Neuroprotective Effect of Rapamycin (Autophagy Enhancer) in Transgenic SOD1–G93A Mice of Amyotrophic Lateral Sclerosis

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Background: The autophagy is the major route for lysosomal degradation of misfolded protein aggregates and oxidative cell components. We hypothesized that rapamycin (autophagy enhancer) would prolong the survival of motor neuron and suppress the disease progression in amyotrophic lateral sclerosis (ALS). Methods: A total of 24 transgenic mice harboring the human G93A mutated SOD1 gene were used. The clinical status involving rotarod test and survival, and biochemical study of ALS mice model were evaluated. Results: The onset of symptoms was significantly delayed in the rapamycin administration group compared with the control group. However, after the clinical symptom developed, the rapamycin exacerbated the disease progression and shortened the survival of ALS mice model, and apoptosis signals were up-regulated compared with control group. Conclusions: Even though further detailed studies on the relevancy between autophagy and ALS will be needed, our results revealed that the rapamycin administration was not effective for being novel promising therapeutic strategy in ALS transgenic mice and exacerbated the apoptosis.

Key Words: Autophagy, Rapamycin, Amyotrophic lateral sclerosis, ALS

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by progressive motor weakness and muscular wasting caused by degeneration of upper and lower motor neurons. Oxidative injury, misfolded protein aggregate, altered axonal transport, impaired energy and calcium metabolism, excess of glutamate activity, and malfunctioning mitocho-
dria have been proposed to be implicated in motor neuron death in ALS, even if the definite pathomechanism of ALS is uncertain. Among them, the recent awareness of altered transactive response (TAR) DNA binding protein 43 (TDP-43), misfolded SOD1, and FUS protein in both familial ALS and sporadic ALS suggests that ALS might be a proteinopathy. Most of these protein aggregates are physiological substrates of the autophagy pathway, which is reported as a key cellular response that degrade mutated or aggregated proteins and removes damaged mitochondria including neurons in the central nervous system. In previous studies on neurodegenerative disorder, the autophagy is the major route for lysosomal degradation of misfolded protein aggregates and oxidative cell components including SOD1, alpha-synuclein, huntingtin, and altered mitochondria. Interestingly, most of the identified mutant proteins involved in familial ALS lead to an impairment of the autophagy pathway. Thus, the study of the autophagy pathway and its therapeutic application would be important in ALS.

Given that apoptosis and autophagy play a central role in motor neuron degeneration and can contribute to neuronal death through distinctive route in ALS, we hypothesized that up-regulation of autophagy would prolong the survival of motor neuron and suppress the disease progression in ALS. For the pharmacological autophagy enhancer, the rapamycin which is a well known autophagy activating agent, was used in this study.

Methods

All procedures on animals were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines of Seoul National University for the care and use of laboratory animals (SNU-200910-20).

Materials and animals

In order to enhance the autophagy, R-5000 rapamycin, which is a FDA-approved macrolide antibiotics and immuno-suppressant, was purchased from LC Laboratories (C51H79-NO13, M.W. 914.17, 165 New Boston Street Wobum, MA 01801, USA), and was dissolved in DMSO and PBS.

A total of 24 transgenic mice harboring the human G93A mutated SOD1 gene [B6SJL-Tg (SOD1-G93A)1 Gur/J; Jackson Laboratory, Bar Harbor, Me, USA] and 6 wild type mice were used following confirmation of their genotype. The 24 transgenic mice were equally divided into 2 groups; transgenic control group and rapamycin administration group. In the rapamycin administration group, 0.5 ml of PBS was mixed with 4 micrograms of rapamycin per gram of mouse, and injected intraperitoneally into 12 animals 3 days a week beginning 60 days after birth. The other 12 transgenic ALS mice and 6 wild type mice were injected intraperitoneally with 0.5 ml normal saline and 0.5 ml PBS for comparative study. The 6 mice of each group were used for rotarod test and all sacrificed at the disease endpoint described below. And, to investigate the effects of the rapamycin on the apoptosis signals, 6 mice from each of the transgenic ALS mice control group and transgenic ALS mice rapamycin administration group were selected. These animals were sacrificed at 70 (n=2), 90 (n=2) and 110 days (n=2) after birth and used for the evaluation of western blotting. In addition, 6 wild-type mice were also sacrificed at the same age and used in western blotting.

Clinical evaluation of symptoms

Behavioral experiments were conducted in a single-blind fashion. The clinical status of the ALS mice model was evaluated 3 times per week beginning 60 days after birth. Symptom onset was defined as shaking of the limbs when the mouse was suspended in the air by its tail, which may be due to the clinical involvement of the upper motor neuron system. At this stage, clonus, hyperreflexia, and crossed spread of spinal reflexes were also detectable in most mice. The endpoint was defined when the mice were unable to right themselves within 30 seconds after being placed on their sides on a flat surface, and the animals were sacrificed. Rotarod test was conducted in a single-blind fashion. Mice were subjected to a 1-week learning period 53 days after birth, during which they were able to perform on an accelerating rotarod. The rotarod test was performed over 3 days a week. The time spent walking on the rotarod was measured, and the time evaluated through 3 repeats was averaged. When the mean time was less than 10 seconds, it was defined as rotarod failure.

The activities of apoptotic signals

To evaluate the effect of the rapamycin on the ALS mice model, western blotting of caspase-3 and caspase-8 antibodies of apoptosis signals was performed. For western blotting, mice were anesthetized with pentobarbital sodium and intracardiac perfusion of PBS was carried out. Subsequently, the spinal cord of each mouse was quickly removed, cooled in ice-cold...
artificial CSF for 5 minutes, and stored in a −80 °C freezer. The spinal cord was homogenized with a 1.0 ml homogenizer and type B pestle in a 10:1 volume/weight buffer containing 10 mM Tris (pH 7.4), 10 mM EGTA, 250 mM sucrose, 2 μg/mL aprotinin, 5 μg/mL leupeptin, 2 μg/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Tissue lysates were centrifuged at 17,000×g for 10 min, and the supernatants were used for the evaluation of caspase-3 and caspase-8 (Cell Signaling Tech, Beverly, MA, USA).

Statistical analysis
The data are expressed as mean±standard error. The data were analyzed with Mann-Whitney statistical method, and difference with p-values less than 0.05 were considered statistically significant. Cumulative probabilities of symptom onset, rotarod failure, and disease endpoint were analyzed with the Kaplan-Meier survival analysis.

Results

Results of the rotarod test showed that coordinate and strength in G93A transgenic mice was increasingly impaired over time; however the motor function deficits of ALS transgenic mice were significantly alleviated by 15 weeks in G93A mice administrated with rapamycin (A). In the rapamycin administration group, the behavioral function using rotarod test was significantly better in early stage of ALS (Mann-Whitney U test, *p<0.05) (A). However the decline of the rotarod function was more rapid in the rapamycin administration group than the control group after 15 weeks (A). Similarly, the onset of symptoms was significantly delayed by the rapamycin administration (B), whereas the time of rotarod failure (C) and disease end point (D) were shortened in the rapamycin administration group compared with the control group.

Figure 1. Results of rotarod test showed that coordinate and strength in G93A transgenic mice was increasingly impaired, however the motor function deficit was alleviated by 15 weeks in G93A mice administrated with rapamycin (A). In the rapamycin administration group, the behavioral function using rotarod test was significantly better in early stage of ALS (Mann-Whitney U test, *p<0.05) (A). However the decline of the rotarod function was more rapid in the rapamycin administration group than the control group after 15 weeks (A). Similarly, the onset of symptoms was significantly delayed by the rapamycin administration (B), whereas the time of rotarod failure (C) and disease end point (D) were shortened in the rapamycin administration group compared with the control group.
with rapamycin (Fig. 1A). Statistical analysis of the rotarod test identified the remarkable improvement of the motor function deterioration by the rapamycin administration in the ALS transgenic mice (Fig. 1A). In addition, the onset of symptoms was significantly delayed in the rapamycin administration group compared with the control group (Table 1, Fig. 1B; $p=0.015$). These findings indicate that the rapamycin may have neuroprotective effects on the ALS mice model with G93A mutant human SOD1 gene in the initial period of ALS progression.

However, unexpectedly, the time of rotarod failure and disease end point (survival) were shortened in rapamycin administration compared with the control group, although the results were not statistically significant (Table 1, Fig. 1C, D). Consequently, the decline of the rotarod function was more rapid in the rapamycin administration than the controls (Fig. 1A).

The effects of the rapamycin on the apoptosis were investigated using caspase-3 and 8 antibodies. With the rapamycin administration, the levels of cleaved caspase-8 were increased in the spinal cord of 110-day-old G93A transgenic ALS mice compared with control, whereas the levels of cleaved caspase-3 was not significant (Fig. 2). The expression levels of these proteins were also verified by band analysis of western blot (fold change of caspase-8; 1.034762 vs. 1.272831, fold change of caspase-3; 1.346501 vs. 1.345572 between control and rapamycin administration). These findings indicate that the extrinsic apoptosis pathway may be activated by the rapamycin administration, and also these results are consistent with the behavior test.

**Discussion**

This study was designed to investigate the therapeutic effect of rapamycin on the motor performance, onset of symptoms and survival of ALS in the mouse model carrying the human G93A mutated Cu/Zn-SOD1 gene, and conclusively to identify the possibility as a novel promising therapeutic strategy in ALS. In summary, the rapamycin administration in ALS mice model improved the behavioral performance and delayed the onset of symptom relative to the control group in the pre-symptomatic stage of ALS (Fig. 1A, B). Therefore, these results revealed that the autophagy might be related to disease progression, and suggested that the rapamycin administration could potentially be a promising neuroprotective strategy by the presymptomatic stage of ALS. However, after the clinical symptoms of motor weakness appeared in the ALS mice, the rapamycin administration inconsistently exacerbated the disease progression and shortened the survival of ALS mice model (Table 1, Fig. 1C, D).

Autophagy, which is a major degradation pathway for various intra-cytosolic, aggregated and disease-causing protein and organelles which are associated various neurodegenerative diseases involving ALS, has received significant attention as a novel therapeutic pathomechanism in recent years, and has possible implication in several human diseases such as cancer and neurodegenerative disorders. Autophagy is negatively regulated by mTOR (mammalian target of rapamycin), whose activity can be inhibited by rapamycin, a lipophilic macrolide antibiotic that is a well established inducer of autophagy. Rapamycin forms a complex with the immunophilin FK506-binding protein 12 (FKBP12), which then sta-

### Table 1. Summary of behavior studies involving symptom onset, rotarod failure, and disease endpoint. The onset of symptoms was significantly delayed in the rapamycin administration group compared with the control group. However the time of rotarod failure and disease end point were shortened in rapamycin treatment group relative to the control group, although the results were not statistically significant

<table>
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<tr>
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<th>Tg mice control</th>
<th>Rapamycin treatment</th>
<th>Mann-Whitney U test</th>
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<tbody>
<tr>
<td>Onset of symptom (days)</td>
<td>102.5±1.8</td>
<td>109.3±1.3</td>
<td>0.015</td>
</tr>
<tr>
<td>Rotarod failure (days)</td>
<td>123.0±3.3</td>
<td>119.2±1.2</td>
<td>0.394</td>
</tr>
<tr>
<td>Disease endpoint (days)</td>
<td>128.0±1.9</td>
<td>125.3±1.3</td>
<td>0.310</td>
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</tbody>
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Values are presented as mean±SD.

**Figure 2.** With the rapamycin administration, the levels of cleaved caspase-8 were increased in the spinal cord of 110-days-old G93A transgenic ALS mice, whereas the level of cleaved caspase-3 was not significant between two groups.
bilizes the raptor-mTOR association and inhibits the kinase activity of mTOR. Autophagy upregulation with rapamycin also protects cells against proapoptotic insults by clearing mitochondria, which are endogenous autophagy substrates. And, the down-regulation or partial inhibition of autophagy sometimes provokes or aggravates neurodegeneration. Of course, the excessive activation of autophagy sometimes produces self cannibalism, an auto-digestion process that may lead to autophagic cell death. Thus, the beneficial or detrimental contribution of autophagy in the pathogenesis and progression of ALS strictly depends on time and quantitative consideration.

On the basis of the theoretical roles of autophagosis, we assumed that the rapamycin administration would be effective in ALS mice via up-regulation of autophagy. However, our results showed that the rapamycin administration might be rather ineffective on ALS transgenic mice and exacerbated the apoptosis pathway. The previous research revealed that treatment with rapamycin accelerated the motor neuron degeneration, reduced the survival of the ALS mice, and additionally demonstrated that rapamycin treatment in the ALS mice causes more severe mitochondrial impairment, higher Bax levels and greater caspase-3 activation. These findings suggest that selective degeneration of motor neurons is associated with the impairment of the autophagy pathway and that rapamycin treatment may exacerbate the pathological processing through apoptosis and other mechanisms in the ALS mice. However, the precise role and detailed pathomechanism of autophagy in ALS were not defined in previous documents. Interestingly, in our study, we verified the increased extrinsic apoptotic signals via western-blot of cleaved caspase 8 in the rapamycin administration group. However, we did not find the significant change of intrinsic apoptotic signal in the biochemical study. Therefore, further studies on these inconsistent results will be necessary for verifying the relevance of intrinsic and extrinsic apoptosis with autophagy.

The limitations of this study are that the number of mice used in this study was too small to identify the statistical significance, and more detailed research using different manners of administration with rapamycin was not performed. In addition, the numbers of cell biomarkers on motor neuron and cellular signals used in the study was relatively small.

Nevertheless, our study identified that the rapamycin administration in ALS transgenic mice might potentially be neuroprotective by the presymptomatic stage, however conclusively, accelerated the motor neuron degeneration and reduced the survival of the ALS mice via exacerbating the pathological apoptosis processing.

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