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Immunohistochemical Study on the Distribution of Glycogen Synthase Kinase (GSK) 3β in the Central Nervous System of SOD1^{G93A} Transgenic Mice

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Abstract : In the present study, we investigated influences of glycogen synthase kinase (GSK) 3β on the development and/or progression of amyotrophic lateral sclerosis (ALS).

We used transgenic mice expressing a human Cu/Zn superoxide dismutase mutant (SOD1^{G93A}) as an *in vivo* model of ALS and examined expressional changes of GSK3β immunohistochemically in the spinal cord, brain stem and cerebellum.

With these experiments we demonstrate that the neurons in these regions of symptomatic SOD1^{G93A} transgenic mice showed increased GSK3β immunoreactivities compared with wild-type SOD1 transgenic mice. In contrast to symptomatic SOD1^{G93A} transgenic mice, few GSK3β immunoreactivity changes were detected in 8w- and 13w-old presymptomatic SOD1^{G93A} transgenic mice.

These data suggest the possibility that GSK3 functions as a modulating factor of apoptosis-related alterations in ALS and that GSK3β exert differential functions in the development and/or progression of ALS. But the exact functional significances of these changes require further elucidation.

Key words : Glycogen synthase kinase (GSK) 3β, Amyotrophic lateral sclerosis (ALS), SOD1^{G93A} transgenic mice, Spinal cord, Brain stem, Cerebellum

Introduction

Amyotrophic lateral sclerosis (ALS), a progressive, fatal neurodegenerative disease, is characterized by the selective loss of motor neurons in the spinal cord, brainstem, and motor cortex. Accumulating evidences have confirmed that the etiologies of sporadic and familial ALS share common mechanisms and that the study of familial cases can provide understandings of sporadic cases (Al-Chalabi and Leigh 2000). Up to 10% of cases of ALS are familial, and 20～25% of these are linked to dominantly inherited mutations in Cu/Zn superoxide dismutase (SOD1) (Al-Chalabi and Leigh 2000). A variety of studies, including investigations with SOD1-mutant transgenic mice (Gurney et al. 1994, Ripp et al. 1995), established that mutant SOD1 causes motor neuron disease through gain of one or more toxic properties (Cleveland and Rothstein 2001, Julien 2001). Although the mechanisms where-

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by mutant SOD1 causes selective motor neuron death is not yet known, several hypotheses have been postulated: enhancement of protein nitrosylation by mutant SOD1; enhanced peroxidase activity; exposure of toxic copper at the active site of SOD1; and accumulation and aggregation of altered or abnormal proteins including SOD1 (Al-Chalabi and Leigh 2000).

Besides these hypotheses, recently microtubule-associated protein, tau which is well known as a major component of the neurofibrillary tangle in Alzheimer's disease and β-amyloid precursor protein (β-APP) which is closely related with the amyloid plaque in Alzheimer's disease are suspected be involved in the development and/or progression of ALS. To support their involvements, tau immunoreactive astrocytic and neuronal inclusions were founded in ALS patients (Yang et al. 2003) and increased β-APP immunoreactivity was recognized in the anterior horns of ALS patients with short clinical courses or with mild depletion of anterior horn neuron (Sasaki and Iwata 1999). Glycogen synthase kinase (GSK) 3 is a kinase closely related with tau and β-APP metabolism, which has two isoforms (GSK3α and GSK3β) (Frame and Cohen 2001). GSK3 is implicated in the phosphorylation of tau and in pathologic states suspected to produce hyperphosphorylated tau which is unable to bind to microtubule and makes aberrant filament assembly such as neurofibrillary tangle (Hanger et al. 1992, Mandelkow et al. 1992, Jope and Johnson 2004).

In addition to the GSK3's effects on tau and β-APP, GSK3 is involved in the regulation of neuronal apoptosis (Frame and Cohen 2001, Jope and Johnson 2004) and modulates activities of cAMP-response-element-binding protein (CREB), whose cofactor, CREB binding protein (CBP), expression was reported to be changed in an ALS animal model by our previous work (Chung et al. 2003). GSK3 seems to be able to influence on the development and/or progression of ALS by various mechanisms. With this hypothesis, we used transgenic mice expressing a human SOD1 mutant ($SOD1^{G93A}$) as an *in vivo* model of ALS (Gurney et al. 1994, Ripps et al. 1995) and examined expressional changes of GSK3β immunohistochemically in the spinal cord, brain stem, and cerebellum of $SOD1^{G93A}$ transgenic mice for the first time.

Materials and Methods

Twelve $SOD1^{G93A}$ transgenic and ten wild-type (wt) SOD1 transgenic mice developed by Gurney et al. (1994) were used. They were bred by 'The Jackson Laboratory' (Bar Harbor, ME) under the strain designations B6SJL-TgN ($SOD1^{G93A}$) 1 Gur/J and B6SJL-TgN (SOD1) 2 Gur/J for $SOD1^{G93A}$ transgenic and wtSOD1 transgenic mice, respectively. B6SJL-TgN (SOD1) 2 Gur/J strain carries the normal allele of the human SOD1 gene, and has been reported that the SOD1 protein level is the same as in the transgenic strain carrying the $SOD1^{G93A}$ transgene. This strain serves as a control for the B6SJL-TgN ($SOD1^{G93A}$) 1 Gur/J. Animals were sacrificed at the age of 8 weeks (w), 13w (presymptomatic) and 18w (symptomatic). Clinical symptoms were manifested in 18w mutant transgenic mice. The first signs of hind limb paresis appeared in 16～18w mice carriers of $SOD1^{G93A}$. When suspended from the tail, these mice did not extend symmetrically both hind limbs, as normal mice do. The weak limb was closer to the body. Subsequently, the weakness of one hind limb progressed to paralysis of this limb and soon thereafter the other hind limb became paralyzed. At that stage both hind limbs were dragged as the mouse moved around the cage. The animals used in this experiment were treated according to the 'National Institute of Health Guide for the Care and Use of Laboratory Animals' (NIH publication No. 80-23, revised in 1996).

The mice were perfused transcardially with cold phosphate buffered saline (PBS, 0.02 M, pH 7.4), and then with ice-cold 4% paraformaldehyde for 10 min at

a flow rate of 5∼6 mL/min. Brains were immediately removed and sliced into blocks 4∼6 mm thick. Spinal cords were also removed and sliced into cervical, thoracic, and lumbar segments of 3∼10 mm in length. These blocks were immersed in a cold fixative for 12 h and then cryoprotected in a series of cold sucrose solutions of increasing concentrations. Frozen sections were cut at 40 μm in the coronal plane. Immunohistochemistry was performed in accordance with the free-floating method described earlier (Shin et al. 2000). Goat polyclonal anti-GSK3β antibodies (sc-18257, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as primary antibodies. To confirm specificities of the primary antibodies, preadsorption test was performed. Preadsorption test was performed using the same method used for immunohistochemistry, but some sections was reacted without primary antiserum as a negative control, other sections were exposed to anti-GSK3β antibody that had been preadsorbed for 24 hours with GSK3β blocking peptide, and another sections were exposed to anti-GSK3β antibody as a positive control.

Sections from each SOD1^{G93A} and wtSOD1 transgenic mice were stained together eliminating conflicts between different experimental conditions. To determine whether GSK3β immunoreactivity changes observed in SOD1^{G93A} transgenic mice are statistically significant, we randomly selected five unit areas at each region of SOD1^{G93A} (n=15) and wtSOD1 transgenic mice (n=12), and determined staining densities of these areas using NIH image program (Scion Image), then calculated averages of signal densities per unit area at various regions of each animal. Finally, with these averages, a student t-test was performed to investigate whether changes in GSK3β immunoreactivity were statistically significant (*$p < 0.05$, Table 1).

Table 1. Changes in mean densities of GSK3β immunoreactivity in the spinal cord, brain stem, and cerebellum of SOD1^{G93A} mutant transgenic mice

Area	wtSOD1 (12 animals)	SOD1^{G93A} mutant (15 animals)
Spinal cord		
Anterior horn	95.27±5.51	117.43±9.51*
Brain stem		
Hypoglossal nucleus	95.07±9.37	103.29±11.98*
Facial nucleus	93.37±13.48	101.57±6.33
Pontine reticular nucleus	93.93±4.66	105.23±5.22*
Cerebellum		
Cerebellar cortex	87.23±3.90	89.33±2.23
Deep cerebellar nuclei	89.62±5.97	113.47±5.10*

Mean density is the sum of the gray values of all the pixels in the section that was divided by the number of pixels within the selection. Values are the mean±standard deviations. Student's t-test was performed (*$p < 0.05$).

Results

In preadsorption test, sections reacted without primary antibody and different samples exposed to anti-GSK3β antibody which had been preadsorbed with GSK3β blocking peptide did not exhibit any immunoreactivities, while sections reacted with anti-GSK3β antibody showed definite GSK3β-immunoreactivity. With these antibodies, many GSK3β-immunoreactive neurons were found in the cervical spinal cord of 8w- and 13w-old wtSOD1 transgenic mice. Compared with 8w- and 13w-old wtSOD1 transgenic mice, there was little GSK3β-immunoreactivity change in 8w- and 13w-old presymptomatic SOD1^{G93A} transgenic mice (Fig. 1A-D). GSK3β immunoreactivity was increased significantly in the cervical spinal cord of symptomatic SOD1^{G93A} transgenic mice, compared with wtSOD1 transgenic mice and these increases were more prominent in the anterior horn (Fig. 1E-H). In high power views, these increased GSK3β immunoreactivity (Fig. 1F) were found to be due to increased immunoreactivity in the neurons (Fig. 2H). Although data are not presented, the thoracic and lumbar spinal cord of symptomatic SOD1^{G93A} transgenic mice showed similar changing patterns.

GSK3β immunoreactivity was also observed to be significantly increased in the brain stem of symptomatic SOD1^{G93A} transgenic mice (Fig. 2A, B). As in the

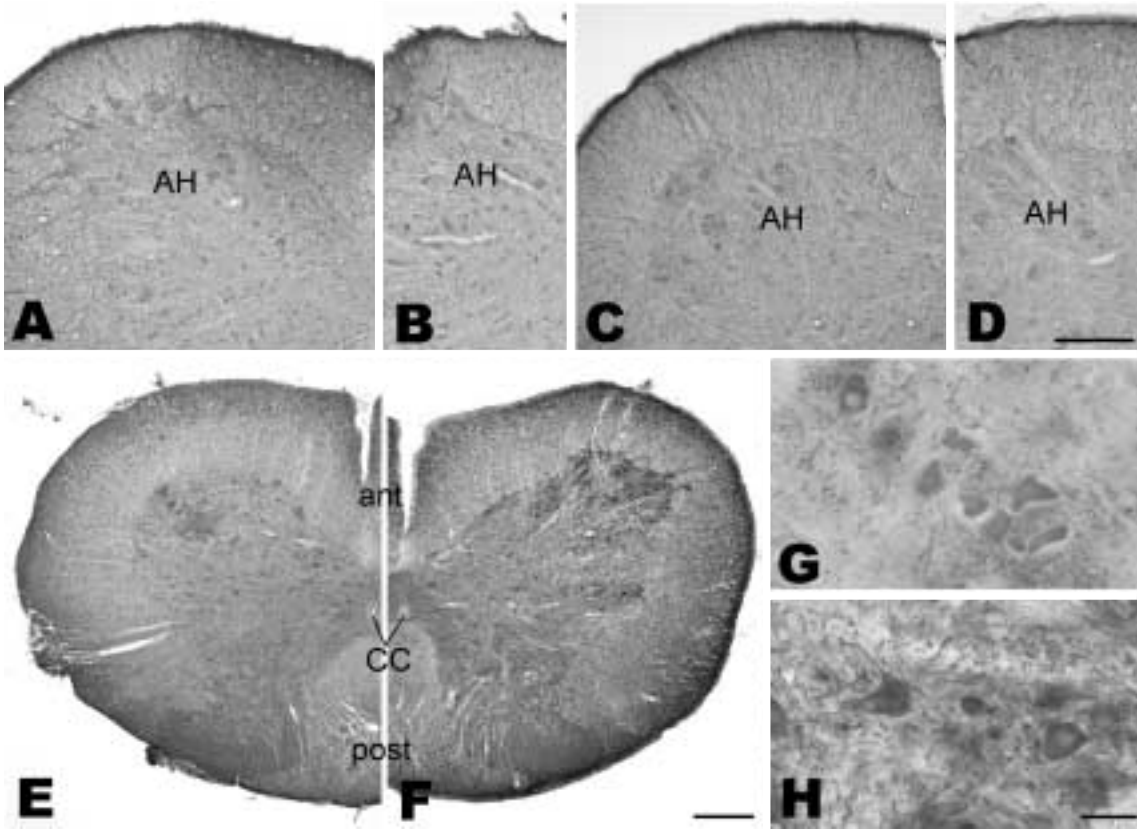

Fig. 1. GSK3β-immunoreactivity changes in the cervical spinal cord of presymptomatic and symptomatic SOD1^{G93A} transgenic mice. In the anterior horn of the cervical spinal cord of both groups, GSK3β-immunoreactivity was observed in the neurons. There was no GSK3β-immunoreactivity change in 8w- and 13-w old SOD1^{G93A} transgenic mice (A, C), compared with 8w- and 13w-old wtSOD1 transgenic mice (B, D). In the cervical spinal cord of symptomatic SOD1^{G93A} transgenic mice, GSK3β-immunoreactivity was increased (F). In high power views of the anterior horn, increase of GSK3β-immunoreactivity was confined to the neurons (H). AH, anterior horn; ant, anterior; CC, central canal; post, posterior. Scale bar=100 μm (A-D); 200 μm (E, F); 40 μm (G, H).

spinal cord, increases of GSK3β immunoreactivity took place in neurons (inset in Fig. 2B). The cerebellar cortex of both wtSOD1 and symptomatic SOD1^{G93A} transgenic mice had few GSK3β-immunoreactive neurons (Fig. 2C, D). In contrast to the cerebellar cortex, neurons in the deep cerebellar nuclei of both groups showed definite GSK3β immunoreactivity and like other regions GSK3β immunoreactivity was observed to be significantly increased in the neurons (Fig. 2E, F, Table 1) of symptomatic SOD1^{G93A} transgenic mice.

Discussion

By this work, we demonstrated that neurons in the spinal cord, brain stem, and cerebellum of symptomatic SOD1^{G93A} transgenic mice show increased GSK3β immunoreactivities (Figs. 1, 2. Table 1), while GSK3β immunoreactivity changes were hardly detected in 8w- and 13w-old presymptomatic SOD1^{G93A} transgenic mice (Fig. 1A-D). Regarding that GSK3β immuno-

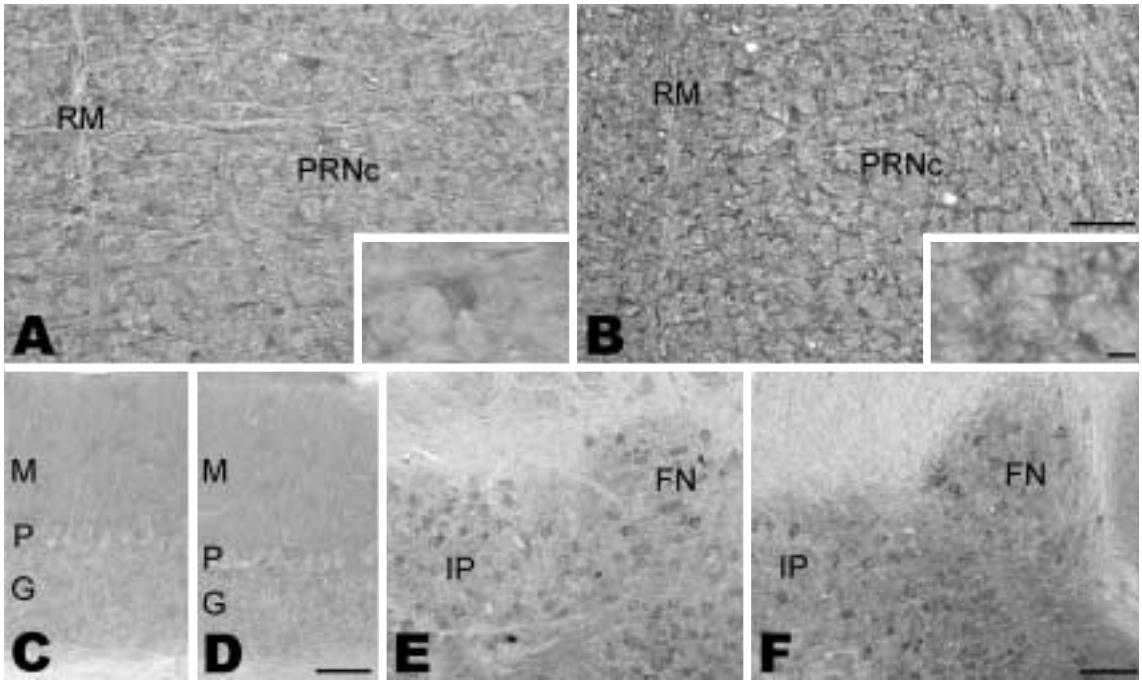

Fig. 2. Increased GSK3β-immunoreactivity in the brainstem and cerebellum of symptomatic SOD1^{G93A} transgenic mice. Compared with wtSOD1 transgenic mice (A), GSK3β-immunoreactivity was increased in symptomatic SOD1^{G93A} transgenic mice (B). Increase of GSK3β-immunoreactivity was confined to the neurons (inset in B). Insets in A and B show detailed GSK3β-immunoreactive cell morphologies. In the cerebellar cortex of wtSOD1 (C) and symptomatic SOD1^{G93A} transgenic mice (D), GSK3β-immunoreactive neurons were hardly observed. In the deep cerebellar nuclei of wtSOD1 transgenic mice, GSK3β were observed to be expressed in the neurons (E). In symptomatic SOD1^{G93A} transgenic mice, GSK3β-immunoreactivity was increased in the neurons (F). FN, fastigial nucleus; G, granular layer; IP, interposed nucleus; M, molecular layer; P, Purkinje cell layer. PRNc, pontine reticular nucleus, caudal part; RM, nucleus raphe magnus. Scale bar=100 μm (A, B, E, F); 20 μm (Insets in A, B); 50 μm (C, D).

reactivity changes were not detected before the development of ALS symptoms, increases of GSK3β immunoreactivity seem to coincide with symptom developments. As GSK3β distribution patterns in the spinal cord and brain stem of mice has not been reported, their distribution patterns observed in this study can not be compared with the previous studies. But in the cerebellum of 5w-old rat, GSK3β was reported to be expressed by the granular layer (Takahashi et al. 1994). Although these are not in accord with the present results, these may be resulted from differences in species. As GSK3β expressions in the brain are greatly altered during development (Takahashi et al. 1994), these can be caused by different ages of animals.

GSK3 has two closely related isoforms, which are encoded by different genes. These two isoforms share 97% sequence similarity within their kinase catalytic domains but outside of the kinase domains, their sequences differ substantially (Frame and Cohen 2001, Jope and Johnson 2004). As GSK3 activity is tightly regulated by many mechanisms (Grimes and Jope 2001, Tanji et al. 2002) and is involved many different cellular signal pathways, i.e., Wnt, Hedgehog, and insulin signaling (Frame and Cohen 2001, Jope and Johnson 2004), these substantial sequence differences outside of the kinase domains suggest that these two

isoforms are differentially regulated. In addition, their different localizations in cells (Hoshi et al. 1995) support they are differentially regulated and differentially exert their functions although little is known about isoform-specific functions. In the present study, it was found that GSK3β expressions were differentially changed in symptomatic $SOD1^{G93A}$ transgenic mice. Therefore GSK3β seem to be differentially regulated during the development and/or progression of ALS and accordingly make different effects on the neurons, which need to be further elucidated.

GSK3 makes tau be unable to bind to microtubule and form aberrant filament assembly (Hanger et al. 1992, Mandelkow et al. 1992, Jope and Johnson 2004). These functions suggest GSK3 may be a key enzyme that regulates tau and β-APP metabolisms in the neurons. In the present study, GSK3β was found to be significantly increased in the neurons of symptomatic $SOD1^{G93A}$ transgenic mice and previously it was reported that tau (Yang et al. 2003) and β-APP immunoreactivities (Sasaki and Iwata 1999) are increased in the neurons of ALS patient. Therefore, increases of tau and β-APP in ALS patient could be mediated by increased GSK3β in the neurons. Also regarding GSK3β which elicits neurotoxicity of Aβ peptide (Takashima et al. 1993, Jope and Johnson 2004) are increased in the neurons of symptomatic $SOD1^{G93A}$ transgenic mice, it can be postulated that increased GSK3β in the neurons might elicit neurotoxicity of increased β-APP (Sasaki and Iwata 1999) and facilitate the neuronal death in the development and/or progression of ALS.

For the first time, we demonstrate that neurons in the spinal cord, brain stem, and cerebellum of symptomatic $SOD1^{G93A}$ transgenic mice show increased GSK3β immunoreactivities, while few GSK3β immunoreactivity changes were detected in 8w- and 13w -old presymptomatic $SOD1^{G93A}$ transgenic mice. These data suggest the possibility that GSK3 functions as a modulating factor of apoptosis-related alterations in ALS and that GSK3β exert differential functions in the development and/or progression of ALS. But the exact functional significances of these changes require further elucidation.

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SOD1^{G93A} transgenic mice의 중추신경계에서 glycogen synthase kinase (GSK) 3β의 분포에 관한 면역조직화학적 연구

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간추림 : 본 연구에서 glycogen synthase kinase (GSK) 3β가 근위축성측삭경화증(ALS)의 발병과 진행과정에서 어떤 영향을 미치는지 알아보고자 하였다.

실험동물로는 ALS의 *in vivo* model인 SOD1^{G93A} transgenic mice를 이용하였고, 면역조직화학적 방법을 사용하여 GSK3β의 발현변화를 연구하였다.

연구결과, 증상을 보이는 SOD1^{G93A} transgenic mice의 척수, 뇌줄기, 소뇌의 신경세포에서 증가된 GSK3β 면역염색성을 나타냈다. 이와 대조적으로, 증상을 보이기 전인 8주령과 13주령의 SOD1^{G93A} transgenic mice에서는 GSK3β 면역염색성을 거의 관찰할 수 없었다.

본 연구결과는 GSK3가 ALS의 apoptosis와 관련된 변화의 조절인자로서 작용할 가능성을 제시하였고, 또한 ALS의 발병과 진행과정에서 다르게 작용할 가능성도 보여주었다. 그러나 이러한 변화에 대한 정확한 기능적인 의미에 대해서는 더 많은 연구가 이루어져야 할 것이다.

찾아보기 낱말 : glycogen synthase kinase (GSK) 3β, 근위축성측삭경화증(ALS), SOD1^{G93A} transgenic mice, 척수, 뇌줄기, 소뇌

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