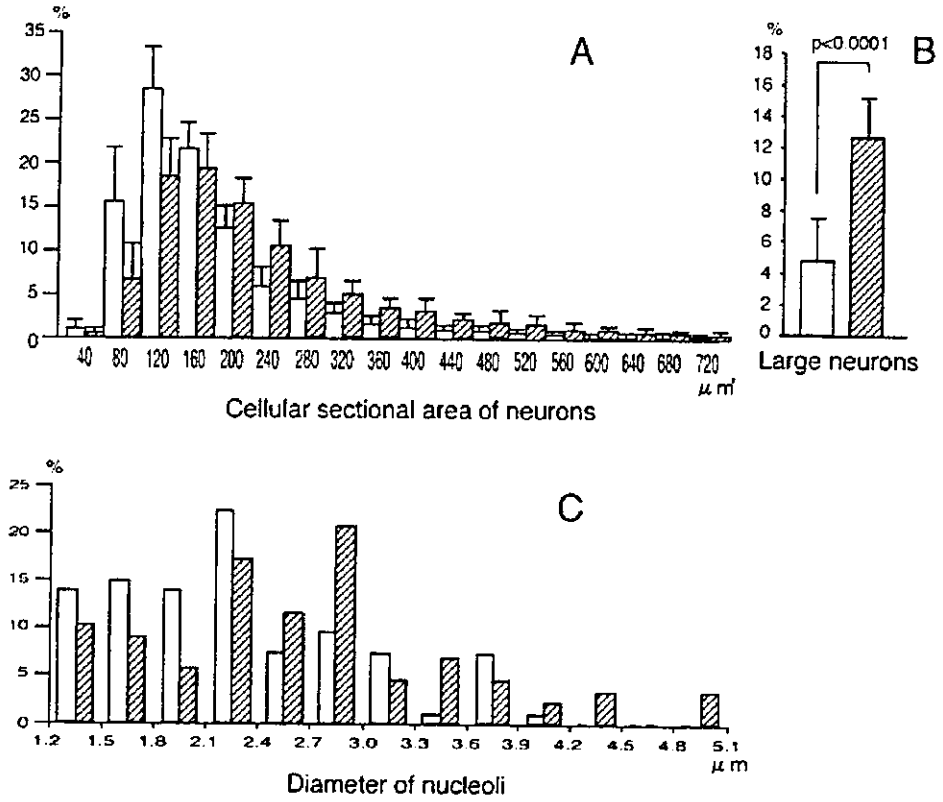

Fig. 3 Distribution of the sectional area of the neurons in the ventral gray matter of the 5th cervical cord. The frequency distribution of the sectional area of the neurons shows one peak at 120 μm^2 in *kl/kl* mice but 160 μm^2 in WT mice, indicating smaller neurons in the *kl/kl* mice (**A**). The number of large neurons with a cross-sectional area over 400 μm^2 is significantly lower in *kl/kl* mice than WT mice (**B**). The frequency distribution by 0.3-μm increments of the diameter of nucleoli of neurons in the ventral spinal gray matter shows the highest peaks at 2.1–2.4 μm in *kl/kl* mice but at 2.7–3.0 μm in WT mice (**C**). *Open bars*: *kl/kl* mice, *hatched bars*: WT mice, *error bars*: SD

Amount of cytoplasmic RNA in AHCs

The integrated OD value of the cytoplasmic RNA in the large AHCs stained with pyronin Y in *kl/kl mice* was 64.4% of that seen in the WT mice (Table 2, Fig. 4).

[Table 2 will appear here. See end of document.]

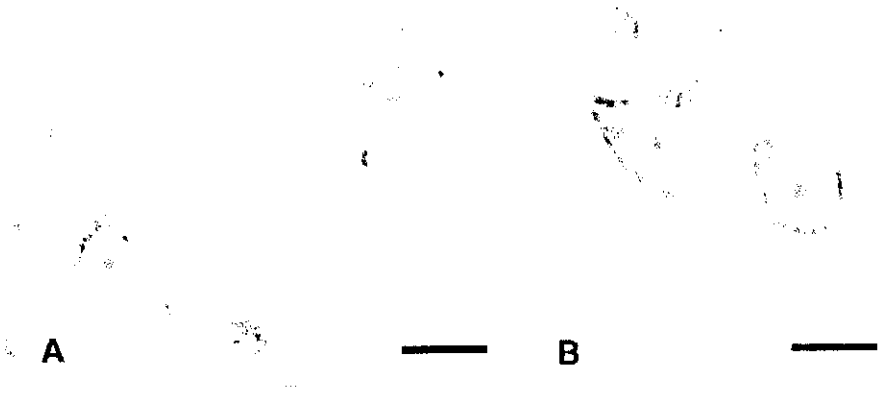

Fig. 4 Pyronin Y-stained AHCs in *kl/kl* (**A**) and WT (**B**) mice. *Bars* 20 μm

Ultrastructural findings of the AHCs

For *kl/kl* mice, the rER was severely fragmented, and the size of cisternae of the rER was reduced. The number of the attached and free ribosomes was noticeably reduced in the AHCs as compared to those in WT mice, while mitochondria, nuclear membrane, karyoplasm and nucleolus in the AHCs in these mice appeared normal (Fig. 5A, B).

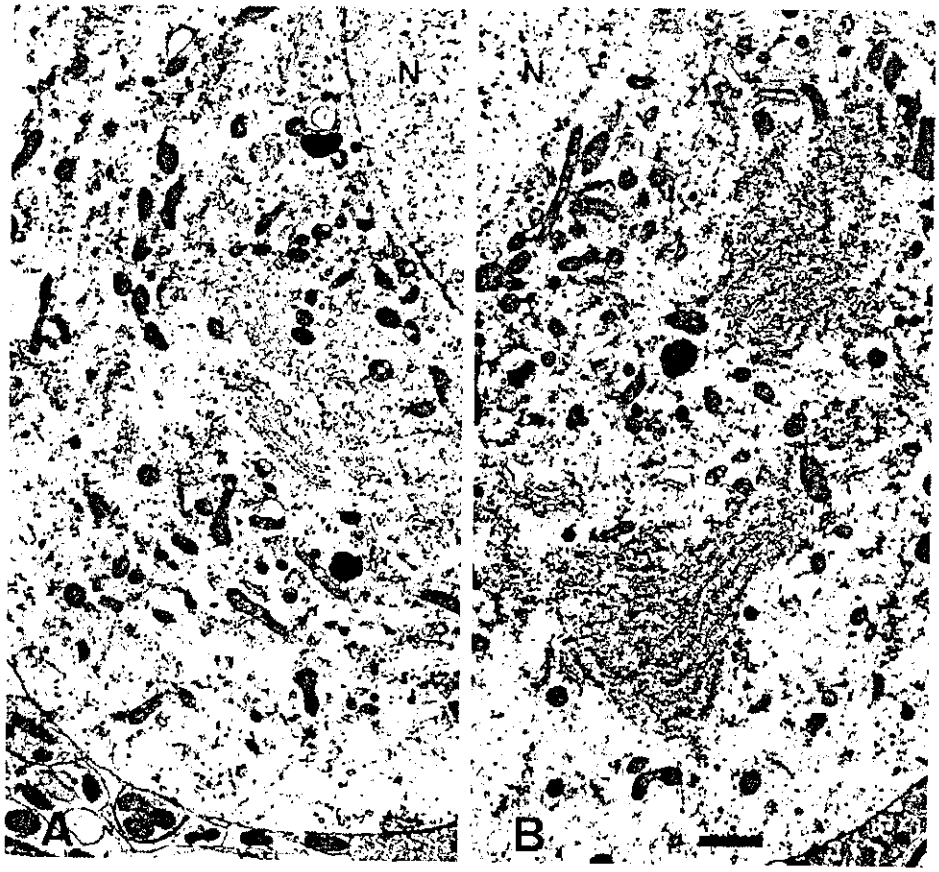

Fig. 5 Ultrastructure of the AHCs. The rERs are severely fragmented, and the amount of rER and the number of free ribosomes sre obviously lower in the neurons of *kl/kl* mice (**A**) as compared with those in WT mice (**B**), whereas mitochondria, nuclear membrane, and nuclear karyoplasm and nucleolus in the AHCs in *kl/kl* mice appear unchanged (*rER* rough endoplasmic reticulum, *N* nucleus). Uranyl-lead staining. *Bar* 1 μm

Transcription activity of rRNA gene in the AHCs

AgNOR-positive areas were clearly and exclusively observed in dark fine granules within the nucleus and in the nucleolus. The ratios of AgNOR-positive areas to cross-sectional areas of the nucleus were significantly lower in the neurons of *kl/kl* mice than of WT (Fig. 6A–C).

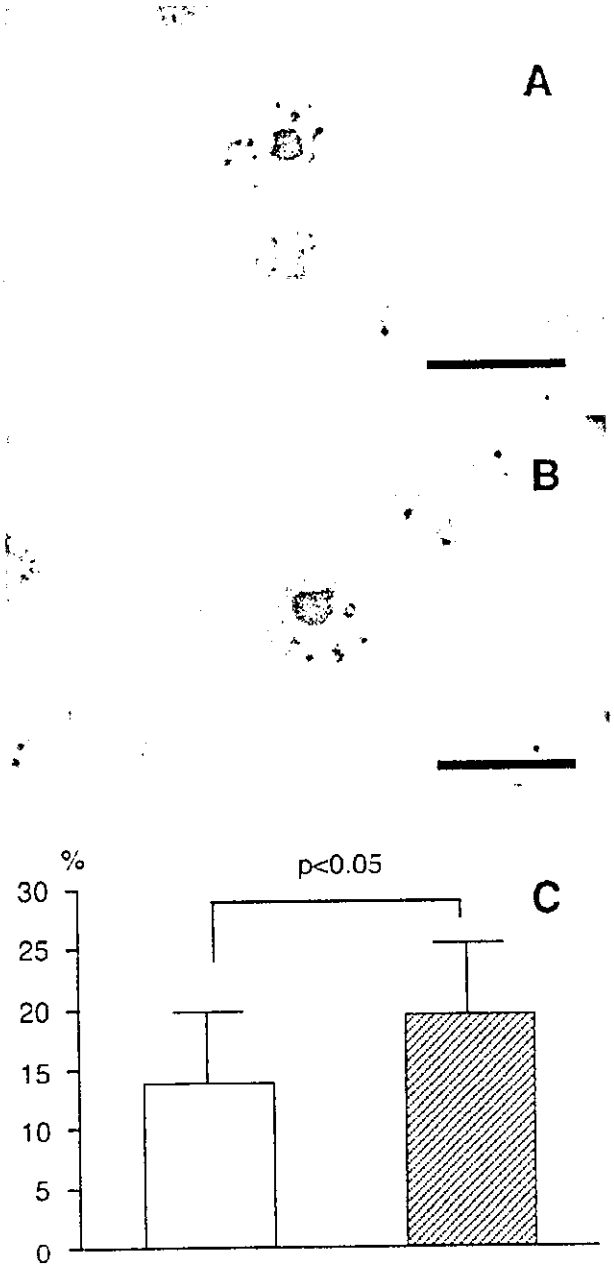

Fig. 6 Transcription activity of rRNA gene in the AHCs. AgNOR-positive area in large AHCs of the *kl/kl* mice (**A**) and WT mice (**B**). The ratio of this area to cross-sectional cellular area of the nucleus is significantly lower in *kl/kl* mice (*open bar*) than WT mice (*hatched bar*) (*error bars* indicate SD) (**C**) (*rRNA* ribosomal RNA, *AgNORs* Silver staining of nucleolar organizer region-associated proteins)

Discussion

The *klotho* gene carries two varieties of mRNA. The majority is mRNA coding a membrane-type Klotho protein, from which a transmembrane-type Klotho protein is translated [27, 29]. The

transmembrane-type protein is composed of an N-terminal signal sequence and a C-terminal transmembrane domain, and between them, two domains (KL1, KL2) homologous to β-glucosidase hydrolyzing steroid β-glucuronides [39]. The other mRNA variety gives rise to strap codons, which translate an approximately half-length secretory Klotho protein. The secretory Klotho protein has only a signal sequence and KL1 domain. It is not known whether Klotho proteins have enzymatic activity. The *klotho* gene is reported to be strongly expressed in the kidneys, and weakly in the brain. *Klotho* gene expression has not been reported in the lungs, bones, or skin, which show severe pathological changes in *kl/kl* mice. These facts suggest the possibility that pathological findings in *kl/kl* mice are not a simple phenotype of the *klotho* gene, but that the secretory Klotho protein may have some function whereby pathological changes are suppressed in WT mice [27, 29].

Our present investigation on *klotho* gene expression revealed that the gene was observed strongly in WT and weakly, but significantly, in *kl/kl* mice after 40 cycles in RT-PCR in the spinal cord. This result demonstrates that *kl/kl* mice are not null but severe hypomorph mice of the Klotho protein, and that the decrease of this protein may induce various morphological alterations observed in the present study.

The differential AgNOR staining is believed to stain certain proteins combined specifically with an rRNA gene, and the quantity of the proteins (AgNOR-positive areas) is believed to reflect the transcription activity of the rRNA gene [4, 6, 14]. To date, AgNOR stainability has been reported to be an index of phenomena including protein synthesis [4, 12], tumor proliferation [7, 8, 11, 31, 32, 34, 43], long-term changes during development [12], brain augmentation due to learning [42], decline in the aging brain [22, 23], and age-related reduction among dermal fibroblasts [8, 38].

The present light microscopic study showed a loss of Nissl substance (chromatolysis) in the AHCs of *kl/kl* mice. Reduction of integrated OD value of pyronin Y-positive material in the cytoplasm of AHCs in *kl/kl* mice indicates a decrease of the cytoplasmic RNA content, and ultrastructural investigation of AHCs revealed a reduction of the number of attached and free ribosomes and of rER in the mice in the present study. The ratio between the activity of rRNA gene transcription and the size of the nucleus in the anterior horn cells were evaluated. The results indicated that the depletion of the activity of rRNA gene transcription was not proportional to overall cell size. Thus, decreased transcription activity of the rRNA gene in the spinal neurons observed in the present study may cause a decrease in cytoplasmic RNA, ribosomes and rER of the AHCs. The reduction of the amount of the ribosomes and rER might induce small neurons in

the AHCs of the spinal cord with sparing the posterior horn in *kl/kl* mice. To determine the cause and mechanism, the amount of Klotho protein should be examined both in the ventral and posterior horns. In addition, regarding the mechanism of reduction of rRNA gene transcription activity, it should be elucidated whether or not the rRNA gene decreases in the chromosomal DNA; some genes are unavailable in the hybridization after being covered by proteins or other cross-linkers [13], and the RNA polymerase I, TFIIA, TFIIB, TATA box-binding protein, and CRE-binding protein exist normally in the AHCs [2].

A decreased number of small neurons and the absence of chromatolysis have been reported in the AHCs of aged humans [5, 26, 37, 40]. These findings differ from those observed in the *kl/kl* mice in the present study. Thus, the pathological mechanisms occurring in the spinal cord in aged humans and *kl/kl* mice are considered to be basically dissimilar. Further examination of the spinal cords in fetus and newborn, to analyze possibly overlapping developmental disturbance, is needed to determine the cause of the severe reduction in the volume of the spinal cord in *kl/kl* mice.

Kl/kl mice show a marked reduction in body weight. Thus, developmental retardation may exist in the mice. However, the brain is relatively well developed and preserved. Neuronal population and the size of neurons in the brains did not show any apparent differences from those of the controls. In addition, the number and size of the neurons in the posterior horn in the spinal cord appeared to be preserved. These findings indicate that normal development and selective degeneration possibly occurred in the AHCs. It is hardly conceivable that selective developmental retardation occurred in the AHCs, and that developmental retardation of the skeletal muscles induce anterior horn degeneration showing rER reduction. There have been no reports that any congenital muscle dystrophy in mice or humans induces rER reduction in the AHCs.

On the other hand, a decreased number of large neurons and reduced amount of the cytoplasmic RNA, rER and ribosomes, as observed in the spinal cord of *kl/kl* mice, have been noted in the motor neurons in the spinal cord and brain stem of patients with classic ALS [15, 16, 28]; however, Bunina bodies and spheroids were absent in these mice. An accumulation of neurofilaments in the AHCs, as in patients with classic ALS [9, 24, 35], was also reported in the peripheral nerve axon in *kl/kl* mice [41]. Decrease of rER (chromatolysis) and accumulation of neurofilaments have been considered to be early changes in the AHCs in patients with classic ALS [17, 20]. Reactive astrocytosis observed in the anterior horn of the *kl/kl* mice in the present study relates to a degenerative process, and has been reported in the ventral horn in patients with classic ALS. This resemblance shows that *klotho* gene insufficiency causes neuronal dysfunction, and might indicate that the *klotho* gene is involved in the pathological mechanism of classic ALS. Mutant SOD mice

may be a good tool for the research of familial ALS [10], and further study is needed to evaluate whether the *kl/kl* mice, which are senescence-accelerated mice showing decreased rER, ribosomes, and cytoplasmic RNA in the AHCs, is a new animal model of AHC degeneration, and can provide clues to understanding the etiology of classic ALS.

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Table 1 The body and brain weight of *kl/kl* and WT mice at the age of 7 weeks. Values represent mean ± SD (*kl/kl* homozygotes with the *klotho* mutation, *WT* wild-type)

	Body weight (g)	Brain weight (g)
kl/kl (male, *n*=17)	7.6±1.1*	0.37±0.02*
WT (male, *n*=17)	24.4±1.58	0.47±0.03

*$P<0.0001$

Table 2 The integrated optical density values (mean ± SD) of the cytoplasmic RNA in the large AHCs stained with pyronin Y in *kl/kl* and WT mice. Measurement was performed in 100 randomly distributed AHCs with nucleolus (10 AHCs/mouse,10 mice each) (*AHC* anterior horn cell)

	Integrated OD value
kl/kl (male)	5,424±3,450* (n=100)
WT (male)	8,427±6,988 (n=100)

*P<0.05

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The nature of the parkinsonism-dementia complex and amyotrophic lateral sclerosis of Guam and magnesium deficiency for two generations

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Abstract

The parkinsonism-dementia complex (PDC) and amyotrophic lateral sclerosis (ALS) were the fatal neurological diseases, showing very high incidence during 1950–1970 and dramatic decrease after 1970 on Guam. Through the research, the present author insisted that; (1) NFTs in Guam ALS patients are merely a background feature widely dispersed in the population, (2) Guam ALS and PDC are basically different diseases, and (3) Guam ALS occurs initially as classic ALS. As pathogeneses of the diseases, intake of low calcium (Ca) and magnesium (Mg) and high aluminum water and of some plant excitatory neurotoxin has been speculated. To elucidate the pathogenesis, the author performed an experiment exposing rats to low Ca and/or Mg intake for two generations, so as to follow the actual way of human living on the island, since several generations live continuously in the same environment. In the experiment, severe loss of dopaminergic neurons was identified exclusively in the substantia nigra in 1-year-old rats of continuous low Mg intake (1/5 of the normal control food) for two generations. The present study indicates that continuous low Mg intake for two generations induces exclusive loss of dopaminergic neurons in in rats, and may support the Mg hypothesis in the pathogenesis of PDC of Guam.

Keywords: Guam; Parkinsonism-dementia complex; Amyotrophic lateral sclerosis; Magnesium deficiency

1. Introduction

The Chamorro population on Guam in the western Pacific Ocean had represented about fifty times of the annual incidence rate of amyotrophic lateral sclerosis (ALS) as compared with the average in the world during 1950–1970 [1], and the rate of the parkinsonism-dementia complex (PDC) [2,3], which is a disease reported exclusively in Guam, Kii peninsula in Japan and west New Guinea, was also very high in the almost same period in Guam. Malamud et al. [4] and Hirano et al. [5] proposed that the ALS of Guam and Guam PDC are a single disease entity, and that Guam ALS is a disease different from classic ALS, because (i) the topographic distribution of NFTs and neuronal loss is similar to that of Guam PDC, (ii) patients with combined PDC and ALS (PDC–ALS) have been identified, and (iii) ALS as well as PDC patients are sometimes admixed within the same family. Regarding to the definition and pathogenesis of the diseases, the author intends to elucidate the nature of these fatal diseases, such as PDC and ALS, and performed an experimental study using rats exposing low magnesium (Mg) and/or calcium (Ca) for two generations.

2. Epidemiology

The maximum annual incidence rate of ALS of Guam from 1945 to 1955 was reported to be about 60–70 per 100,000 for men and 30–40 per 100,000 for women, and that of PDC in Guam from 1950 to 1970, to be about 60 for men and 20 for women [6,7]. The annual incidence rate of ALS was quite different among villages, from 0 to 250 per 100,000 population [1]. Although precise epidemiologic study is scant on Guam before the World War II, patients with ALS and/or PDC might exist on Guam from the far past, but it is evident that the annual incidence rate of these diseases on Guam has decreased remarkably after the World War II within short duration.

The mortality rate of PDC in Chamorro people on Saipan, a northern island of Guam, whose genotypic

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composition is similar to Guam Chamorro, was strikingly low suggesting an environmental risk factor [8]. Filipino migrants to Guam are susceptible to the disease further supporting an environmental over genetic etiology [9]. The increased risk to spouses of affected individuals in a longitudinal case-control study also strongly implicated environmental factors [10]. Since 1965, the incidence rate of Guam PDC has been decreasing, especially in men, but has remained at about 10–25 per 100,000 when last estimated for the period of 1980–1990 [6,7]. These findings suggest that environmental factors in combination with possible genetic risk factors may predispose to Guam PDC and account for the decreasing incidence in recent years. Similarly, the incidence rate for ALS has markedly decreased in recent years and is now similar to the rate in the rest of the world, namely about 3–5 per 100,000 [11].

3. Clinical symptoms

3.1. Parkinsonism-dementia complex

Patients with PDC are characterized by rigidity, tremor, bradykinesia and dementia in the fifth to sixth decade of life, and progression to a vegetative state with pelvicrural flexion contractures within 4–6 years [2,12]. About 40% of the patients with PDC, have clinical evidence of diabetes mellitus and hypertension before the onset of PDC [13].

3.2. Amyotrophic lateral sclerosis and ALS–PDC

Clinical symptoms of the patients with ALS of Guam have been reported to be essentially similar to those of the classic ALS. Average of onset of the disease was 47 years in male and 42 years in female, and spasticity had been reported to be the single initial feature in 13% of the patients [14], and 10% of the ALS patients lived at least 10 years [14]. Pyramidal tract sign was remarkable, but many patients lacked lower motor neuron signs of the lower extremities. Patients with long duration of the disease showed marked spasticity in the legs [15]. It has been reported that 5% of the patients with ALS subsequently developed the total clinical pictures of PDC (ALS–PDC), while 38% of the original PDC patients eventually developed typical ALS (PDC–ALS). Those ALS–PDC and PDC–ALS patients showed mixture of the symptoms of ALS and PDC [14].

4. Neuropathological findings

4.1. Parkinsonism-dementia complex

4.1.1. Macroscopic findings

The brain weight of PDC patients is reduced to be about 1070 g [16]. The cerebrum shows diffuse atrophy accentuated in the frontal and temporal lobes. The thickness of the cerebral cortex is generally reduced, especially in the hippocampus and parahippocampal gyrus. The hemispheric white matter is diffusely atrophic. The basal ganglia and thalamus are less severely deteriorated. The midbrain and pons show as severe atrophy as in the cerebrum (Fig. 1). There is marked depigmentation of substantia nigra and locus ceruleus. The volume of the cerebellum is preserved [16,17].

4.1.2. Neuronal loss and neurofibrillary tangles (NFTs)

The topographic distribution of neuronal loss and NFTs roughly coincide with that of brain atrophy [16,18–20]. Severe loss of neurons is seen in the CA1, and severe to moderate loss is observed in the temporal, insular and frontal cortices. Many NFTs are observed in the CA1, parahippocampal gyrus, temporal neocortex, and frontal cortex. The NFTs are predominantly distributed in the superficial layers of the cerebral cortex [21]. A great number of granulovacuolar degeneration and many Hirano bodies are seen in Ammon's horn. Except in a few cases, there are only small numbers of senile plaques [3,16,20,22]. Curly fibres/neuropil threads are rarely seen [16,22]. The cerebral white matter shows severe atrophy, but myelin pallor and threads are not remarkable in the cerebral white matter of most cases.

The number of large neurons in the neostriatum and the nucleus accumbens decreases to 40 and to10% of the control level, respectively. Large neurons correspondingly decrease in the basal nucleus of Meynert [23]. The globus pallidus shows a moderate neuronal loss and density of NFTs. Many alpha-synuclein positive neuronal inclusions and neurites are observed chiefly in the amygdaloid nucleus, and frequently coexist with tau-positive pretangles and NFTs in the same neurons [24]. The thalamus shows moderate neuronal loss and NFTs in the lateral nucleus and mild loss of neurons with some NFTs in the medial nucleus. Severe loss of neurons and many NFTs are present in the hypothalamus [3,16,18].

Severe loss of pigmented and nonpigmented neurons and presence of NFTs are observed in the substantia nigra [3,16,25], ventral tegmental area, locus ceruleus and superior central nucleus. Lewy bodies are rare. The pedunculopontine and pontine nuclei show many NFTs with mild neuronal loss [19].

Purkinje and granule cells are preserved in number. Although a small number of NFTs are observed in the dentate nucleus; no neuronal loss is evident and no grumose degeneration is seen [16]. No marked degeneration is observed in the cerebellar and spinal white matter. The spinal anterior horn cells appear shrunken, but are not reduced in number. A small number of NFTs composed of STs are observed in the intermediate zone and posterior horn, and occasionally in the anterior horn [3,16,19].

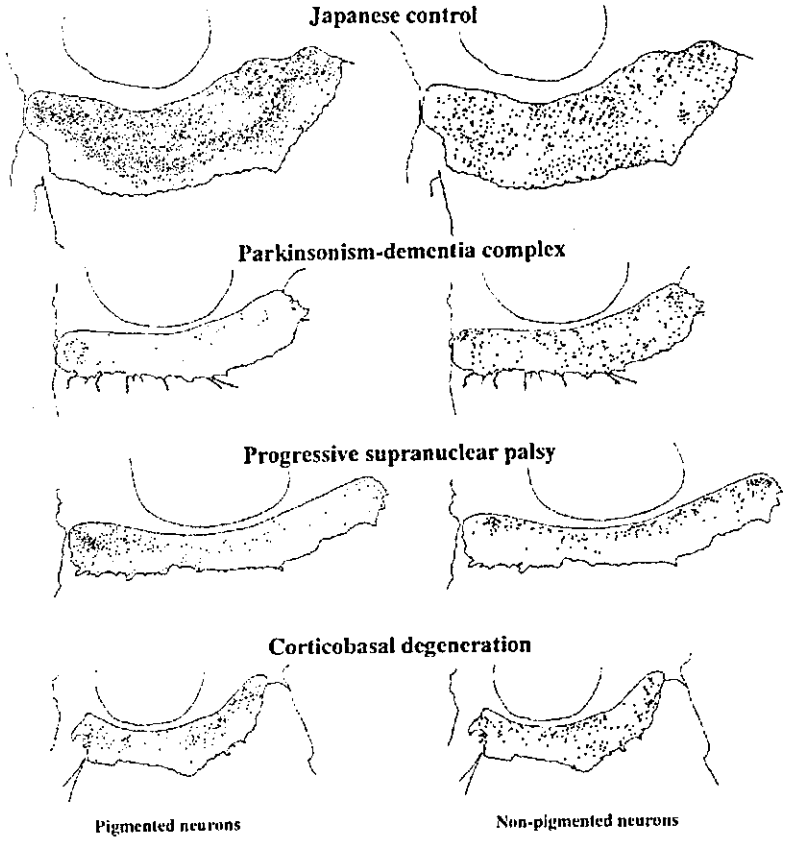

Fig. 1. Midbrain of the parkinsonism-dementia complex. Atrophy of midbrain and severe depigmentation of the substantia nigra was seen in a patient with PDC (right) comparing with an age-matched control Guamanian (left).

4.1.3. Ultrastructure and biochemistry of NFTs

NFTs are immunopositive for tau [16], and are mostly composed of paired helical filaments (PHFs) and partly of straight tubules (STs) in the Ammon's horn [19,26]. The remaining large neurons in the neostriatum frequently contain NFTs composed mainly of PHFs and partly of STs [23]. NFTs in the spinal cord were composed of STs [19]. A major tau triplet, 55, 64 and 69 kDa, and a minor variant at 74 kDa are the components of tau protein of NFTs in Guam PDC [27,28].

4.1.4. Glial inclusions

Tau-immunopositive and Gallyas-positive glial inclusions are observed in the patients with PDC. Granular hazy inclusions are observed in the astrocytes predominantly in the amygdaloid nucleus, motor cortex, and inferior olivary nucleus [29]. Coiled/crescent bodies are present in the oligodendroglia of the anterior nucleus of the thalamus, motor cortex, midbrain tegmentum, and the pyramids [29].

4.2. Amyotrophic lateral sclerosis and ALS–PDC

Essential neuropathological findings of the patients with ALS on Guam are those reported in the classic ALS. Neuronal degeneration is fundamentally restricted to the upper and lower motor neuron system. Lateral and anterior corticospinal tracts in many of the patients with ALS showed degeneration with preservation of the posterior funiculus. Bunina bodies and skein inclusions are frequently observed in the spinal anterior horn cells and facial and hypoglossal nuclei [16,19,20,30]. Ubiqutinated inclusion bodies (so-called motor neuron disease-inclusion) in the dentate gyrus and neuronal loss in the subiculum are not remarkable.

However, in addition to these findings, patients with ALS on Guam frequently showed NFTs and neuronal loss in the areas whose topographic distribution is similar to that of PDC [19].

5. Differential diagnosis and the nature of PDC and ALS on Guam

5.1. Parkinsonism-dementia complex

The large amount of NFTs with relatively small number of neuropil threads and glial tangles in Guam PDC is different from the widespread threads and glial tangles in

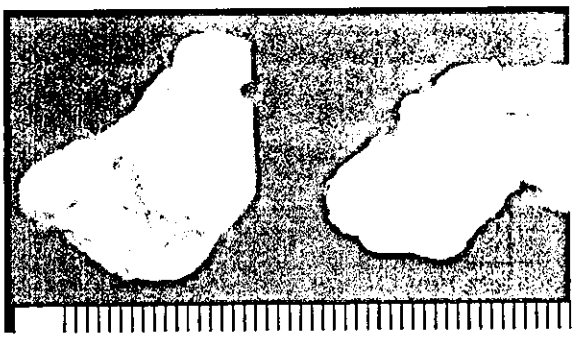

Fig. 2. Topographic distribution of loss of neurons in the substantia nigra. (Cited from Ref. [25]).

the grey and white matter found in progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). The mild loss of neurons in the subthalamic nucleus, absence of grumose degeneration in the dentate nucleus and rare tuft-astrocytes in Guam PDC are different from PSP. Topographic distribution of loss of neurons in the substantia nigra is different from those in PSP and CBD [25] (Fig. 2). The absence of astrocytic plaques and ballooned neurons, and the small number of pretangles and foamy spheroid bodies in Guam PDC are different from CBD [20]. Astrocytic inclusions in PEP have been reported to be restricted to within the third ventricle wall and the periaqueductal area. Granular hazy astrocytic inclusions have been exclusively reported in Guam PDC [20,29].

5.2. Amyotrophic lateral sclerosis and ALS–PDC

As described above, it has been proposed that the ALS of Guam and Guam PDC are a single disease entity, and that Guam ALS is a disease different from classic ALS [4,5]. To elucidate the fundamental differences and similarities of the neuropathological features and etiopathogenesis of PDC and ALS of Guam, the author conducted a topographic and quantitative investigation of NFTs in 61 areas of the brains in 7 Guam ALS patients, 6 PDC patients, 3 ALS-PDC combined patients, and 20 non-ALS non-PDC Guamanians. NFTs were observed in 75% of non-ALS non-PDC Guamanian subjects, and in 86% of Guam ALS patients. The numbers of NFTs in the non-ALS non-PDC subjects and in ALS patients were the same, and less than that of PDC patients. The number of NFTs in ALS-PDC was the same as in PDC [19]. These findings indicate that;

(1) NFTs broadly occur in Guamanians living on Guam,
(2) NFTs in Guam ALS patients are merely a background feature widely dispersed in the population,
(3) Guam ALS and PDC are basically different diseases,
(4) Subtraction of the NFTs and related neuronal loss from the neuropathological findings of Guam ALS reveals findings of classic ALS,
(5) ALS–PDC patients are considered to be combined cases of ALS and PDC. In other words, association of NFTs to the non-ALS non-PDC subjects and patients with ALS over the certain threshold level, brings them to PDC or ALS–PDC.
(6) Guam ALS occurs initially as classic ALS.

Summary of the discussion [19,20]:

(1) Guam ALS − NFT = Classic ALS
(2) Classic ALS + NFT = Guam ALS
(3) Classic ALS + many NFT = ALS–PDC
(4) Guam ALS = Initially classic ALS
(5) Guam ALS ≠ PDC

6. Pathogenesis

6.1. Genetics

No mutation of the tau gene has been found in PDC patients [31]. The percentage of subjects with the homozygous tau allele A0 is somewhat higher in Chamorro controls and PDC patients than Caucasian controls [32]. The representation of the G-to-C mutation in exon 9 of the CYP2D6 gene, linked to a slower metabolism of exogenous toxins, is higher in Chamorro control subjects and PDC patients than in Caucasian controls, and the apolipoprotein E2 allele frequencies in Chamorro controls and Guam PDC patients are considerably lower than those in Caucasian controls [33].

6.2. Environmental factors and experimental study

Infectious causes have been contradicted by the intense study by NIH, USA. Eventually intake of low Mg and Ca and high Al water [34] and of some plant neurotoxins (from cycad flour) [35], and a certain genetic predisposition [36] have been proposed. Recently another neurotoxin hypothesis from cycad rumphii via flying fox dish was proposed. The paper stressed high condensation of the toxin in bats [37].

Based on these possible pathogeneses proposed, experimental studies focusing on low Mg and Ca and high Al, and on plant neurotoxins have been explored. Repeated oral administration of alpha-amino-beta-methylaminopropionic acid (L-BMAA), the proposed toxic factor within flour of cycad circinalis, to macaques produces chromatolysis of Betz cells, simple atrophy of spinal anterior horn cells and neuritic swelling in the substantia nigra [35], and a low-Ca, high-Al diet in monkeys induces neurofibrillary pathology characterized by accumulation of phosphorylated neurofilaments in the anterior horn cells [38]. A low-Ca and Mg, high-Al diet in mice for long duration of 11–31 months, induces loss of neurons and occurrence of tau-immunopositive neurons in the cerebral cortex [39]. Despite decades

of research, no animal model completely recapitulates PDC or ALS. However, most of these experiments used adult or infantile animals in one generation.

7. Nigral degeneration in rats with Mg deficiency for two generations

Symptoms of the PDC and ALS occur at 50 or 60th in the life, and the patients with the diseases cannot to start to intake the water or the cycad flour at the ages. The present author performed an experiment using rats with long duration exposure of low Ca and/or Mg intake for two generations, in order to reproduce the actual way of life on the island, i.e. several generations live in the same circumstances.

7.1. Materials and methods

Wistar albino rats were used. For elucidation of the critical period, which will induce lesions in the rats later, five groups with different exposure time were settled. Group (i): starting exposure before mating till birth of the pups, group (ii): starting before mating until 1 month following the pups' birth, group (iii): starting before mating until the pups were 1-year-old, group (iv): starting 2 months after the birth of the pups until 1-year-old, finally group (v): after 2 month of the pups to 1 year.

In the present study, the foods were compounded with these trace metals in six different ratios. Food decreased Ca and Mg into two steps, about half and one-fifth of the normal control feed. Nos. #1 and 2 are groups fed low concentration of Ca, # 3 and 4 are groups of low Mg, and #5 and 6 are groups of both low Ca and Mg. Distilled and deionized milli-Q water was given to drink. The food and water were given ad libitum. Number of experimental groups are 60, and examined number of rats was totally about 600.

For these groups, the pups were sacrificed at embryonic day 16, and birth day, one month, six months, 1 year after birth by perfusion fixation through the aorta by 4% paraformaldehyde (PFA) or 2.5% glutaraldehyde (GA) in 0.1 M phosphate buffer (PB) (pH 7.2) after deep anesthesia of ethyl ether. At sacrifice, the blood was taken, and the mineral content, such as Ca, Mg, iron, potassium, chloride and phosphorus within serum was examined.

Light microscopic examination was performed on the central nervous system using 5-μm-thick sections of paraffin-embedded tissues stained with hematoxylin and eosin and Küver-Barrera. An electron microscope (Hitachi H-9000) was used to examine the substantia nigra. The GA fixed tissues were post-fixed by 1% osmium tetroxide, dehydrated through a graded ethanol series and then embedded in Epon 812. Toluidine-blue-stained 1-μm-thick sections were examined with the aid of a light microscope, and ultrathin sections were stained with lead citrate and uranyl acetate and then examined using the electron microscope at 100 kV.

7.2. Results

Histological examination of the brain and spinal cord revealed no evident alteration in animals of E16 and birthday in each group. In the group #4, central nervous system was not remarkable till 6 months, but neurons and neuropile of the cerebral cortex were slightly atrophic at 1 year. The most significant changes were observed in the substantia nigra at 1 year. The substantia nigra of the rats of group (iii) in #4, which was continuous Mg deficiency till 1 year, showed marked atrophy. The neurons decreased in number, and appeared small in size in the rats of Mg deficiency. Immunohistochemistry for tyrosine hydroxylase (TH) revealed that the size and number of TH-immunopositive neurons were small, and the number of TH-immunopositive dendrites or axons of the substantia nigra decreased severely in 1-year-old rats in the group #4.

Electron microscopic observation showed decreased number of mitochondria, rough endoplasmic reticulum and free ribosomes, and nuclear membrane indentation in the remaining neurons of the substantia nigra of 1-year-old rats in Mg deficiency (group (iii), #4). Similar but less evident changes were observed in the groups of (iii) in #4 at 6 months, and (ii) in #4 at 1 year. There were no evident changes in the substantia nigra in each group at 6 months.

GFAP-immunopositive reactive gliosis was observed in the substantia nigra in rats of group (iii) in #4 at 1 year. Lewy bodies, neurofibrillary tangels and senile plaques were not evident in each group, revealed by immunohistochemistry for alpha-synuclein, tau, ubiquitin and beta-peptide.

The spinal cord in each group was unremarkable.

7.3. Discussion

It has been reported that dietary Mg deficiency plays a major role in the pathogenesis of ischemic heart disease in humans, congestive heart failure, cardiac arrythmia, vascular complications of diabetes mellitus, pre-eclampsia and hypertension. Mg deficiency, as a possible pathogenesis of neurological diseases, had been speculated in the PDC and ALS in Chamorro population on Guam. Findings reported in the present study lead to the conclusion that Mg deficiency for two generations in rats induces the degeneration of substantia nigra by involving the mitochondria, rER, and free ribosomes in the neurons.

Degree of loss of neurons in the substantia nigra, and developmental retardation were more evident in the group of low Mg intake than the group of both low Mg and Ca intake. The finding shows that solely Mg deficient diet is more fundamental than evenly deficient diet of both Mg

and Ca for the degeneration of the neurons and physical development.

In the present study, motor neurons, such as anterior horn cells were unremarkable though the substantia nigra showed evident loss of neurons. This might indicate that the pathogenesis of ALS of Guam is different from that of PDC of Guam, as reported in neuropathological studies of the patients by the present authors.

Though neurofibrillary tangles were not identified, this study may support the Mg hypothesis for pathogenesis of PDC of Guam, and may indicate the possibility of prevention/treatment of parkinsonism by Mg intake.

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Oxidized galectin-1 advances the functional recovery after peripheral nerve injury

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Abstract

Oxidized galectin-1 has been shown to promote axonal regeneration from transected-nerve sites in an in vitro dorsal root ganglion (DRG) explant model as well as in in vivo peripheral nerve axotomy models. The present study provides evidence that oxidized galectin-1 advances the restoration of nerve function after peripheral nerve injury. The sciatic nerve of adult rats was transected and the distal nerve was frozen after being sutured into a proximal site with four epineurial stitches. An osmotic pump delivered oxidized galectin-1 peripherally to the surgical site. Functional recovery was assessed by measurement of the degree of toe spread of the hind paw for 3 months after the sciatic nerve lesion. The recovery curves of toe spread in the test group showed a statistically significant improvement of functional recovery after day 21 by the application of oxidized recombinant human galectin-1 (rhGAL-1/Ox) compared to the control group. This functional recovery was supported by histological analysis performed by light microscopic examination. The regenerating myelinated fibers at the site 21 mm distal to the nerve-transected site were quantitatively examined at 100 days after the operation. The frequency distribution of myelinated fiber diameters showed that exogenous rhGAL-1/Ox increased the number and diameter of regenerating myelinated fibers; the number of medium-sized (6–11 μm in diameter) fibers increased significantly ($P < 0.05$). These results indicate that oxidized galectin-1 promotes the restoration of nerve function after peripheral nerve injury. Thus, rhGAL-1/Ox may be a factor for functional restoration of injured peripheral nerves.

Keywords: Oxidized galectin-1; Axonal regeneration; Sciatic nerve injury; Remyelination; Myelinated fiber; Functional recovery; Toe spread

Initiation of neural restoration after axotomy has been thought to be regulated by neurotrophic factors [1,17], however, it remains unclear what signal prompts the axons to send out new processes in response to nerve injuries [1]. Recently, we discovered that oxidized galectin-1 promotes initial axonal growth after axotomy in peripheral nerves [6,7,10]. Galectin-1 is a member of a family of β-galactoside-binding lectins and is intensely expressed in dorsal root ganglion (DRG) neurons, spinal cord motoneurons and Schwann cells in normal sciatic nerves of adult rodents [3,6,9,16]. Galectin-1 contains six cysteine residues and exhibits lectin activity in its reduced form [11,15]. However, our structural–activity relationship study revealed that galectin-1 promotes axonal regeneration only in its oxidized form, which contains three intramolecular disulfide bonds [10]. Therefore, it is necessary to distinguish between oxidized galectin-1, which promotes

Abbreviations: rhGAL-1, recombinant human galectin-1; rhGAL-1/Ox, oxidized recombinant human galectin-1; PBS, phosphate-buffered saline; DRG, dorsal root ganglion

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axonal regeneration, and galectin-1, which shows lectin activity.

Using in vivo peripheral nerve regeneration models, we have demonstrated that the application of oxidized recombinant human galectin-1 (rhGAL-1/Ox) to the injured region promotes axonal growth [6]. Conversely, treatment with a functional blocking galectin-1 antibody strongly inhibits the restoration. These experiments show that oxidized galectin-1 is an essential factor for initiating axonal regeneration in injured peripheral nerves.

In the present study, we examined whether or not local administration of exogenous rhGAL-1/Ox advances the restoration of nerve function using a rat injured sciatic nerve model. The degree of toe spread [4] was measured for 3 months after the sciatic nerve lesion in order to assess functional recovery. Histological and quantitative studies [14] were also conducted after the functional assessment to evaluate the regeneration of myelinated fibers.

rhGAL-1/Ox was prepared as described previously [6,10]. Briefly, *Escherichia coli* expressed rhGAL-1 was purified by DEAE-HPLC and rhGAL-1 was oxidized by the air oxidation method using $CuSO_4$ as a catalyst. rhGAL-1/Ox was purified by reversed phase HPLC.A total of 36 adult male Sprague–Dawley rats (10 weeks) were used. The animals were randomly assigned to one of three groups of 12 animals each. In accordance with the guidelines of our institutional Animal Research Committee, we placed the animals two to a gage with a 12-h light:12-h dark cycle, and rat chow and water were available ad libitum.

In a previous study, we have shown that oxidized galectin-1 promotes axonal regeneration together as well as the Schwann cell migration into the acellular nerve or a grafted silicone tube filled with collagen gel [6]. In order to reproduce the appropriate acellular nerve conditions, we introduced a cut and freeze-killed sciatic nerve model. The operation was carried out according to the method previously described [13], with some modification. Briefly, the animals were anesthetized, to avoid unnecessary pain, with intraperitoneal sodium pentobarbital (60 mg/kg). The left sciatic nerve was exposed and transected at the mid-thigh level with microscissors. The distal stump was sutured into the proximal stump with four epineurial stitches of 8–0 Nylon, then 7 mm of distal nerve section was frozen for 10 s with forceps that had been chilled in liquid nitrogen. An osmotic pump (Alza Corp., 2 ml reservoir) was used to deliver test solutions peripherally, either rhGAL-1/Ox or PBS for control. The solutions were delivered at 2.5 μl/h from the polyethylene tube connected to the osmotic pump, which was implanted subcutaneously on the back, for a period of 4 weeks. Rats were divided into three groups of 12 rats each: a control group which was treated with phosphate buffered saline (PBS), and two test groups, one of which received 5 μg/ml of rhGAL-1/Ox solution applied to the surgical site, and one of which received 100 μg/ml of rhGAL-1/Ox solution. These concentrations of rhGAL-1/Ox solution were chosen because 5 μg/ml of rhGAL-1/Ox was found to be effective in a mouse model in our previous studies [3,6], and because a higher dose would be expected to advance functional recovery. No rats showed any toxic effects in reaction to the administration of rhGAL-1/Ox.Functional recovery was evaluated by measuring the degree of toe spread [4], which is defined as the maximum distances between the first and fifth toes (toe spread) and between the second and fourth toes (intermediary toe spread) of the hind paw. Both toe spreads were measured at 1, 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84 days postoperatively with calipers at the tips of the toes by holding the rat's back from behind and pushing the paw slightly to the floor. The distance of the toe spread were not dependent on the pushing strength and reproducibility of the data was very good; note that the pressure exerted on the hind paw remains constant regardless of pushing strength because the rat's pelvis absorbs any extra pressure beyond that which produces the toe spread. All measurements were carried out at least three times, and the values were averaged ($n = 12$ for each group).After the functional assessment, 14 rats (four in the PBS group, four in the 100 μg/ml rhGAL-1/Ox group, two in the 5 μg/ml rhGAL-1/Ox group and four in an unoperated group) were perfused through the heart and fixed with 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate. The fixed sciatic nerve was dehydrated through a graded ethanol series and embedded in EPON 812. Cross sections (1 μm-thick) were cut 21 mm distal to the transection site, then stained with toluidine blue and examined under a light microscope. The quantitative analysis of regenerating myelinated axons was performed as described previously [14]. Briefly, photographs of three randomly chosen areas (16,870 μm^2 each) of the cross sections were taken (×200 magnification). Enlarged prints (×2200 magnification) were made, and a digitizer was used to obtain the mean diameter of the myelinated fibers by averaging the longest and shortest diameters (the latter being perpendicular to the former). The data for the three areas were summed and converted to numerical values per total area of the cross section, and the frequency distribution of the myelinated axon diameters, in 1-μm increments, was determined.Statistical analysis of the experiments was performed using StatView for Macintosh (SAS Institute, Cary, NC). Significant differences between groups were determined by two-way ANOVA. Data are presented as the mean ± S.E.M. $P < 0.05$ between any two groups was considered significant according to the Bonferroni procedure.Motor nerve conduction velocity (MNCV) was measured using 18 rats with the same operation mentioned above: 10 rats were received PBS and eight were received 100 μg/ml rhGAL-1/Ox. MNCV was measured on left sciatic nerve at day 84 after the operation using MEB-7102 instrument (Nihon Koden, Osaka, Japan). Measurements were performed under general anesthesia using halothane and the body temperature of the animals was kept constant at 37 °C. The comparisons between the groups for MNCV were performed using an unpaired *t*-test.

At 12 weeks after the operation, the nerve repair sites and pump delivery systems were intact and there was no dif-