Inducible Nitric Oxide Synthase (iNOS) and Nitrotyrosine Immunoreactivity in the Spinal Cords of Transgenic Mice with a G93A Mutant SOD1 Gene

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Abstract. We performed a prospective, longitudinal immunohistochemical study of the spinal cords of transgenic mice with a G93A mutant SOD1 gene at 4 fixed points in time, using antibodies to inducible nitric oxide synthase (iNOS) and nitrotyrosine. The purpose of this study was to characterize the temporal and topographic distribution of iNOS and nitrotyrosine immunoreactivity in the spinal cord over a certain period, thus illuminating the possible role of increased oxidative damage to the motor system in the neurodegenerative process in this animal model. Specimens from age-matched non-transgenic wild-type mice served as controls. The control mice showed no positive iNOS or nitrotyrosine immunoreactivity in the somata of anterior horn neurons or their neuronal processes at any age. On the other hand, the transgenic mice demonstrated a common immunostaining pattern of iNOS and nitrotyrosine in the anterior horn neurons. When the mice reached the age of 24 wk (early presymptomatic stage), the anterior horn neurons and their neuronal processes were occasionally immunostained for iNOS and nitrotyrosine; at 28 wk (late presymptomatic stage), the anterior horn neurons were not uncommonly immunostained; at 32 wk (early symptomatic stage) and 35 wk (end-stage), positive iNOS and nitrotyrosine immunoreactivity was frequently observed in proliferated reactive astrocytes as well as in the soma of the anterior horn cells. The selective localization of positive iNOS and nitrotyrosine immunoreactivity in the anterior horn neurons suggests that oxidative stress may be involved in the pathomechanism of degeneration of motor neurons in this transgenic animal model.

Key Words: Amyotrophic lateral sclerosis; Immunohistochemistry; Inducible nitric oxide synthase; Nitrotyrosine; SOD1 mutation; Spinal cord; Transgenic mice.

INTRODUCTION

Nitric oxide (NO) is one of the reactive oxygen species and a short-lived, free radical that plays several important biological roles (1-3). So far, 3 distinct NO-synthesizing isoenzymes have been purified and molecularly cloned (4). The first 2 are constitutive cytosolic neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS), and they are both Ca2+-dependent. The third is a cytosolic Ca2+-independent inducible isoform (iNOS) that is induced in many cell types by inflammatory stimuli leading to the production of large quantities of NO (5). nNOS is the main NOS isoform in the brain, as its catalytic activity and protein are identifiable throughout the central nervous system (6, 7). In contrast, iNOS normally is not (8) or is minimally (9) expressed in the brain. However, in pathological conditions such as brain ischemia, NO demonstrates either protective or detrimental characteristics (10), and iNOS expression can increase in brain glial cells (11) and in invading macrophages in response to a variety of injuries (12, 13). As NO is rapidly broken down into more stable oxidation products, nitrotyrosine is widely used as an indicator of NO formation because nitrotyrosine reflects protein nitration by NO-derived peroxynitrite and nitrate (14). Excessive NO formation, which leads to free radical production, has been implicated in the neurotoxicity associated with several neurodegenerative conditions including sporadic and familial amyotrophic lateral sclerosis (ALS) (15-17). Moreover, iNOS and peroxynitrite-mediated tyrosine nitration have been implicated in the pathogenesis of a number of nervous system diseases (18, 19). On the other hand, the alternative hypothesis has been proposed related to the property of NO in irreversible inhibition of mitochondrial function and damage to a variety of mitochondrial components via oxidizing reactions, and the potential for neurodegeneration related to reduced ATP synthesis (20). However, little is known about the expression of iNOS and nitrotyrosine immunoreactivity in the spinal cords of ALS patients (21-25) and of transgenic mice with a G93A mutant SOD1 gene (26, 27).

About 20% of patients with familial amyotrophic lateral sclerosis (FALS) exhibit a SOD1 mutation. Subsequently, various kinds of SOD1 transgenic mice have been produced. Among them, a relatively low expressor line of transgenic mice carrying a mutant human SOD1 gene develops pathological changes that most closely resemble those in human ALS (28). We carried out an immunohistochemical study of the spinal cords of a relatively low expressor line of transgenic mice carrying the mutant SOD1 gene, using anti-iNOS and nitrotyrosine antibodies. The purpose of this study was to characterize the temporal and topographic distribution of iNOS and nitrotyrosine immunoreactivity in the spinal cord over the course of time,
Fig. 1. Normal-appearing large anterior horn neurons are not immunostained by the anti-iNOS antibody, while there is granular positive immunoreactivity in the neuropil of the gray matter at the cervical cord (age 32 wk, non-transgenic littermate).
thus illuminating the possible contribution of increased oxidative damage to the motor system to the neurodegenerative process in this transgenic animal model.

MATERIALS AND METHODS

Experimental Animals and Clinical Assessment

Transgenic mice expressing G93A mutant human SOD1 were used in this study (29). They were originally obtained from the Jackson Laboratory (B6SJL-TgN (SOD1-G93A) 1 Gur®. Bar Harbor, ME) and were backcrossed to a C57BL/6 background by mating hemizygote males with inbred C57BL/6 female mice (C57BL/6CrlScI, Nihon SLC, Shizuoka, Japan) to produce transgenic (Tg) and non-transgenic (non-Tg) littermates. Their backcrossing on to the black 6 background has entirely eliminated the SJL (dysferlin gene associated FSH dystrophy) background used for these studies. The transgenic progeny were identified by polymerase chain reaction (PCR) amplification of tail DNA with specific primers for exon 4 (30).

At around 32 wk of age, the G93A transgenic mice developed progressive muscle weakness and spasticity in one or more limbs beginning with a posterior limb. One to 2 wk later, they could not feed themselves due to severe paralysis expressed by the hyperextension of their hindlimbs. The G93A Tg and non-Tg mice were examined simultaneously. They were identified by polymerase chain reaction (PCR) amplification of tail DNA with specific primers for exon 4 (30).

Histopathological Analysis

Seven Tg and 7 non-Tg wild-type mice were killed at ages ranging from 24 to 35 wk. All mice were deeply anesthetized with ether and perfused intracardially with heparinized saline (pH 7.4) followed by perfusion with ice-cold 4% paraformaldehyde (Katayama Chemical, Osaka, Japan) in 0.1 M phosphate buffer (pH 7.4). The spinal cords were rapidly removed and post-fixed by immersion in the same fixative (5 days, 4 °C). Cross-sections of the spinal cord were embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin.

Immunocytochemistry

The primary antibodies used in this study were as follows: a polyclonal anti-human iNOS antibody (Upstate Biotechnology, Lake Placid, NY; diluted 1:50); a polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology; diluted 1:100), and a monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (Dako; 1:400). We employed the antibody to iNOS, which had been purified from RAW 264.7 cells (macrophages) activated by γ-interferon and bacterial lipopolysaccharide, and which cross-reacts with human iNOS (Upstate Biotechnology). Sections (4-μm-thick) of the paraffin-embedded spinal cords were deparaffinized, treated with nonimmune serum as the blocking reagent, quenched with 3% H2O2, and incubated overnight at 4 °C with the primary antibodies. An autoclave pretreatment (121°C, 10 minutes [min]) was used for iNOS immunostaining. Antibody binding was visualized by the avidin-biotin-immunoperoxidase complex (ABC) method using an Elite ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer’s recommendations. 3,3′-Diaminobenzidine tetrahydrochloride (DAB) was the final chromogen. Selected sections were incubated with the antibody against iNOS, which had been preabsorbed with excess amounts of iNOS (diluted 0.1 mg/ml). Sections from which the primary antibody was omitted served as negative reaction controls. For double immunostaining for GFAP and iNOS, immunoreaction product deposits for GFAP were detected with the ABC method using DAB as the chromogen, and those for iNOS were visualized with the peroxidase-antiperoxidase method using NiCl2-DAB as the chromogen.

Crossed immuno-absorption tests of iNOS against nNOS and eNOS were performed to rule out the possibility that the antibody to iNOS cross-reacted with nNOS and eNOS.

Immunoblotting

For the immunoblotting, the lumbar spinal cords of the Tg and non-Tg mice (n = 4 per group, respectively) were quickly removed from deeply anesthetized animals. Each spinal cord was individually homogenized in a lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.6], 1 mM EDTA, 0.25 M sucrose, 1 mM phenylmethylene sulfonyl fluoride, and 5 μg/ml aprotinin). The solubilized samples were centrifuged at 8,000 g for 15 min...
RESULTS

iNOS Immunoreactivity

Non-Transgenic Littermates: No iNOS immunoreactivity was observed in the somata of the neurons, including the anterior horn neurons and posterior horn neurons (Fig. 1), nor in the blood vessels, white matter, or axons in the anterior and posterior roots. On the other hand, dot-like or granular deposits of iNOS immunoreactivity were recognized in the neuropil of the gray matter, including the anterior horns, posterior horns, and Clarke’s column at all levels of the spinal cord. There was no difference in the expression of immunoreactivity among mice of different ages.

Transgenic Mice: When the mice had reached the age of 24 wk (early presymptomatic stage), no neuronal depletion or vacuolar changes were observed in the anterior horns. Immunocytochemically, most anterior horn neurons and their neuronal processes were not immunostained for iNOS, but some normal-appearing anterior horn neurons showed positive iNOS immunoreactivity in the somata and their neuronal processes (Fig. 2). When the mice had reached 28 wk of age (late presymptomatic stage), we recognized a slight neuronal loss of anterior horn cells and prominent vacuolar changes of various sizes in the neuropil of the anterior horns and anterior roots in the anterior column. Lewy body-like inclusions were occasionally observed in the neuropil of the anterior horns. Normal appearing and, to a lesser extent, degenerated anterior horn cells occasionally showed iNOS immunopositivity in the perikarya and their neuronal processes. There was moderate increase of reactive astrocytes that immunostained for iNOS in the neuropil of the anterior horns. In the neuropil of the anterior horns, GFAP-positive reactive astrocytes were more intensely immunostained for iNOS as compared with controls. At 32 wk (early symptomatic stage), the anterior horns showed a moderate neuronal loss of anterior horn cells accompanied by astrogliosis. Vacular changes were observed in the neuropil of the anterior horns and the anterior roots, and Lewy body-like inclusions were frequently observed within the neuronal processes in the anterior horns. Cord-like swollen axons frequently containing Lewy body-like inclusions were often seen in the anterior horns. At 35 wk (end-stage), there was a severe neuronal loss of the anterior horn neurons accompanied by prominent astrogliosis, and Lewy body-like inclusions were frequently observed in the neuronal processes. However, vacuolar changes were less prominent in the anterior horns. At the symptomatic stage and especially at the end-stage, the immunoreactivity of iNOS was frequently observed in the anterior horn cells (Fig. 3), particularly in neurons (Fig. 4A) showing degenerative changes such as central chromatolysis, simple atrophy and cord-like swollen axons (Fig. 4B). Some normal-appearing anterior horn neurons still remained and were immunostained for iNOS (Fig. 5). Concurrently, reactive astrocytes markedly increased in number and were immunoreactive with the anti-iNOS antibody. Double label immunostaining of GFAP and iNOS demonstrated GFAP and iNOS co-localization on reactive astrocytes (Fig. 6). Some axons in the anterior roots were immunostained for iNOS.

There was no iNOS immunoreactivity in the somata of neurons in the gray matter of the posterior horns and Clarke’s columns, nor in the white matter including that of the corticospinal tracts. The blood vessels were not immunostained for iNOS. Neither the sections processed without the primary antibody nor the sections incubated with the preabsorbed antibody showed any immunoreaction product deposits (Fig. 7).

Nitrotyrosine Immunoreactivity

Non-Transgenic Littermates: There was no nitrotyrosine immunoreactivity in the gray matter including the anterior horn cells, nor in the white matter including that of the corticospinal tracts, at any age (Fig. 8).

Transgenic Mice: When the mice had reached the age of 24 wk, most anterior horn neurons were negatively immunostained for nitrotyrosine, but the somata of some anterior horn cells were immunostained. At 28 wk, it was not uncommon for anterior horn neurons to be immunostained for nitrotyrosine (Fig. 9). Moderately proliferated reactive astrocytes in the anterior horn were immunoreactive with the anti-nitrotyrosine antibody. At the symptomatic stage and especially at the end-stage, the
Fig. 12. Dot blotting of iNOS, nNOS and eNOS. iNOS reacted with its respective antibody (anti-iNOS), but not with anti-nNOS or anti-eNOS. Conversely, nNOS was labeled by anti-nNOS, but not with anti-iNOS or anti-eNOS. eNOS reacted with anti-eNOS, but not with anti-iNOS or anti-nNOS.

Fig. 13. Non-transgenic mice show no detectable bands (lane 1), while samples of transgenic mice reveal a specific band with molecular weight of approximately 130 kDa corresponding to immunoreactive iNOS protein (lane 2, arrow).

DISCUSSION

To our knowledge, this is the first report of iNOS and nitrotyrosine immunoreactivity in the anterior horn cells of SOD1 transgenic mice observed at fixed points in time throughout the course of the disease. We recognized positive iNOS and nitrotyrosine immunoreactivity in normally appearing anterior horn cells as early as the presymptomatic stage. We also found that the immunoreactivity increases with the progression of anterior horn cell degeneration and the proliferation of reactive astrocytes. These findings suggest that oxidative damage in SOD1 transgenic mice may initiate the degeneration of motor neurons and take part in the progression of anterior horn cell degeneration leading to neuronal death.

In pathological conditions, NO exhibits a Janus face with either neuroprotective or detrimental characteristics (32). NO may perform a protective function by scavenging superoxide free radicals generated by excitotoxic mechanisms (33) and may be involved in the regenerative response of the motor neuron (34). On the other hand, the reduced form of NO is able to restrict Ca$^{2+}$ entry into neurons by modifying the activity of the NMDA receptor (35), and this activity protects against neurotoxicity. The administration of the NOS inhibitor nitroarginine has been shown to rescue motor neurons that would otherwise have died in response to ventral root avulsion (36). In the mutant mouse wobbler, abnormal nNOS immunopositivity could be found in the degenerating motor neurons, while nNOS is not normally found in the somatic motor neurons, suggesting that induction of nNOS expression may play a role in motor neuron death (37). Similarly, for spinal somatic motor neurons, induction of nNOS expression has been demonstrated following ventral root avulsion in both the adult (38) and neonatal rat (39), following sciatic nerve lesion in the neonatal rat (40), and in sporadic ALS patients (41).

There is mounting evidence that reactive oxygen species, especially NO, play a pivotal role in the pathogenesis of the neurototoxic process in ALS, and this evidence supports the hypothesis that oxidative stress contributes to the pathogenesis of ALS (24, 25, 42, 43). However, the expression of iNOS immunoreactivity in the spinal cord of ALS patients has not yet been fully elucidated. Chou et al have reported that neurofilamentous accumulations, such as spheroids and conglomerates in the motor neurons of patients with ALS, were immunoreactive with an anti-iNOS antibody (22). Fujita et al reported that iNOS immunopositivity was observed mainly in the reactive astrocytes in the anterior horns, while anterior horn neurons were not immunostained for iNOS (23). On the other hand, anterior horn neurons and their neuronal processes frequently showed positive nitrotyrosine immunoreactivity, and markedly proliferated reactive astrocytes in the anterior horns were immunostained for nitrotyrosine (Fig. 10). Some cord-like swollen axons and some axons in the anterior roots were also immunostained for nitrotyrosine (Fig. 11).

Crossed Immuno-Absorption Tests

Dot blot analysis indicated that the anti-iNOS antibody did not react with nNOS and eNOS (Fig. 12).

Western Blotting for iNOS

A representative immunoblot is exhibited in Fig. 13. Non-transgenic mice showed no detectable bands (Fig. 13, lane 1). In contrast, samples of transgenic mice revealed a specific band with molecular weight of approximately 130 kDa corresponding to immunoreactive iNOS protein (Fig. 13, lane 2, arrow).
other hand, Wong and Strong reported that neither iNOS mRNA nor iNOS immunoreactivity was observed in ALS or control motor neurons (44). Our recent study of ALS patients demonstrated that most normal-appearing anterior horn neurons did not exhibit iNOS or nitrotyrosine immunoreactivity, while many of the degenerated neurons demonstrated positive iNOS and nitrotyrosine immunoreactivity, and the reactive astrocytes in the neuropil of the anterior horns and in the corticospinal tracts were intensely immunostained for iNOS and nitrotyrosine (25).

In the transgenic mice with a G93A mutant SOD1 gene, immunostaining for iNOS was identified only in glial cells and not in neurons (26); iNOS mRNA level and catalytic activity were significantly increased in the spinal cord; and the time at which the spinal cord gliosis and iNOS up-regulation occurred paralleled that of motor neuronal loss (26). Cha et al (27) reported the presence of nitrotyrosine-immunoreactive astrocytes and motor neurons in the spinal cords of mutant SOD1 transgenic mice at the age of 9 months, when clinical symptoms were manifested. The distribution of iNOS and nitrotyrosine immunoreactivity in the anterior horn neurons and reactive astrocytes in the mutant SOD1 transgenic mice in this study is quite similar to that in sporadic ALS patients (25) and the mutant SOD1 transgenic mice (27), and is consistent with the increased iNOS mRNA level in transgenic mice (26). Moreover, in our study, iNOS and nitrotyrosine immunoreactivity was observed in the anterior horn neurons as well as in astrocytes as early as the presymptomatic stage and the immunoreactivity increases with disease progression. The results of our study indicate that the extent of iNOS and nitrotyrosine staining correlates with the degeneration of anterior horn cells and the number of reactive astrocytes and that iNOS and nitrotyrosine expression may be detrimental to the motor neuron system and be involved in the neurotoxic pathomechanism.

As for the positive iNOS immunoreactivity of the anterior horn cells, iNOS is distinct from constitutive NOS (cNOS), and the anti-iNOS antibody does not cross-react with NOS based on the antigen sequence and Western blot experiments (45) including the present study. The same cell may even contain both cNOS activity and iNOS activity, perhaps reflecting the presence of both cNOS and iNOS within a single cell (46), and thus is likely to contain both enzymes under some circumstances. Differences among the immunohistochemical data of various researchers regarding iNOS expression could reflect differences in the level of an expression line, the disease progression occurred much more slowly in the transgenic mutant SOD1 mice in our study than in those reported by Almer et al (26).

Selectively increased iNOS and nitrotyrosine immunoreactivity in the motor system of the spinal cord suggests that NO functions in several specific motor circuits and that free radical-mediated mechanisms (oxidative stress or oxidative damage) may contribute to selective motor neuron degeneration or the selective vulnerability of the motor system. Motor neurons may be a potential target for NO-mediated cell damage. Nitric oxide produced in the degenerated anterior horn neurons and reactive astrocytes may activate other motor neurons in adjacent laminae via diffusion rather than traditional somatotopic synaptic projection (47). Thus, under conditions of excessive NO production, even a small population of neurons and astrocytes could lead to degeneration of a large population of surrounding neurons.

The findings of this study may have potential therapeutic implications. Recent studies have shown that antioxidants and a selective nNOS inhibitor increase survival in the SOD1 transgenic mouse model of FALS (48), whereas Facchinetti et al have reported that nNOS is unlikely to play a major role in the pathogenesis of the G93A transgenic mouse model of familial ALS (49). On the other hand, the protection of neurons has been observed in rodents with iNOS inhibition after induction of ischemia (50). Our results suggest that iNOS inhibitors or antioxidants may represent a valuable target for the development of new therapeutic avenues particularly for the early stage of ALS, since they may slow down neurodegenerative processes in human ALS.

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REFERENCES

INOS AND NITROTYROSINE IMMUNOREACTIVITY IN SOD1 MUTANT

22. Chou SM, Wang HS, Komai K. Colocalization of NOS and SOD1
47. Gally JA, Montague PR, Reeke GN, Edelman GM. The NO hypothesis: Possible effects of a short lived rapidly diffusible signal
in the development of and function of the nervous system. Proc Natl Acad Sci USA 1990;87:3547–51

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