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Loss of *hsp70.1* Decreases Functional Motor Recovery after Spinal Cord Injury in Mice

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Heat shock proteins (HSPs) are specifically induced by various forms of stress. Hsp70.1, a member of the *hsp70* family is known to play an important role in cytoprotection from stressful insults. However, the functional role of Hsp70 in motor function after spinal cord injury (SCI) is still unclear. To study the role of *hsp70.1* in motor recovery following SCI, we assessed locomotor function in *hsp70.1* knockout (KO) mice and their wild-type (WT) mice via the Basso, Beattie and Bresnahan (BBB) locomotor rating scale, before and after spinal hemisection at T13 level. We also examined lesion size in the spinal cord using Luxol fast blue/cresyl violet staining. One day after injury, KO and WT mice showed no significant difference in the motor function due to complete paralysis following spinal hemisection. However, when it compared to WT mice, KO mice had significantly delayed and decreased functional outcomes from 4 days up to 21 days after SCI. KO mice also showed significantly greater lesion size in the spinal cord than WT mice showed at 21 days after spinal hemisection. These results suggest that Hsp70 has a protective effect against traumatic SCI and the manipulation of the *hsp70.1* gene may help improve the recovery of motor function, thereby enhancing neuroprotection after SCI.

Key Words: Spinal cord injury, Neuroprotection, Heat shock protein, Mice

INTRODUCTION

Spinal cord injury (SCI) results in devastating events for patients such as loss of motor function and neuropathic pain. As a consequence of primary mechanical injury to the cord, various pathologic events trigger subsequent secondary injury that can aggravate spinal cord damage [1-3]. Thus, controlling for the secondary injury following SCI is an important technique for limiting the extent of tissue damage and consequent functional impairment [4-6]. Previous studies have shown a number of interrelated factors that may contribute to the secondary injury process, including heat shock proteins [1,7].

The heat shock proteins (HSPs) are a highly conserved proteins that act as molecular chaperones to aid protein transport and assembly of newly synthesized polypeptides [8]. Of the HSPs, Hsp70 is not usually detectable under normal condition and is rapidly induced by various stresses such as hyperthermia, oxidative stress and amino acid analogues [9-12]. Previous studies, both *in vivo* and *in vitro* studies, have suggested HSP70 induction is associated with cell protection from various lethal insults [13-15]. Experimental studies using transgenic mice have shown that Hsp70 overexpression protected neuronal cells from ischemic insult [16,17]. Plumier et al. [16] found that Hsp70 overexpression following permanent focal cerebral ischemia significantly protected the pyramidal neurons in the hippocampus but did not affect the overall infarct area. Rajdev et al. [17] reported that cerebral infarct volume following brain ischemia was significantly lower in Hsp70 overexpressing transgenic mice than in wild-type mice. However, another study using transgenic mice showed no significant difference in infarct size or hippocampal cell survival [18]. Taken together, these may suggest that Hsp70 protects neuronal cells against some, but not all types of central nervous system injury. Recently, several studies demonstrated that Hsp70 plays an important role in the secondary injury cascade after SCI [19,20]. A number of *in vitro* [21,22] and *in vivo* studies [23-26] have suggested Hsp70 has cytoprotective effects in the spinal cord. Because neuroprotection involves several genes including Hsp70 [27], the neuroprotective effects of Hsp70 itself after SCI are difficult to determine. Thus, in the present study, we used *hsp70.1* knockout mice (KO) to investigate whether Hsp70 plays a neuroprotective role in SCI.

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ABBREVIATIONS: SCI, spinal cord injury; HSP, heat shock protein; BBB, Basso-Beattie-Bresnahan motor rating scale.

METHODS

Experimental animals

All experimental procedures were conducted in accordance with guidelines set by the Korea University College of Health Science Animals Research Policies Committee. We obtained *hsp70.1* KO mice used in this study from Dr. Jeong-Sun Seo, at Seoul National University. A previous study has described these KO mice bearing a null-allele at *hsp70.1* and confirmed the absence of *hsp70.1* mRNA expression in them [47]. For this experiment, we used 50 adult mice (8 weeks-old) weighing 25~30 gm at the time of their operation: Thirty *hsp70.1* KO mice and twenty littermate wild type mice (WT). The animals were kept on a 12-hour light /12-hour dark cycle with lights on at 7:00 A.M.

Surgical procedures

Under enflurane anesthesia (by mixture of 3% enflurane and 95% O_2), we shaved the skin overlying the thoracic vertebrae of each subject and disinfected with povidone iodine. After the skin was incised along the midline, a laminectomy on the T12 vertebrae was performed to expose the T13 spinal cord. With a no. 11 blade scalpel, the cord was hemisected on the left side. The wound was closed in anatomical layers, the skin with stainless wound clips. After surgery, the mice displayed contralateral hindlimb paralysis were excluded from the study.

Behavioral tests

Behavioral test for motor function was performed preoperatively and postoperatively (PO) for hindlimbs. The test was performed on each mouse 1 day prior to surgery and 1, 4, 7, 10, 13, 17, and 21 days PO. Hindlimb motor function was assessed using the Basso-Beattie-Bresnahan (BBB) motor rating scale [28]. The BBB has 22 levels from 0 to 21 that systematically and logically follow recovery of hindlimb function, from a score of 0, indicative of no observed hindlimb movements, to a score of 21, representing a normally ambulating mouse. Testing procedures were as follows. Briefly, animals were allowed to move freely on a paper-covered mattress, which makes a noise if the mouse drags its feet. Test sessions were 4 min in duration, and scores were obtained from 2 blinded observers according to the criteria. Left and right hind limbs were assessed separately due to potential asymmetrical recovery.

Analysis of histology

After the tests, the animals were randomly selected from both the KO and WT groups and were subjected to the comparison of histological differences in the spinal segments epicenter to the hemisection. Histological study was conducted on 6 mice in each group 3 weeks after spinal hemisection. During this period, the hemisected mice fully recovered motor function and showed well-established signs of mechanical allodynia, as described in our previous report using a rat model [29]. Mice were deeply anesthetized with sodium pentobarbital and perfused with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The spinal segments including epicenter were removed, post-fixed for 6~8 hr and stored overnight in 30% sucrose. Tissues were embedded in paraffin, cut into 8 μm thick longitudinal sections. The sections were treated with xylene and 95% alcohol. The slices on the slides were incubated in Luxol fast blue and then counterstained with cresyl violet. To quantify the degree of tissue damage following SCI, size of lesion of each section were measured by using a computer-assisted image analysis system (NIH image software). All assessments were performed in a blinded fashion.

Statistical analysis

All values were expressed as mean±SEM. The Mann-Whitney U test was used to compare scores obtained on a given experimental day between 2 groups. The lesion size in the KO and WT groups was compared with t-test. Differences between groups were considered statistically significant if $p<0.05$.

RESULTS

Functional motor recovery after SCI in 70.1 KO and WT mice

Prior to hemisection, locomotor function in all animals was evaluated in both ipsi- and contralateral hindlimbs using the BBB scale via an open field test (Fig. 1). The scores from *hsp70.1* KO mice were not different from those of the WT mice. After hemisection, all mice showed paralysis on the ipsilateral hindlimb, corresponding to a BBB score 0. However, progressive motor recovery was observed with hindlimb joint movement on 4 d PO, and then relatively rapid recovery proceeded until 13 d PO. At that time, the WT mice showed coordinated walking. The mice achieved maximal recovery on 21 d PO. On 1 d PO, mice also showed mild disturbance in contralateral hindlimb, due to walking imbalance resulting from ipsilateral hindlimb paralysis. However, as shown in Fig. 1, the locomotor function of the contralateral hindlimb improved as the ipsilateral hindlimb recovered locomotor function.

Throughout the entire recovery period, there was no significant difference in contralateral side motor function between the two groups. However, on the ipsilateral side, the *hsp70.1* KO mice showed delayed motor function recovery compared to the WT mice. This difference in motor recovery between *hsp70.1* KO and WT mice was statistically significant from 4 d PO up to 21 d PO, except on 17 d PO after spinal hemisection ($p<0.05$). Functionally prominent difference in motor function between *hsp70.1* KO and WT mice was noticed from 7 d PO. At 7 d PO, the *hsp70.1* KO mice scored 10.5±0.65 on the BBB scale, whereas the WT mice scored 14.1±0.31 ($p<0.05$). Although this score difference does not seem great, the difference in terms of functional gain is significant. The *hsp70.1* KO mice exhibited frequent weight-supported plantar stepping, with no forelimb-hindlimb coordination. In contrast, the WT mice showed consistent weight-supported plantar steps and consistent forelimb- hindlimb coordination. Although, the *hsp70.1* KO and WT mice did not show significant locomotor function differences at 17 d PO, the difference retuned on 21 d PO, the last day of the study. At this time, the KO mice displayed frequent to consistent weight-supported plantar steps with consistent forelimb-hindlimb coordination, whereas the WT mice showed consistent weight-sup-

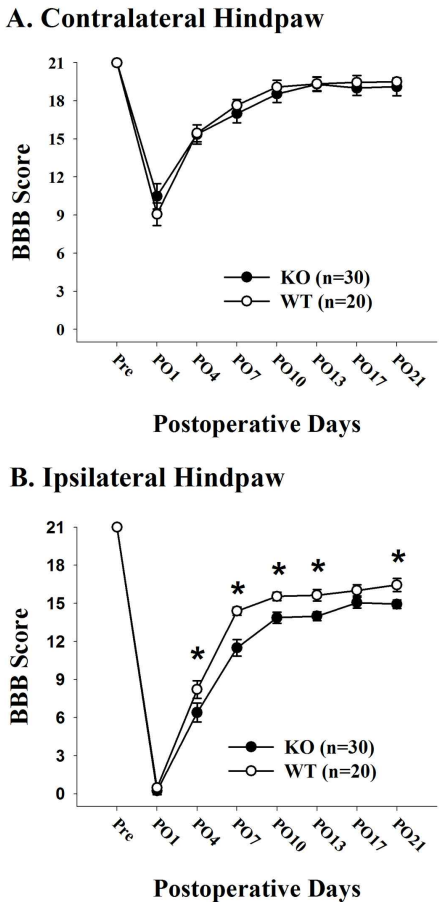

Fig. 1. Motor recovery of hindlimb. Hindlimb motor function on the contralateral (A) and the ipsilateral side (B) was evaluated before SCI and on days 1, 4, 7, 10, 13, 17 and 21 after SCI. Asterisks indicate the values significantly different between the two groups (*$p<0.05$).

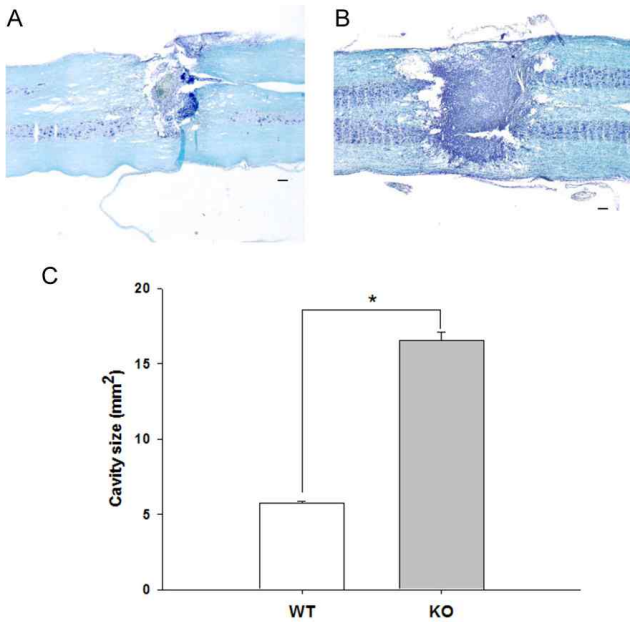

Fig. 2. A representative section of spinal cord segment including epicenter in WT (A) and KO (B) mice. Longitudinal spinal cord sections double-stained with Luxol fast blue and cresyl violet show the injury and atrophy area. The lesion size is significantly different between WT and KO mice (C) (*$p<0.05$). Scale bars, 400 μm.

ported plantar steps, with consistent forelimb-hindlimb coordination.

Lesion size

To assess the lesion size, the longitudinal cord sections were double-stained with Luxol fast blue and cresyl violet. The lesions exhibited scar formation and complete loss of myelinated fiber. Scar formation appearances at the lesion site differed between KO and WT mice 3 weeks after SCI. In the KO mice, scar tissue extended toward the contralateral side of the spinal cord and extended rostrocaudally from the epicenter in the spinal cord. In contrast, the WT mice had less scar formation at the lesion site (Fig. 2). In WT and KO mice, the cresyl violet stained areas were 6.70±0.21 mm^2 and 17.5±024 mm^2, respectively (Fig. 2). There were significant lesion area differences between *hsp70.1* KO and WT mice following spinal hemisection ($p<0.05$).

DISCUSSION

The present data demonstrated that mice lacking *hsp70.1* experienced a poorer functional motor recovery than did WT mice after spinal cord hemisection. We also found that lesion size was significantly larger in *hsp70.1* KO mice than in WT mice. As suggested by several previous studies showing the beneficial effects of pharmacological upregulation of Hsp70 on cell survival, the present study indicates Hsp70 possesses neuroprotective potential after SCI. To our knowledge, this is the first study evaluating the protective role of Hsp70 in SCI by using an *hsp70.1* KO mouse model.

In this study, we examined motor function up to 21 d PO after spinal hemisection because previous reports showed motor function recovery plateaued at 3 weeks after SCI [29,30]. The present results show that *hsp70.1* KO mice experienced delayed and decreased functional motor recovery as compared to WT mice. As a result, the significant difference in locomotor function between *hsp70.1* KO and WT mice was occurred from 4 d PO. However, the prominent functional difference between *hsp70.1* KO and WT mice appeared from 7 d PO. The difference in hindlimb function quality between the two groups was huge at this time point, although the score difference on BBB expressed in numbers could say. KO mice merely placed their limbs in a weighted fashion and showed occasional weighted plantar steps, whereas WT mice displayed nearly consistent plantar stepping with consistent forelimb-hindlimb coordination.

Our findings are consistent with previous studies showing Hsp70 upregulation produced beneficial effects after SCI. Upregulation of Hsp70 via anti-inflammatory drug treatment has improved functional outcomes after SCI [31,32]. Previously, Shin et al. [32] reported that cyclosporine A reduced neurological injury due to spinal cord ischemia in a rabbit model. They concluded this improved outcome after spinal ischemia correlated to overexpression of Hsp70. A significant improvement in neurological function using modified Tarlov's scores was more evident on day 7 PO.

Park et al. [31] also demonstrated significant improvement in motor function at 7 d after SCI in rats pretreated with pioglitazone, a peroxisome proliferator-activated receptor inhibitors. The pioglitazone treated group showed a prominent enhanced expression of Hsp 70. Our result is strongly supported by the earlier study [33] on ischemic insult to the spinal cord. Their study showed that Hsp70 expression in motor neurons created ischemic tolerance after spinal ischemia in rats. Taken together, the preservation of motor functions after SCI may be partially mediated by upregulation of Hsp70.

It is well known that SCI leads to a progressive series of degenerative processes induced by the original insult to the spinal cord. These secondary degenerative processes, which contribute to progressive tissue loss and cavitation at the injury site, are a major cause of motor dysfunction [34]. Accordingly, recent studies on neuroprotection have focused on the inhibition of secondary injury after SCI [5,35-37]. It has been suggested that the induction of Hsp70 is associated with cell protection from various lethal insults both *in vivo* and *in vitro* studies [13-15]. Therefore, in the realm of neuroprotection, Hsp70 might be a useful target molecule for the therapeutic treatment. Previous studies have demonstrated that the induction of Hsp70 may be responsible for secondary processes after SCI [38,39]. Recent experimental evidence showed that upregulation of Hsp 70 with pharmacological inhibition of inflammation achieved a neuroprotective effect [31,32]. In particular, Shin et al. [32] found that neuroprotective effect of cyclosporine A against ischemia was related to overexpression of neural nitric oxide synthase and Hsp70, indicating the role of HSPs in modulating secondary injury. The present finding is consistent with these studies and with the body of work showing neuroprotective role of Hsp70 in ischemic brain and cardiac injury.

In contrast, other studies have suggested Hsp70 plays a different role of in neuroprotection. Reportedly, intrathecal administration of bupivacaine and hypothermia protected neuronal cells in rat SCI and this effect was most likely due to Hsp70 downregulation [40]. Such contradictory reports on the role of Hsp70 in SCI are explicable. First, previous studies have suggested that HSPs have a multifaceted modulatory role in inflammation, from proinflammatory to anti-inflammatory functions [41,42]. Second, the role of Hsp70 seems to depend on the time it is induced [33] and the level of HSP expression [43]. Whereas most studies on HSP roles in SCI have focused on Hsp70, they limited themselves to certain SCI models, particularly ischemic SCI produced by thoracic aorta occlusion. Most of all, it is difficult to determine whether *hsp70*, itself, has a neuroprotective effect after SCI, because several other genes are involved in neuroprotective paradigms [27]. Different subtypes of HSP such as Hsp27 are involved in neuroprotection. Tachibana et al. [44] found that the level of HSP27 gene expression level increased more than 2 times at 24 hours after SCI in a traumatic SCI rat model. Others have similar results [45] suggesting 17-allylamino-17-demethoxygeldanamycin, a potent Hsp90 inhibitor, produced neuroprotective effects by inhibiting HSP90, indicating that Hsp70 may act in concert with other HSPs.

Currently, gene transfer techniques and transgenic animal strain have made it possible to selectively overexpress HSP, to better understand the precise role of Hsp70 in cellular injury. Several studies using transgenic mice have suggested role of Hsp70 in neuroprotection is still controversial [16-18]. Based on these reports, Hsp70 upregulation seems to have a neuroprotective effect on neuronal cells against some, but not all types of central nervous system injury. Most previous studies using transgenic mice were limited to certain brain injury produced by ischemia. Thus, less is known about the precise influence of Hsp 70 on neuroprotection and functional outcomes after traumatic SCI. In the present study, *hsp70* KO mice had larger lesion size than WT mice had. Inducible Hsp70 is encoded from both *hsp70.1* and *hsp70.3* genes, which show high similarity in their coding sequences. The fact that the two inducible *hsp70* genes differ from each other in the 5'- and 3'-untranslated regions [46], suggests they might be under differential regulation. However, the deletion of *hsp70.1* results in a remarkable decrease in Hsp70 expression [47]. Taken together, these findings also strongly suggest that Hsp70 is a cytoprotective protein within the spinal cord and may be responsible for neuroprotection after SCI.

In summary, the present results demonstrated *hsp70.1* deletion results in a remarkable decrease in functional outcome and an increase in lesion size after SCI. The present report supports the ideas that Hsp70 mainly has a neuroprotective effect after SCI. Further molecular mechanisms must be investigated for the clinical application of HSP.

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