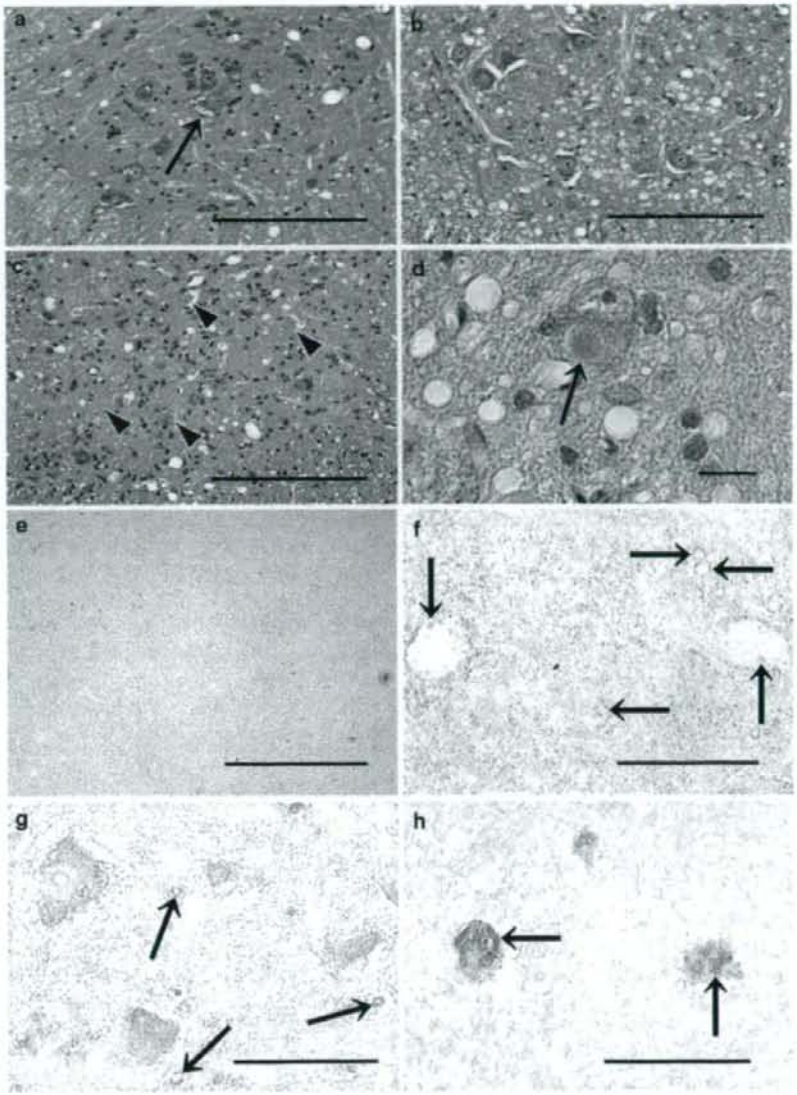

Fig. 3 Neuropathological findings of G1H-G93A mice as a gold-standard ALS model. **a** The number of anterior horn cells at 90 days of age is not decreased significantly; approximately 10 anterior horn cells can are observed, although an anterior horn cell with intracytoplasmic vacuolation is evident (*arrow*). **b** The numbers of anterior horn cells at 100 days of age are slightly decreased; about eight anterior horn cells can be seen, whereas abundant vacuoles are observed. **c** At 120 days, when the mouse is quadriplegic and moribund, there is severe loss of anterior horn cells and prominent inclusion pathology (*arrowheads*), although the vacuolation pathology is less marked than that at disease onset (in Fig. **b**). There is an inverse correlation between the number of vacuoles and the number of inclusions. **a–c** H&E. Scale bar **a** (also for **b, c**) 200 μm. **d** Light micrograph of a round neuronal LBHI (*arrow*) in a spinal anterior horn cell of a G1H-G93A mouse. This LBHI is observed in the cytoplasm of an anterior horn cell, and is composed of eosinophilic core with paler peripheral halo. Vacuolation pathology is evident in the neuropil. H&E. Scale bar **d** 20 μm. **e-h** Immunostaining with antibody against human SOD1 in the spinal cord anterior horn of the G1H-G93A mouse. This antibody (MBL, Nagoya, Japan) recognizes only human SOD1, i.e., mutant human G93A-SOD1. **e** Littermate mouse, showing no expression of G93A-SOD1. **f** In a 90-day-old G1H-G93A mouse, G93A-SOD1 is expressed mainly in the neuropil, and sometimes expressed intensely within the rims of vacuoles in the neuropil and motor neuron cytoplasm (*arrows*). However, it is not expressed in the motor neurons. **g** In a 110-day-old G1H-G93A mouse, G93A-SOD1 is expressed within the motor neuron cytoplasm in addition to the vacuole rims in the neuropil (*arrows*). **h**: In a 120-day-old G1H-G93A mouse, LBHIs are strongly positive for G93A-SOD1 (*arrows*). Scale bar **e** (also for **f–h**) 10 μm

with more frequent focusing on lower motor neuron pathology. This can be explained mainly in terms of the anatomical difference in the corticospinal tract system between humans and rodents: the main corticospinal tracts in the human spinal cord are the lateral and anterior columns, while the main spinal cord pyramidal tracts in rodents are the dorsal columns. Another major consideration is that even at necropsy with end-stage pathology, SOD1-mutated FALS, which is the prototype for these models, demonstrates only slight or mild corticospinal tract degeneration. Although there are a relatively few reports, some are pertinent. One group has reported that 110-day-old G1H-G93A mice demonstrate degeneration of the corticospinal and bulbospinal systems, in which 53% of corticospinal, 41% of bulbospinal and 43% of rubospinal neurons are lost (the bulbospinal neuron system in mice comprises three systems: rubospinal, vestibulospinal and reticulospinal neurons) [103]. Another group has reported that G85R mice at the end stage show progressive axonal degeneration of corticospinal tracts in the dorsal and lateral columns of the spinal cord [101].

Inclusion pathology

Ever since Hirano et al. [42] emphasized the presence of neuronal LBHIs in the anterior horn cells of FALS patients with posterior column involvement in 1967, and the author discovered Ast-HIs in 1996 [53], LBHI/Ast-HI have been considered pathognomonic features of mutant SOD1-linked FALS with posterior column involvement, which is the prototype form of human mutant SOD1 transgenic rodents, and in these transgenic rodents neuronal LBHIs are frequently observed in the soma (Fig. 3d) and neurites, although rarely in axons. Although Ast-HIs are sometimes found in only long-surviving patients with SOD1-mutated FALS [53, 54], they are frequently seen in G85R mice and H46R rats as well as in both G1H-/G1L-G93A mice and G93A rats at terminal stage [56, 59].

The author has examined six different lines of G1H-/G1L-G93A mice, G85R-148 mice, L84V mice, and H46R-4 and G93A-39 rats by electron microscopy, and all show almost identical ultrastructural features of neuronal LBHIs and Ast-HIs. Interestingly, neuronal LBHIs observed in both SOD1-mutated rodents and humans have a similar ultrastructure, being composed mainly of randomly oriented granule-coated fibrils approximately15–25 nm in diameter and granular materials. Ast-HIs seen in both SOD1-mutated rodents and humans also have the same ultrastructure. Therefore, the essential ultrastructural common components of neuronal LBHIs and Ast-HIs in SOD1-mutated rodents and humans are granule-coated fibrils about 15–25 nm in diameter and granular materials. As the inclusions develop, the granule-coated fibrillar component increases and the amount of granular material decreases, suggesting that the former might be derived from the latter [57].

It is of considerable interest that LBHIs/Ast-HIs observed in SOD1-mutated rodents are light- and electron-microscopically identical to those in patients with SOD1-mutated FALS. The presence of LBHI/Ast-HI is a morphological hallmark of cells affected by mutant SOD1 [58], and the formation of LBHI/Ast-HI is reported to be correlated with disease severity and progression [90]. However, the mechanism by which SOD1 mutation in vivo leads to the formation of 15–25-nm granule-coated fibrils as an essential component of LBHI/Ast-HI remains poorly defined. An important clue for explaining the formation of the granule-coated fibrils as an ultrastructural hallmark of mutant SOD1 has been reported: the same granule-coated fibrils as those in SOD1-mutated cells in vivo are induced by endoplasmic reticulum (ER) stress in vitro using neuroblastoma cells overexpressing human mutant L84V SOD1 [100]. Transgenic mice with L84V SOD1 show aberrant aggregation of the ER in association with early-stage neuronal LBHIs, suggesting that the LBHIs might arise as a result of ER dysfunction [100]. Collectively, the presence of LBHI/Ast-HI is a light-microscopical hallmark of SOD1-mutated cells, and the 15–25-nm granule-coated fibrils as an essential component of the LBHI/Ast-HI provide ultrastructural authentication of SOD1-mutated cells. In marked contrast, Bunina bodies are not found in the transgenic rodents bearing mutant SOD1.

Vacuolation pathology

With regard to vacuolation pathology, although transgenic rodents expressing mutant SOD1 exhibit vacuoles of various sizes in neurons and neuropil, similar features are not evident in autopsy cases of mutant SOD1-related FALS, which is the prototype of the mutant SOD1 transgenic rodent. This vacuolation pathology is also undetectable in mutant SOD1-unrelated FALS and SALS. Ultrastructurally, the vacuolation is evident in somata, dendrites and axons of motor neurons. At an early stage, these alterations occur in the rough ER and mitochondria. In particular, perinuclear vacuoles in somata at the early stage are derived from dilated ER cisternae. As the disease progresses, the number of mitochondria-derived vacuoles increases, while the number of vacuoles originating from the ER decreases. Mitochondria-derived vacuoles originate through expansion of the mitochondrial intermembrane space and extension of the outer mitochondrial membrane [40]. These vacuoles are apparently more abundant at disease onset, and decline thereafter; vacuole formation itself is reported to be related to disease onset rather than disease progression [61]. There are abundant vacuoles and few LBHIs in the early course of degeneration in G1L-G93A and G1H-

G93A mice (Fig. 3b), and mice at the terminal stage show less abundant vacuoles and many LBHIs (Fig. 3c), i.e., there is a significant inverse correlation between the numbers of vacuoles and LBHIs [91]. Another important finding in G1H- and G1L-G93A mice at the presymptomatic stage is fragmentation of the Golgi apparatus in the spinal cord anterior horn cells [69].

Contribution of mutant SOD1 in each cell type

Transgenic rodents overexpress human mutant G93A SOD1 in all cells because the transgene is driven by a non-cell-specific endogenous promoter. On the other hand, there are transgenic mice in which mutant SOD1 expression is driven by a neuron-specific promoter such as the neurofilament light chain. In transgenic mice whose anterior horn cells specifically overexpress mutant SOD1, neither motor neuron impairment nor degeneration is evident [80]. Transgenic mice that overexpress the mutant SOD1 transgene in neurons after birth also do not show motor neuron pathology [65]. Some other types of transgenic mice overexpress mutant G86R SOD1 only in astrocytes under control of the GFAP promoter. Despite the fact that these mice develop astrocytosis, they show no motor neuron degeneration and develop normally [34]. Although neurons or astrocytes play very important role in ALS pathogenesis, it is of considerable interest that mutant SOD1, when over expressed either in neurons or astrocytes, does not sufficiently contribute to the onset of ALS. In culture study, conversely, astrocytes expressing mutated SOD1 kill spinal primary and embryonic mouse stem cell-derived motor neurons [73]. In addition to neurons and astrocytes, microglia are closely related to neuron injury not only in ALS but also other neurodegenerative disorders. Approaches such as the use of a deletable mutant SOD1 transgene have demonstrated that diminishing mutant SOD1 within microglia has little effect on the early disease phase but sharply slows later disease progression: i.e., SOD1 mutated motor neurons are a determinant of onset and early disease, and mutant accumulation within microglia accelerates disease progression [9]. Interestingly, microglia themselves have a double-edged sword effect; wild-type microglia can extend the survival of G93A mice with PU.1 knockout mice (which are unable to develop myeloid and lymphoid cells) by using bone marrow transplantation [6]. Since retraction of motor axons from synaptic connections to muscle is among the earlier presymptomatic morphological findings in SOD1-mutated mice, muscle itself is also a likely primary source of mutant SOD1 toxicity. However, use of a deletable mutant gene to eliminate mutant SOD1 from muscle does not affect disease onset or survival: SOD1-mutant-mediated damage within muscles is not a significant contributor to non-cell-autonomous pathogenesis in ALS [66].

Relation between mutant SOD1 and disease progression

Unlike patients with SOD1-mutated FALS, transgenic rodents bear both human mutant SOD1 and native endogenous rodent SOD1. Native endogenous rodent SOD1 catalyzes the conversion of the superoxide radical to hydrogen peroxide and molecular oxygen. Even overinduced human mutant SOD1 also detoxifies the superoxide radical, which is a source of reactive oxygen species generated from aerobic organisms, and protects cells, including motor neurons, from oxidative injury. Based on the gain-of-function theory, human mutant SOD1 itself acts as a cytotoxic factor, and in G1H-G93A mice human mutant G93A-SOD1 shows cytotoxicity for motor neurons. In 90-day-old G1H-G93A mice that show no significant motor neuron loss and only slight vacuolation pathology, G93A-SOD1 is already present but its expression level is not marked, and immunohistochemically it is expressed mainly in the neuropil, sometimes being expressed intensely within the rims of vacuoles in the neuropil and motor neuron cytoplasm (Fig. 3e,f). In contrast, motor neurons do not express mutant SOD1 (Fig. 3e,f). In 100-day-old G1H-G93A mice that demonstrate a slightly decreased number of motor neurons and prominent vacuolation pathology, mutant G93A-SOD1 is highly expressed in comparison with the level at 90 days of age. At 110 days, the mice that show loss of anterior horn cells with some inclusions and vacuole formation also exhibit high expression of mutant G93A-SOD1, which morphologically is located within the motor neuron cytoplasm and the vacuole rims in the neuropil (Fig. 3e,g). End-stage G1H-G93A mice that show severe motor neuron loss as well as vacuolation and inclusion pathologies demonstrate high expression of mutant G93A-SOD1, and immunohistochemically mutant G93A-SOD1 is aggregated and sequestered into the LBHIs, which are strongly positive for mutant G93A-SOD1 (Fig. 3e, h). Considered in connection with the abundance of neuropil vacuoles and few LBHIs at the early stage, and the fact that mice at the terminal stage show many LBHIs and less abundant vacuoles, as well as the accumulation of mutant G93A-SOD1 in vacuoles at the early stage and marked aggregation of mutant G93A-SOD1 in LBHIs at the late stage, it is possible that cytotoxic mutant G93A-SOD1 within vacuoles at the early stage leaks into the neurons and then aggregates within neurons as LBHIs with disease progression. Along with disease progression, there is a breakdown of cytotoxic mutant SOD1 sequestration in vacuoles, and the mutant SOD1 aggregates in motor neurons, resulting in their degeneration/death.

Development of rats with human mutant SOD1

The ultimate aim of developing transgenic rodents expressing human mutant SOD1 are as follows: to gain an

understanding of the mechanism of motor neuron death in the presence of mutant SOD1, and to test new ALS therapies. Freshly obtained mouse spinal cord including the nerve roots, cauda equina and filum terminale, weighs only about 110 mg and is approximately 4 cm in length. In order to perform more extensive analysis of the mechanism of motor neuron death and to devise new ALS therapies that are difficult or impossible to explore using the small spinal cord of the mouse, transgenic rats expressing human mutant SOD1 have been developed [2, 43, 72]. As rats are a larger species than mice, they are easier to use in studies involving manipulations of spinal fluid (e.g., implantation of intrathecal catheters for chronic therapeutic studies and CSF sampling) and the spinal cord (e.g., direct administration of viral and cell-mediated therapies).

Relationship between ALS and TDP-43

The 43-kDa TAR DNA-binding protein (TDP-43) is localized to the nucleus. Originally, TDP-43 was identified as a component of ubiquitinated inclusions in frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and ALS [3, 74]. Analyses of TDP-43 immunohistochemistry in SALS (two patients), mutant SOD1-unrelated FALS (two patients) and ALSD (one patient) have shown TDP-43-immunoreactive inclusions such as SLI/RHI in the anterior horn cells of the spinal cord [95]; as mentioned above, SLI/RHI are characteristic morphological structures in ALS, and mutant SOD1-unrelated FALS is neuropathologically indistinguishable from SALS. TDP-43 immunoreactivity has also been detected in the motor neurons of the hypoglossal nucleus in 4 patients with FTLD-MND/ALSD and 11 patients with ALS; TDP-43-positive structures include SLI/RHI [26]. Although Bunina bodies are a pathognomonic structure in SALS, mutant SOD1-unrelated FALS and FTLD-MND/ALSD, Bunina bodies themselves are negative for TDP-43 [95]. With regard to mutant SOD1-related motor neuron death, LBHIs, which are characteristic structures in mutant SOD1-related FALS with A4T (one patient) and D101Y (one patient) reportedly do not express TDP-43 [95]. In SALS (one patient) as well as mutant SOD1-related FALS with A4T (one patient) and I113T (one patient), TDP-43 is mislocalized from the nucleus to the cytoplasm [83]. Especially, one case of SALS was reported to show ubiquitin-positive RHIs with TDP-43 staining pattern [83]. By marked contrast, human mutant G93A, G37R and G85R SOD1-transgenic mice do not show any TDP-43 abnormalities including either TDP-43-positive inclusions or TDP-43 mislocalization [83]. It could be stated that in general, TDP-43 contributes to mutant SOD1-unlinked motor neuron degeneration, whereas mutant SOD1-linked motor neuron degeneration may not be essentially related to TDP-43 abnormality; in particular, human mutant SOD1 transgenic mice do not show TDP-43 abnormality [83].

Mouse models of ALS2

As already mentioned, SOD1 mutations have been identified as a cause of autosomal dominant FALS [22, 84]. Mutation in a second ALS-related (ALS2) gene has also been identified as the cause of a rare autosomal recessive form of juvenile-onset ALS, also referred to as ALS2 [7, 37, 102], as well as juvenile-onset primary lateral sclerosis (PLS) [37], and infantile-onset ascending hereditary spastic paralysis (HSP) [24, 29, 35]. In humans, the ALS2 gene is located on chromosome 2 at position 33.2, and encodes a protein called ALS2 protein or alsin. ALS2 protein is produced in a wide range of normal tissues, with the highest amounts in the brain and spinal cord. ALS2 protein is composed of 1,657 amino acids with three predicted guanine nucleotide exchange factor (GEF)-like domains [37, 102]: an N-terminal regulator of chromatin condensation (RCC 1)-like domain (RLD) homologous to GEF for Ran GTPase [77], middle Dbl homology (DH) and pleckstrin homology (PH) (DH/PH)-like domains resembling GEF for Rho GTPase [87], and a C-terminal vacuolar protein sorting 9 (VPS9)-like domain similar to GEF for Rab5 GTPase [79]. This ALS2 protein is particularly abundant in motor neurons. ALS2 protein is preferentially associated with the cytoplasmic face of the endosomal membrane [79]. Although the function of ALS2 protein in motor neurons is unclear, it may play an important role in regulating cell membrane organization and the movement of molecules within motor neurons. Therefore, it would be expected to play a role in the development of axons and dendrites. It is unclear how and why loss of ALS2 protein function causes the ALS2-linked diseases: ALS2, juvenile-onset PLS, and infantile-onset ascending HSP. In order to gain insight into the physiological role of ALS2 protein and the pathogenesis of ALS2-linked diseases, four types of ALS2 knockout mice have been successfully developed.

The ALS2 knockout mice with disruption of exon 3 of the murine ALS2 gene reported by Cai et al. [13] show a higher anxiety response as well as an age-dependent deficit of motor coordination and learning. Histopathologically and biologically, ALS2 knockout mice are characterized by a lack of neuropathological abnormality, no alteration of peripheral nerve conduction or electromyography features, susceptibility to oxidative stress, and increased susceptibility to glutamate receptor-mediated excitotoxicity. In the ALS2 knockout mice reported by Hadano et al. [38], exon 3 of the murine ALS2 gene is disrupted by inserting a stop codon. These mice demonstrate no obvious developmental,

reproductive or motor abnormalities. However, histopathologically and biologically, they are characterized by an age-dependent decrease in the size and number of ventral motor axons and cerebellar Purkinje cells, astrocytosis and microglial activation in the spinal cord and brain, motor unit remodeling and fiber redistribution in skeletal muscle, and slightly affected endosomal dynamics. ALS2 knockout mice with disruption of both exons 3 and 4 of the murine ALS2 gene reported by Devon et al. [25] show mild hypoactivity. Neuropathologically, at the age of 12 months, they show significantly smaller cortical motor neurons, and in addition, marked diminution of Rab5-dependent endosome fusion activity and disturbance in endosomal transport of the insulin-like growth factor 1 and BDNF receptors. ALS2 knockout mice with disruption of exon 4 of the murine ALS2 gene reported by Yamanaka et al. [101] demonstrate slowed movement without muscle weakness and progressive axonal degeneration in the lateral spinal cord. Significantly, all four of these ALS2 knockout murine models show no human ALS2-like symptoms and are not neurologically analogous to humans with ALS2.

Among previously reported human patients with ALS2-linked disease, the members of a Tunisian family with 138delA ALS2 gene mutation showed development of spasticity in all limbs between 3–10 years of age [7, 37, 102], and their clinical symptoms might be classifiable as part of a spectrum of HSP rather than typical ALS. The members of a Kuwaiti family with 1425_1426delAG ALS2 gene mutation showed infantile-onset spastic paralysis without lower motor neuron involvement at 1–2 years of age [37]. Members of a Saudi Arabian family with1867_1868delCT ALS2 gene mutation developed PLS between 1–2 years of age [33, 102]. Up to now, eight additional ALS2-linked diseases have been reported [24, 29, 30, 35, 62], and a major common characteristic is infantile-onset spastic paralysis, reflecting upper motor disturbance. Although lower motor neuron impairment has been reported in a limited number of patients with ALS2 gene mutation, the majority of ALS2 gene mutations appear to be linked to upper motor neuron diseases from the viewpoint of human clinical data of 11-type ALS2-linked diseases. Although to the author's knowledge there has been no reported autopsy case of ALS2, detailed neuropathological data from human ALS2 autopsy cases would clarify this point. In this context, although the author is unable to address the similarities and differences between human ALS2 and ALS2 animal models from a neuropathological viewpoint, it might be concluded that data from ALS2 knockout mice and ALS2-linked diseases mentioned above would become more valuable for clarifying the pathogenesis of human ALS2 if detailed human ALS2 autopsy data were also available.

Animal models based on cytoskeletal abnormalities

Animal models based on neurofilament abnormalities

The neuron cytoskeleton consists of three major filaments: actin microfilaments, microtubules, and neurofilaments. Neurofilaments biochemically comprise three different isoforms known as neurofilament triplet proteins: light subunit (68 kDa), medium subunit (160 kDa), and heavy subunit (200 kDa). Ultrastructurally, neurofilaments are approximately 10 nm in diameter, but in cross-section they appear tubular in structure with a narrow central electron-lucent core, and have fine side arms. Their size places them in the so-called "intermediate filaments" morphologically.

Neurofilament-lacking mice

Mice lacking any of the neurofilament triplet protein genes show no developmental problems [82]. However, mice lacking the neurofilament light subunit show a lack of intermediate filament structure, axonal hypotrophy, and aggregation of neurofilament medium and heavy subunit in motor neurons, although significant motor neuron loss is not evident [5]. Mice lacking the neurofilament medium subunit show axonal atrophy without significant motor neuron loss, and reduce contents of neurofilament light subunit [28]. Mice without the neurofilament heavy subunit also exhibit axonal atrophy without significant motor neuron loss [47]. Therefore, model mice lacking any of the neurofilament triplet protein subunits are not compatible with human ALS patients, as no significant motor neuron loss is evident.

Transgenic mice expressing the human wild-type neurofilament gene

Transgenic mice expressing the human wild-type neurofilament heavy chain gene show defective axonal transport and axonal atrophy in association with ultrastructural diminution of cytoskeletal components, the smooth endoplasmic reticulum, and mitochondria [16]. In mice showing a high level of expression, neurofilament aggregation is observed in the cytoplasm of neurons and proximal axons [17]. However, this transgenic mouse model shows no significant motor neuron loss [16]. Like neurofilament heavy chain-type transgenic mice, the neurofilament light chain-type transgenic mice show accumulation of neurofilaments in the neurons and axonal degeneration without significant motor neuron loss [99]. Therefore, transgenic mouse models expressing the human wild-type neurofilament gene bear no histopathological resemblance to human ALS in terms of significant motor neuron loss.

Transgenic mice expressing the human mutant neurofilament light chain gene with the L394P

Transgenic mice expressing the human mutant neurofilament light chain gene with the L394P develop neurological symptoms of muscle weakness. Unlike mice expressing the human wild-type neurofilament gene, these mice show significant motor neuron loss [64]. From this viewpoint, this mouse model is closely similar to human ALS on the basis of neurofilament pathology, although in human ALS there is no mutation of the neurofilament light chain gene with L394P [27].

Transgenic mice expressing the peripherin gene

Peripherin is a 58-kDa type III intermediate filament protein, which has been reported to be a component of ubiquitinated inclusion bodies in motor neurons of ALS patients [39]. As the name "peripherin" indicates, the protein exists mainly in the peripheral nervous system, and only a small amount is expressed with a selective distribution in the central nervous system. Overexpression of peripherin in transgenic mice leads to loss of spinal cord anterior horn cells and formation of inclusions that are immunoreactive for peripherin [4].

Transgenic mice expressing the dynamitin gene

The dynein/dynactin-complex is a type of motor protein responsible for minus-end-directed movement along the microtubule and plays an important role in fast retrograde-related axonal transport. Supporting the hypothesis that impairment of retrograde axonal transport causes motor neuron death, point mutations of the p150 subunit of the dynactin gene have been reported in ALS patients [70]. Experimentally, on the basis of this retrograde axonal transport impairment theory, mice overexpressing dynamitin, which is a subunit of dynactin, have been produced, and these mice show disruption of the dynein/dynactin complex, leading to inhibition of retrograde axonal transport. Histologically, such dynamitin-overexpressing mice show motor neuron loss [63].

Concluding remarks

Human ALS pathology exhibits a variety of cytopathological features including Bunina bodies, SLIs/RHIs, LBHIs/Ast-HIs, and NFCIs in addition to motor neuron degeneration. Among various rodent models of ALS, rodents with mutant SOD1 recapitulate motor neuron degeneration and SOD1-immunoreactive LBHIs/Ast-HIs, both of them found in SOD1-mutated FALS patients. Even with these similarities, human ALS pathology is different from that of rodents carrying SOD1 mutation, because (1) ALS is not a single entity but rather a heterogeneous syndrome, (2) relevant anatomical structures are different between humans and rodents, and (3) human pathology generally deals with only the terminal stage of the disease. In spite of these differences, motor neuron degeneration in rodent models provides us with opportunities to analyze the motor neuron degeneration process in detail and even to test therapeutic attempts. It is necessary to be aware not only of the similarities but also of differences between these ALS models and human ALS, because they are complementary.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research (c) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (SK: 17500229), a Grant for Research on Psychiatric and Neurological Disease and Mental Health from the Ministry of Health, Labor and Welfare of Japan (SK), and a Grant from The Research Group on Development of Novel Therapeutics for ALS of the Ministry of Health, Labor and Welfare of Japan (SK).

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（資料 3.）

平成 20 年度　総括研究報告書

研究成果に関する一覧表およびその刊行物

厚生労働科学研究費補助金（こころの健康科学研究事業）
研究報告書

筋萎縮性側索硬化症に対する肝細胞増殖因子を用いた画期的治療法の開発

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研究要旨：本研究の目的は神経難病でも最も苛酷な筋萎縮性側索硬化症（ALS）に対して肝細胞増殖因子（Hepatocyte Growth Factor; HGF）を用いた画期的治療法を開発することとそれに関わる基盤研究を進めることにある。ALS の病因研究および治療研究には変異 Cu/Zn superoxide dismutase（SOD1）導入 ALS ラットが重要な役割を果している。私共はこの ALS ラットを用いて運動ニューロンに対し神経栄養因子作用を有する recombinant human HGF（rhHGF）の髄腔内持続投与で ALS に対する有効性を示してきた。多くの神経栄養因子のなかでもこの様に変異 SOD1 トランスジェニック動物による ALS モデルに対して明確な治療効果を示したものは少なく、この有効性を ALS 患者に臨床応用する意義と必要性がある。しかも、臨床応用の最も可能性の高いルートとしての髄腔内投与での効果が ALS ラットで確認されたので、霊長類（マーモセット）に対する髄腔内投与での安全試験および容量設定を開始した。マーモセットによる ALS モデルは確立されていないので、rhHGF の安全試験および臨床用量決定には慶応大学の岡野らが確立したマーモセットによる脊髄損傷モデルを用いた。ALS ラットおよび脊髄損傷ラットで効果が確認された容量（400μg/4weeks）の rhHGF をマーモセット頸髄圧挫損傷モデルに対して損傷後よりくも膜下腔に持続投与したところ、著明な損傷範囲の縮小および良好な運動機能回復が得られた。同時に安全性も確認中である。ヒト ALS 患者に対する rhHGF による治療は医薬品機構との安全性相談が終了し、現在の安全性試験計画をクリアできればフェーズ 1 の治験に進めることを確認している。rhHGF はわが国発の ALS 治療薬候補としてスーパー特区（代表　岡野栄之）に選定された。

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A. 研究目的

筋萎縮性側索硬化症（ALS）は運動ニューロンの選択的な細胞死が惹起されて、全身の筋萎縮と脱力が進行する原因不明の難治性神経筋疾患である。しかも 2～3 年の経過で呼吸筋の麻痺をきたす極めて予後不良な疾患であるが、現状では有効な治療法がない。ALS の病因と病態の解明を行ない、そ

れを基盤にした新規治療法の開発が世界的に切望されている。

わが国で発見された神経栄養因子である肝細胞増殖因子（Hepatocyte Growth Factor; HGF）は、運動ニューロンに対する強力な保護作用が知られており、私たちは遺伝子工学的に ALS マウスにおける HGF の運動ニューロン死に対する抑制効果を確認している。さらには ALS の臨床応用を目指し、私たちが開発した大型 ALS 動物モデルである変異 Cu/Zn superoxide dismutase （SOD1）導入 ALS ラットに対して HGF 蛋白の髄腔内投与実験を行い、その有効性も確認している。

すでに ALS ラットに対してヒトリコンビナント HGF 蛋白（rhHGF）の髄腔内持続投与で有効性を示したので、霊長類を用いて HGF の髄腔内投与による安全性を検証すると共に臨床用量の設定を行う。その結果を元に、ALS 患者に対する治験フェーズ 1 に進む。同時に HGF の治療効果の機序を特にミクログリアの特性変化に注目して明らかにする。

B. 研究方法

1）HGF 投与によるミクログリアの特性変化の分子機序の検討

ALS モデル動物として SOD1(G93A)発現するトランスジェニックマウス(ALS-Tg)、そのコントロールとして同一年齢の野生型 Littermate を用いた。これと神経特異的に HGF を発現する HGF-Tg マウス（HGF-Tg）を交配することで、WT, ALS-Tg, HGF-Tg, ALS/HGF-Tg を作成した。組織解析は、動物を深麻酔後、脊髄をすみやかに取り出し、アルコール系列により組織を固定後、パラフィン包埋し、切片を作成した。組織片は、Nissl 染色、および免疫染色(Iba-1, Mac2 および P2Y12 抗体)を施行した。

2）コモンマーモセットの脊髄損傷モデルに対する rfHGF の髄腔内投与

成体メスのコモンマーモセットの第 5 頚椎高位に 20g の重錘を 5cm の高さから落として圧挫損傷を作製した。直後より第 7 頚椎高位のくも膜下からカテーテルを挿入し、rhHGF 400μg を髄腔内に 4 週間持続投与した（n=6）。対照群には PBS を投与した(n=5)。術後 12 週まで Bar grip test にて上肢筋力を、独自に開発した Open field scoring にて上肢の神経学的機能を評価した。損傷後 1・3・12 週に頚髄 MRI（7.0 tesla）を撮像し、損傷範囲を同一個体で経時的に評価した。また、拡散テンソル投射路撮影（Diffusion tensor tractography; DTT）にて脊髄軸索を描出し途絶の程度を評価した。損傷後 12 週目に脊髄を採取し腫瘍形成の有無を含めた免疫組織学的検討を行った。

3）治験に関するプロトコールの作成

ALS 患者を対象した治験を行うために、東北大学トランスレーショナルリサーチセンターと共にプロトコールの検討を行った。

（倫理面への配慮）

すべての遺伝子操作は本学 DNA 組換え実験指針に従い、また動物実験は同動物実験指針に従った上で動物愛護面に配慮しかつ利用動物数を極力減らすように務めた。

C 及び D. 研究結果及び考察

1）HGF 投与によるミクログリアの特性変化の分子機序の検討

組織解析の結果、SOD1(G93A)を発現する ALS モデルトランスジェニックマウス（ALS-Tg）の脊髄運動ニューロンは、経時的に死細胞数が増え、回復する事はなかった。特に運動ニューロン死のおこる advanced stage になると、ALS-Tg の脊髄中には、resting microglia のマーカー陽性細胞数が極端に減少し、逆に Mac2 陽性細胞数が著明に増加した。その免疫染色性の変化は相反的であり、Mac2 陽性細胞数が増殖してくる、もしくは血液中から recruit されてくる事に加えて、resting microglia から Mac2 陽性細胞への特性変化がおこっている可能性が示唆された。

一方で、ALS/HGF-Tg においては、初期から ALS-Tg の advanced stage に相当する時期までの間、resting microglia の数が多く、逆に Mac2 陽性細胞数が少なかった。このことから、HGF は、resting microglia から活性化型ミクログリアへの特性変化を抑制、あるいは積極的に resting な状態にもどす可能性が示唆された。

HGF が ALS 病態進行時のミクログリアに直接作用してミクログリアの特性修飾を行う事が示唆され、発症後 HGF 投与の効果そのものに加えて、発症後 HGF 投与による ALS 治療の分子基盤が明確となった。

2）コモンマーモセットの脊髄損傷モデルに対する rfHGF の髄腔内投与

rhHGF 投与群で Bar grip test、Open field scoring いずれの評価法においても対照群に比べ有意に良好な運動機能回復が認められた。観察期間中に異常行動は認めなかった。

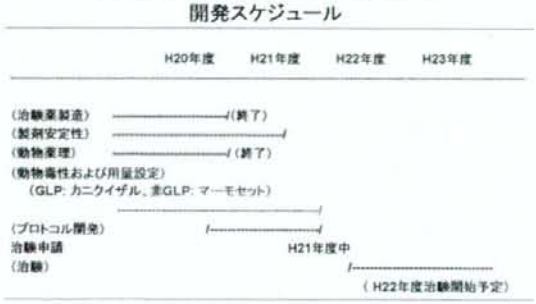
損傷後3週目までの MRI 像では両群間に明らかな差は認められなかったものの、12週目には T1-low、T2-high を示す異常信号領域が rhHGF 投与群で著明に縮小していた。また DTT の結果より、rhHGF 投与群で有意に多い脊髄軸索が描出された。LFB 染色の結果から、rhHGF 投与群で空洞形成面積が縮小し、髄鞘化面積が有意に保たれていた。次に皮質脊髄路を示す calmodulin-dependent protein kinase 2-α (CaMK2-α)陽性線維が、損傷部より尾側においても有意に良好に保たれていることが明らかとなった。

また、脊髄灰白質後角における Calcitonin gene-related peptide (CGRP) 陽性の C 線維分布に両群間で有意な差を認めなかった。rhHGF 投与群で腫瘍形成は1例も認めなかった。

3）治験に関するプロトコールの作成

rhHGF による ALS 治療は医薬品機構との安全性相談が終了し、現在の安全性試験計画をクリアできればフェーズ1の治験に進めることを確認した。東北大学トランスレーショナルリサーチセンターと共にプロトコールの検討を行っている。

（参考）ヒトリコンビナントHGF蛋白製剤
開発スケジュール



E. 結論

本研究の目的は神経難病でも最も苛酷な筋萎縮性側索硬化症（ALS）に対して肝細胞増殖因子（HGF）を用いた画期的治療法を開発することとそれに関わる基盤研究を進めることにある。臨床応用の最も可能性の高いルートとしての髄腔内投与での効果がALSラットで確認されたので、霊長類（マーモセット）に対する髄腔内投与での安全試験および容量設定を開始した。マーモセットによるALSモデルは確立されていないので、HGFの安全試験および臨床用量決定には慶応大学の岡野らが確立したマーモセットによる脊髄損傷モデルを用いた。

マーモセット脊髄損傷モデルに対して損傷後よりrhHGFをくも膜下腔に持続投与し、損傷範囲の著明な縮小ならびに有意に良好な運動機能の回復を認めた。霊長類脊髄損傷に対してもラットと同じ体重比の容量で有効性が確認され、また腫瘍形成や異常行動が認められなかったことから、本治療法がヒトALSに対し有効かつ安全な治療法となり得る可能性が大きく示唆された。さらに今回はげっ歯類では評価困難な、霊長類で特に発達した上肢の機能に注目することで、rhHGFのくも膜下腔持続投与により霊長類に対しても著明な治療効果が得られることを明らかとした。

rhHGFによるALS治療は平成21年度中の治験届けの提出を目指している。わが国発のALS治療薬候補としてスーパー特区（代表　岡野栄之）に選定された。

F. 健康危険情報

特になし

G. 研究発表

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ほか

H. 知的財産権の出願・登録状況

1. 特許登録（申請中）

発明の名称：脊髄損傷治療薬剤

発明者：岡野栄之 戸山芳昭 中村雅也 岩波明生 北村和也 中村敏一 船越洋

整理番号：P10001205

申請日： 2007.2.28

PCT 出願： PCT/JP2007/053804

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧

原著論文

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Neuroscience Letters 448 (2008) 125–129

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Hepatocyte growth factor suppresses ischemic cerebral edema in rats with microsphere embolism

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ARTICLE INFO

Article history:
Received 21 August 2008
Received in revised form
25 September 2008
Accepted 8 October 2008

Keywords:
Cerebral ischemia
Edema
Ion
Hepatocyte growth factor

ABSTRACT

The present study was aimed at determining whether human recombinant hepatocyte growth factor (HGF) ameliorates cerebral edema induced by microsphere embolism (ME). Rats were injected with 700 microspheres (48 μm in diameter). Continuous administration of HGF at 13 μg/3 days/animal into the right ventricle was started from 10 min after embolism to the end of the experiment by using an osmotic pump. On day 3 after the ME, the rats were anesthetized, and their brains were perfused with an isotonic mannitol solution to eliminate constituents in the vascular and extracellular spaces. Thereafter, tissue water and cation contents were determined. A significant increase in tissue water content of the right hemisphere by ME was seen. This ME-induced increase in water content was associated with increases in tissue sodium and calcium ion contents and decreases in tissue potassium and magnesium ion contents of the right hemisphere. The treatment of the animal with HGF suppressed the increases in water and sodium and calcium ion contents, but not the decreases in potassium and magnesium ion contents. These results suggest that HGF suppresses the formation of ischemic cerebral edema provoked intracellularly in rats with ME.

It is well recognized that ischemia-induced cerebral edema (ischemic cerebral edema) is a common complication in stroke patients [15]. In experimental animals, the degree of ischemic cerebral edema is dependent on the type and period of ischemia [32] or ischemia/reperfusion [2], and is associated with several pathophysiological alterations including disruption of the blood–brain-barrier (BBB) [23], changes in the threshold of cerebral blood flow [12], and an increase in hydrostatic pressure [17].

We have demonstrated various pathophysiological aspects of microsphere embolism (ME)-induced cerebral ischemia in the rat. For example, ME elicited decreases in substrates for cerebral energy production [28] and mitochondrial activity [29], and caused alterations in neuronal transmitter metabolism [27] as well as learning and memory dysfunction [26]. Although cerebral edema is considered to occur after the onset of permanent arterial occlusion [10,13], it remains unclear whether the ischemic cerebral edema occurs under our experimental conditions for ME. Accordingly, we determined whether ME-induced cerebral edema could be detectable after the embolism.

Hepatocyte growth factor (HGF) and its receptor c-Met were found to be expressed in the central nervous system, including endothelial cells, and to function in an variety of ways [1,11,24,25]. Accumulating evidence indicates that HGF has been shown to have organotropic action leading to regeneration from and protection against ischemic brain injury [19,22]. Particularly, we have described profound effects of HGF on the ME-induced hyperpermeability of the brain and learning dysfunction [7]. Accordingly, the present study was designed to determine whether treatment with HGF would affect the ME-induced ischemic cerebral edema. Since, it remains unclear whether ME-induced cerebral edema occurs intracellularly or extracellularly, we focused on the development of intracellularly provoked cerebral edema in this study.

Male Wistar rats weighing 220–250 g were used as the experimental animals. The animals were freely given food and water according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the Guideline for Experimental Animal Care, issued by the Prime Minister's Office of Japan. The study protocol was approved by the Committee of Animal Care and Welfare of Tokyo University of Pharmacy and Life Sciences.

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0304-3940/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2008.10.026