National University, Chuncheon, Gangwon 200-701, Republic of Korea; 3Institute of Integrative Traditional and Western Medicine, Medical College, Yangzhou University, Yangzhou, Jiangsu 225001, P.R. China; 4Department of Anatomy and Cell Biology, College of Veterinary Medicine, Seoul National University, Seoul 151-742; 5Department of Neurosurgery, Dongtan Sacred Heart Hospital, College of Medicine, Hallym University, Hwaseong, Gyeonggi 445-170; 6Department of Emergency Medicine, Sacred Heart Hospital, College of Medicine, Hallym University, Anyang, Gyeonggi 431-796; 7Department of Emergency Medicine, Kangnam Sacred Heart Hospital, College of Medicine, Hallym University, Seoul 150-950; Departments of 8Emergency Medicine, Chunchon Sacred Heart Hospital, and 9Physiology, College of Medicine, Hallym University, Chuncheon, Gangwon 200-702, Republic of Korea

Received April 10, 2014; Accepted July 22, 2014

DOI: 10.3892/mmr.2014.2800

Abstract. Expression of c-Fos in the spinal cord following nociceptive stimulation is considered to be a neurotoxic biomarker. In the present study, the immunoreactivity of c-Fos in the spinal cord was compared between young adult (2-3 years in dogs and 6 months in mice) and aged (10-12 years in dogs and 24 months in mice) Beagle dogs and C57BL/6J mice. In addition, changes to neuronal distribution and damage to the spinal cord were also investigated. There were no significant differences in neuronal loss or degeneration of the spinal neurons observed in either the aged dogs or mice. Weak c-Fos immunoreactivity was observed in the spinal neurons of the young adult animals; however, c-Fos immunoreactivity was markedly increased in the nuclei of spinal neurons in the aged dogs and mice, as compared with that of the young adults. In conclusion, c-Fos immunoreactivity was significantly increased without any accompanying neuronal loss in the aged spinal cord of mice and dogs, as compared with the spinal cords of the young adult animals.

Introduction

Unexplained pain has been reported as a common complaint of the elderly, and age-related patterns in pain prevalence are complex (1,2). Neuropathic pain, one of the most common types of chronic pain, results from the abnormal processing of sensory input due to damage caused by disorders of the nervous system, such as spinal cord injury, and increases with advancing age (3,4).

c-Fos is a cellular proto-oncogene, belonging to the immediate early gene family. c-Fos has been used as a relative marker of neuronal activity in the brain following numerous types of brain insult (5-7). Furthermore, c-Fos expression in the spinal cord is considered to be a neurotoxic biomarker which has been detected in the dorsal spinal neurons, following nociceptive stimulation (8-12) and repeated swim stress (13).

Previous studies regarding age-related physiological changes, have focussed on spinal cord-specific changes. A loss of myelin and axonal involution have been observed in the aged rat spinal cord (14), and expression levels of substance P, a major neurotransmitter of primary afferent nociceptive fibers, and somatostatin have been shown to decrease in the spinal cord of the aged rat (15,16). Little is currently known about the changes in c-Fos expression in the spinal cord during the process of normal aging.

The present study compared the age-related changes in the immunoreactivity of c-Fos in the spinal cords of the young adult and aged Beagle dog and C57BL/6J mouse. Both the Beagle and C57VL/6J mice are considered to be good animal models to study aging (17-21).
Materials and methods

Experimental animals. Clinically and neurologically healthy male Beagle dogs and male C57BL/6J mice were used in the present study. Young adult dogs, aged 2-3 years, and aged dogs, aged 10-12 years were used (n=7/group); alongside young adult mice, at 6 months, and aged mice, at 24 months (n=14/group). The animals were maintained in conventional housing under adequate temperature (23°C) and humidity (60%) conditions, with a 12 h light/12 h dark cycle, and free access to food and water.

Animal handling and care followed the guidelines of the current international laws and policies [National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals, NIH Publication no. 85-23, 1985, revised 1996], and the experimental protocol was approved by the Institutional Animal Care and Use Committee of Kangwon National University (approval no. KW-130424-3). All of the experiments were conducted to minimize the number of animals used, and to avoid animal suffering.

Tissue processing for histology. For histochemical analysis, the young adult and aged dogs and mice (n=7 in each group) were anesthetized with a mixture of Zoletil 50 (8 mg/kg; Virbak Korea, Seoul, Korea) Xylazine (2 mg/kg; Bayer Korea, Seoul Korea) and pentobarbital sodium (40 mg/kg; JW Phar. Co., Ltd., Seoul, Korea), respectively. Anesthesia was followed by a transcardial perfusion with 0.1 M phosphate-buffered saline (PBS, pH 7.4; Sigma-Aldrich, St. Louis, MO, USA), followed by 4% paraformaldehyde (Samchun Chemicals, Pyeongtaek, Korea) in 0.1 M phosphate buffer (pH 7.4). The cervical (C6-C8) and lumbar (L5-L6) spinal cord regions were harvested from the animals and postfixed, in the same fixative, for 12 h. The spinal cord tissues were cryoprotected by infiltration with 30% sucrose (Junsei Chemical Co., Ltd., Tokyo, Japan) over 30% sucrose (Junsei Chemical Co., Ltd., Tokyo, Japan) overnight. Subsequently, the frozen tissues were serially sectioned at 30 μm using a cryostat (Leica, Wetzlar, Germany) and the sections were then placed into six-well plates containing PBS.

Fluoro-Jade B (F-J B) histofluorescence staining. F-J B histofluorescence staining procedures were conducted according to previous methods (22). Briefly, the sections were immersed in a solution of 80% ethanol containing 1% sodium hydroxide, followed by immersion in 70% ethanol. The sections were then transferred into a solution of 0.06% potassium permanganate, prior to staining with a 0.0004% F-J B solution (Histochem, Jefferson, AR, USA). The sections were then incubated with diluted mouse anti-NeuN antibodies and streptavidin peroxidase complex (1:200; Vector Laboratories, Burlingham, CA, USA) following dehydration. The sections were then incubated with pre-immune serum in place of a primary antibody. The negative control test was carried out with pre-immune serum in place of a primary antibody. The negative control test resulted in the absence of immunoreactivity in all structures.

Western blot analysis for c-Fos. To confirm the changes in the c-Fos expression levels in the cervical spinal cord region between the young adult and aged mice, the mice spinal cord tissues (n=7 in each group) were used for western blot analysis. Briefly, the tissues were homogenized in 50 mM PBS (pH 7.4), containing ethylene glycol tetraacetic acid (pH 8.0), 0.2% NP-40, 10 mM ethylenediaminetetraacetic acid (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM sodium fluoride, 150 mM sodium chloride, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (DTT; Santa Cruz Biotechnology, Inc.). Following centrifugation at 16,000 x g for 20 min, the protein concentration of the supernatants was determined using a Micro
Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). Aliquots containing 20 µg of total protein were boiled in a loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue, and 30% glycerol. Subsequently, the aliquots were loaded onto a polyacrylamide gel, and separated by electrophoresis, after which the blots were transferred to nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). The membranes were incubated with 5% non‑fat dry milk in PBS containing 0.1% Tween‑20, followed by an incubation with the primary antibody for 2 h. The membranes were then incubated with peroxidase ‑conjugated donkey anti ‑goat immunoglobulin G (Sigma‑Aldrich). An Enhanced Chemiluminescence kit (Pierce Biotechnology) was used to visualize the blots. Western blot analysis was repeated three times. Following exposure of the membranes, the blots were scanned and densitometric analysis for the quantification of the bands was performed using Scion Image software (Scion Corporation, Frederick, MD, USA), which was used to determine the ROD. A ratio of the ROD was calibrated as a percentage, with the young adult group designated as 100%.

Statistical analysis. The difference of the mean ROD between the groups was statistically analyzed using a Student t‑test. A P<0.05 was considered to indicate a statistically significant difference.

Results

NeuN‑immunoreactive neurons. NeuN‑immunoreactive neurons were shown to be distributed throughout the grey matter of the cervical and lumbar spinal cord regions in the young adult and aged dogs (Fig. 1A‑1D). There were no significant differences in the number of NeuN‑immunoreactive neurons between the young adult and aged dogs, however the number of NeuN‑immunoreactive neurons was shown to be slightly decreased in the cervical and lumbar regions of the aged spinal cord, as compared with the number in the young adult spinal cord (data not shown).

Similarly to the dogs, the number of NeuN‑immunoreactive neurons in the cervical and lumbar spinal cord regions was not significantly different between the young adult and aged mice (data not shown).

F‑J B positive cells. To examine the extent of neuronal degeneration of the spinal cord, F‑J B staining was performed in the young adult and aged dogs and mice. F‑J B positive cells were not observed in the cervical and lumbar spinal cord regions of the young adult and aged dogs (Fig. 1E‑H) or mice (data not shown).

C‑Fos immunoreactivity. In the young adult dogs, moderate c‑Fos immunoreactivity was observed in the neurons in the whole grey matter of the cervical and lumbar spinal cord regions (Fig. 2A, 2b, 2C and 2f). The c‑Fos immunoreactivity was generally found to be contained within the nuclei of the spinal neurons (Fig. 2b and 2f). In the aged dogs, the c‑Fos expression pattern was similar to that in the young adult dogs (Fig. 2B, 2c, 2d, 2D, 2g, and 2h); however, the c‑Fos immunoreactivity in the nuclei of the neurons in the aged dogs was significantly higher as compared with that in the young adult dogs (Fig. 2c, 2d, 2g, 2h and 2E) (P<0.05).

c‑Fos immunoreactivity was also observed in the nuclei of the spinal neurons of the cervical and lumbar spinal cord.
Figure 2. (A and B) c-Fos immunohistochemistry in the cervical and (C and D) lumbar spinal cord regions of (A and C) young adult and (B and D) aged dogs. c-Fos immunoreactivity (arrows in "c, d, g and h") was increased in the aged group. Strong immunoreactivity was observed in the nuclei of the spinal neurons. Scale bars = (A-D) 500 µm, (a-h) 50 µm. (E) Relative optical density as a percentage of c-Fos-immunoreactive structures in the young adult and aged spinal cord (n=7 per group; *P<0.05 versus the adult group). The bars indicate the means ± standard error of the mean. DH, dorsal horn; VH, ventral horn; ROD, relative optical density.

Figure 3. (A and B) c-Fos immunohistochemistry in the cervical and (C and D) lumbar spinal cord regions of (A and C) young adult and (B and D) aged mice. c-Fos immunoreactivity (arrows in "c, d, g and h") was increased in the aged group. Strong immunoreactivity was observed in the nuclei of the spinal neurons. Scale bars = (A-D) 50 µm, (a-h) 200 µm. (E) Relative optical density as a percentage of c-Fos-immunoreactive structures in the young adult and aged spinal cord (n=7 per group; *P<0.05 versus the adult group). The bars indicate the means ± standard error of the mean. DH, dorsal horn; VH, ventral horn; ROD, relative optical density.
The present findings support previous research that showed that low levels of c-Fos immunoreactivity was observed in young adult animals (28-30). However, other studies have previously reported that the basal expression of c-Fos in the dorsal horn was decreased in the aged rat spinal cord (31), which is not consistent with the findings of the present study. This discrepancy may result from differences in experimental methods.

It has previously been reported that c-Fos and astrocytes become activated by a combined stress of non-thermal irradiation and the toxic effects of picrotoxin in the rat brain (32). Activated astrocytes are associated with several neuropathic and cancer pain states, through the release of neurotoxic substances including pro-inflammatory cytokines (33-35). In addition, it has previously been reported that pro-inflammatory cytokines, including interferon-γ, and interleukins (IL)-1β and -2, were markedly increased, without any significant neuronal loss, in the spinal cord of the aged dog (26,36). It has also been reported that increasing age can enhance the expression levels of tumor necrosis factor-α, the IL-6 family of cytokines, chemokine receptor-2 and pro-inflammatory chemokines following ischemic stroke in the aged rat (37). Therefore, based on these data and the results of the present study, c-Fos expression in the spinal cord may be triggered by different stress factors, including chronic inflammatory activity.

In conclusion, the present study observed that c-Fos immunoreactivity in the aged dog and mouse spinal cords was markedly increased, as compared with young adult animals. This finding implicates that the increase in the expression of c-Fos in the aged dog spinal cord may be associated with aging-related changes in the aged spinal cord.

Acknowledgements

The authors would like to thank Mr. Seung Uk Lee for his technical help in this study. This research was supported by the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2010-0010580) and by the ICT R&D program of MSIP/IITP (10033634).

References


