Decreased Amount of Collagen in The Skin of Amyotrophic Lateral Sclerosis in The Kii Peninsula of Japan

Tomomi Tsukie[1], Hiroaki Masaki[1], Sohei Yoshida[2], Mikio Fujikura[1], Seiitsu Ono[1]

Abstract-

Purpose: The Kii Peninsula of Japan, together with Guam and West New Guinea, has one of the highest incidences of amyotrophic lateral sclerosis (Kii ALS) in the world. There is a controversy whether the etiology is the same or not between sporadic ALS and Kii ALS. Skin studies from patients with sporadic ALS have shown unique pathological and biochemical abnormalities. However, there has been no report of collagen content of the skin of Kii ALS patients.

Methods: The skin tissues from Kii ALS patients were studied by electron microscopy and their collagen contents were examined.

Results: On electron microscopy the most conspicuous finding in Kii ALS was the smaller diameter of collagen fibrils. The collagen content per dry weight (mg) of the samples in Kii ALS was significantly decreased ($p<0.001$) than in controls. In Kii ALS patients the more severely affected pathological samples showed the greater decrease. In addition, there was a significant negative correlation ($r=-0.88$, $p<0.01$) between the collagen content and duration of illness in the Kii ALS patients, but there was no such correlation in controls.

Conclusion: These results indicate that the metabolism of skin collagen might be affected in the disease process of Kii ALS.

Key Words: collagen, skin, Kii peninsula, hydroxyproline, amyotrophic lateral sclerosis

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative motor neuron disease characterized by muscle weakness, atrophy, spasticity and eventually by respiratory failure. There is a degeneration and loss of motor neurons in the spinal cord, brainstem, and cerebral cortex. So far studies of sporadic ALS skin have shown unique pathological and biochemical abnormalities in collagen, elastic fibers, and the ground substance [1-3]. The

From the [1]Department of Neurology, Teikyo University Chiba Medical Center, 3426-3 Anesaki, Ichihara, Chiba 299-0111, Japan; [2]Department of Health Sciences, Kansai University of Health Sciences, 2-11-1 Wakaba, Kumatori-cho, Sennan-gun, Osaka 590-0482, Japan.

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Correspondence to: Seiitsu Ono, MD. Department of Neurology, Teikyo University Chiba Medical Center, 3426-3 Anesaki, Ichihara, Chiba 299-0111 (Japan)
E-mail: ono@med.teikyo-u.ac.jp

lack of bedsore formation even in the terminal stages in ALS patients is considered characteristic [4,5].

Two villages of the Kii Peninsula of Japan, Hobara in the Mie Prefecture and Kozagawa in the Wakayama Prefecture, are the high-incidence foci of ALS in the world, together with Guam and West New Guinea [6]. A distinct form of disease, parkinsonism-dementia complex (PDC), has also been known to occur at a very high prevalence rate in the same areas. In spite of differences in clinical manifestations, the same neuropathological changes occur in ALS and PDC [7]. The clinical features and neuropathogical findings of the ALS and PDC cases in this area (Kii ALS/PDC) are almost identical to ALS/PDC of Guam [8]. Since manifestations of Kii ALS and PDC frequenthy overlap in a single patient or in members of a family, they are suspected to be part of a spectrum of a single disease entity – Kii ALS/PDC – as is ALS/PDC of Guam [9-11]. Epidemiologically, Kii ALS/PDC differs from Guamanian ALS/PDC in its continuing high incidence and prevalence rates in the Kii area after the 1990s, in contrast to the marked decline in high incidence rates on Guam [12-14]. Many ALS patients from the Hobara area in Kii Peninsula and Guam Island have familial tracts, but those from the Kozagawa area in Kii Peninsula are almost always sporadic cases [15].

The clinical manifestations of Kii ALS consisted of amyotrophy, pyramidal tract signs, and bulbar palsy, but without dementia or parkinsonism, and were similar to those of classic ALS [16]. The initial symptoms were weakness in the extremities, gait disturbance and dysarthria although the range of duration of illness was longer than that of sporadic ALS. A certain number of patients with Kii ALS showed rigidity, resting tremor and parkinsonian hands besides muscle weakness and wasting in the extremities, hyperreflexia and positive Babinski reflexes [16]. Neuropathological features of Kii ALS were characterized by classical ALS pathology and the appearance of neurofibrillary tangles (NFTs), especially in the temporal cortex, hippocampus, amygdalia, hypothalamus, brain stem and spinal cord [6]. Intracytoplasmic inclusion bodies, including Bunina bodies, ubiquitin-immunopositive inclusions (Lewy body-like inclusions and skein-like inclusions), and neurofilamentous accumulations were also observed [16].

In previous reports, we studied the reticular dermis in patients with Kii ALS and controls. By light microscopy, collagen bundles in Kii ALS dermis were smaller in quantity and more loosely woven than in controls [17]. Electron microscopy revealed a significant negative correlation between the duration of illness and the diameter of collagen fibrils in patients with Kii ALS. There was also a marked increase in amorphous material separating collagen bundles in the ground substance.

There has been no report of collagen content in the skin of Kii ALS patients. In this communication, we present the amount of collagen of skin from patients with various stages of Kii ALS and compare these with controls matched for biopsy site, age and gender.

METHODS

Patients

Our subjects were 7 patients with Kii ALS (mean age±SD, 59.6±8.7 years; range, 46-74 years), and 12 controls were patients with other neurologic or muscular disorders (mean age±SD, 62.1±7.1 years; range, 49-73 years) (Table 1). All Kii ALS patients were from a high incidence area of Kii ALS, Kozagawa area in Wakayama Prefecture, Japan, and were sporadic. The diagnosis of ALS was made according to the El Escorial criteria of the World Federation of Neurology and all Kii ALS patients in whom weakness and/or atrophy started in the upper limbs had clinically definite ALS [18]. None of the patients with Kii ALS demonstrated abnormalities in cognitive functions on the Mini-Mental State Examination and parkinsonism. Informed consent was obtained from all patients with Kii ALS and all the control subjects. All persons gave their informed consent prior to their inclusion in the study. This study has been approved by the ethics committee of Teikyo University School of Medicine and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

The age and gender of both groups were comparable, and biopsy sites were free of atrophic or hypertrophic scars, recent bruises, or induration. Special care was taken to minimize stress on the tissue when taking the biopsy.

Tissue processing

Three 3-mm biopsy specimens of skin overlying the left biceps were obtained following local anesthesia with

Table 1. Clinical summary

Case no.	Age (years)	Gender	Diagnosis	Duration of illness (years)
Kii ALS				
1	74	F		0.6
2	56	M		1.0
3	46	M		1.0
4	60	F		1.2
5	61	M		2.0
6	65	M		3.0
7	55	M		4.5
Controls				
1	49	M	Sjögren syndrome	0.5
2	55	F	Mitochondrial encephalomyopathy	1.0
3	65	M	Parkinson disease	1.5
4	56	M	CIDP	2.0
5	58	F	Polymyositis	2.0
6	73	M	Parkinson disease	2.5
7	62	M	Corticobasal degeneration	2.8
8	71	F	Multiple system atrophy	3.0
9	58	M	Multiple system atrophy	3.5
10	65	M	Progressive supranuclear palsy	4.0
11	71	F	Polymyositis	4.0
12	63	M	Mitochondrial encephalomyopathy	5.0
13	56	M	Myotonic dystrophy	6.0
14	68	M	Multiple system atrophy	7.0

CIDP = chronic inflammatory demyelinating polyradiculoneuropathy

1% procaine hydrochloride. One third of the specimens was placed in half-strength Karnovsky's fixative for electron microscopic studies and the rest was stored at -80°C until use.

Electron microscopy

Small pieces of skin were placed in 0.1 mol/l cacodylate buffer, and fixed for 2-4 h at 4°C. Samples were washed in buffer, post-fixed in 1% OsO4 in distilled water for an additional hour, dehydrated through a graded series of alcohols, and embedded in Epon by means of conventional methods. Ultrathin (gold-silver) sections (approximately 80 nm) were cut in a plane perpendicular to the skin surface and stained with uranyl acetate and lead citrate. Electron micrographs were taken below the epidermal-dermal junction, within the reticular dermis of all specimens.

Measurement of collagen

All operations were carried out at 4°C. After the subcutaneous fat and hair were carefully removed, the samples were minced by razor blade and pulverized under liquid N2 by Spex Freezer Mill (Spex, Inc., Metuchew, NJ, USA). These were then washed with cold 0.015 M N-trismethyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.4 and cold distilled water by centrifugation at 2000 × g for 30 min, which was repeated 3 times, and lyophilized.

Hydroxyproline (Hyp) is one of the collagen-associated amino acids and is present in very few other proteins [19,20]. Appreciable amounts of Hyp have been found almost exclusively in collagen, and its presence has been used as a criterion for identifying collagen [21,22]. In interstitial collagen, approximately 300 residues of Hyp are present in one molecule of collagen [23]. In the present study, therefore, the collagen content was determined from

a value of 300 residues of Hyp per mole of collagen [23].

Approximately 0.5mg of each sample was hydrolyzed with 6N HCl in vacuo, after flushing with N2 for 24 h at 115°C. The hydrolysates were evaporated by speed vacuum (Savant Instruments, Hicksville, NY, USA) and the residues were dissolved in 500μl of water and filtered with a 0.22μm membrane. An aliquot of the hydrolysate (approximately 50μl) was analyzed for its Hyp on a Varian 5560 liquid chromatograph configured as amino acis analyzer (AA911 column, Interaction, Mountain View, CA, USA) using ninhydrin with color development at 135°C in a stainless steel reaction coil [24]. The values of collagen amount were expressed as nmoles per mg dry weight.

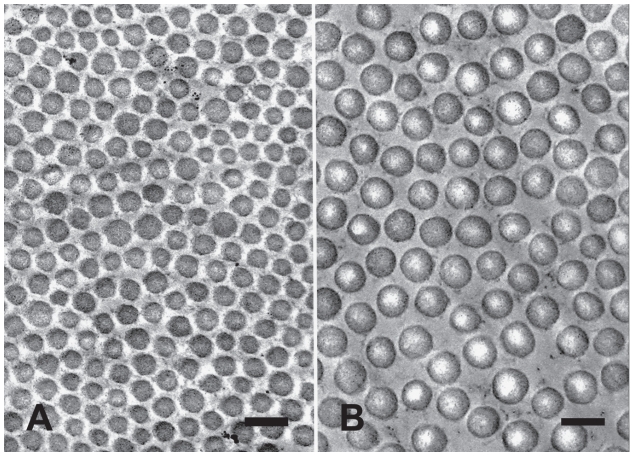
Statistical analysis

Statistical comparisons were made by the two-tailed Student's t test with $p<0.05$ as the significance level. Correlations were calculated by the least-squares method. Results were expressed as the mean±SD.

RESULTS

Electron microscopy

In cross-sections, collagen fibrils appeared smaller in diameter in Kii ALS patients than in controls, but there was no structural abnormality of collagen fibrils (Graph 1).



Graph 1. Cross section of collagen fibrils of the reticular dermis from (A) a patient with Kii ALS (case 5) and (B) a control (case 5). Note marked decrease of the diameter of collagen fibrils in (A) as compared with (B). Bars=150nm.

In Kii ALS patients, some areas showed collagen bundles that were fragmented and widely serapated by amorphous material. The fibrils were often randomly oriented with respect to the accumulations of amorphous material. The longer the duration of Kii ALS, the more marked these findings were. In controls, these findings were not observed, and all collagen fibrils were densely packed and parallel in collagen bundles. These findings were in good agreement with the previous report [17].

Measurement of collagen

The collagen content per dry weight (mg) of the samples in Kii ALS (0.79±0.26 nmol/mg) was significantly decreased ($p<0.001$) than in controls (1.53±0.20 nmol/mg) (Figure 1). In Kii ALS patients the more severely affected pathological samples showed the greater decrease. In addition, there was a significant negative correlation (r=-0.88, $p<0.01$) between the collagen content and duration

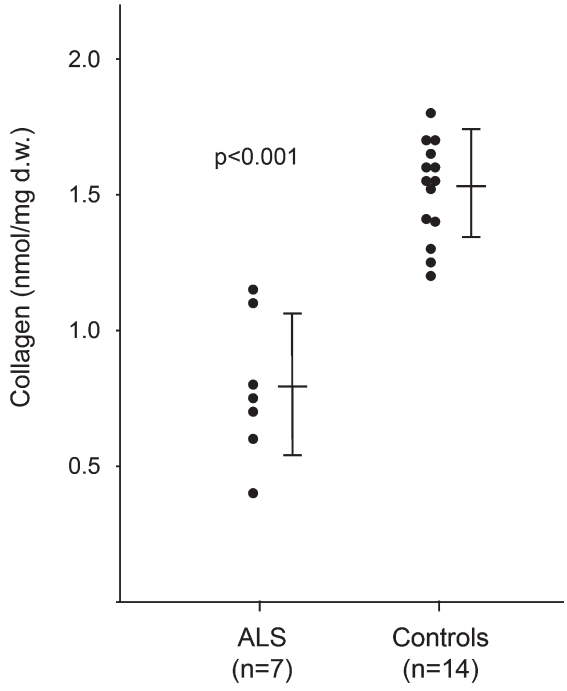


Figure 1. The collagen content of patients with Kii ALS and controls. 1 mol of collagen is based on a value of 300 residues of hydroxyproline per mol of collagen. The collagen content is significantly lower ($p<0.001$) in Kii ALS patients than in controls. Bars show mean±SD. d.w.: dry weight of the sample.

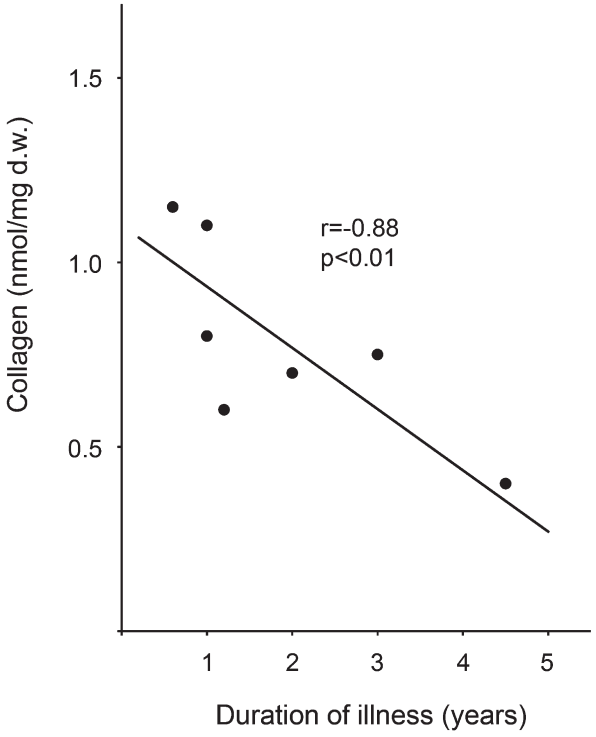


Figure 2. Correlation of duration of illness to the collagen content in patients with Kii ALS. There is a significant negative correlation (r=-0.88, $p<0.01$) between the collagen content and duration of illness in patients with Kii ALS. d.w.: dry weight of the sample.

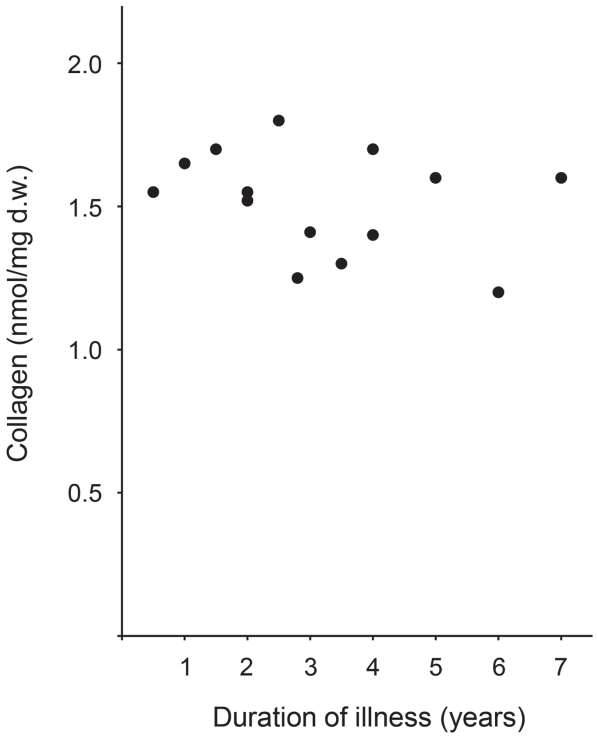


Figure 3. Correlation of duration of illness to the collagen content in controls. There is no correlation between the collagen content and duration of illness in controls. d.w.: dry weight of the sample.

of illness in the Kii ALS patients (Figure 2), but there was no such correlation in controls (Figure 3); in controls, age, gender, duration, severity of illness, muscle atrophy and weakness, and physical condition did not alter the level of collagen.

DISCUSSION

There are many hypotheses about the pathogenesis of sporadic and Kii ALS, including glutamate-induced excitotoxic injury, oxidative damage, exposure to toxins such as compounds in cycad seeds or minerals, disorganization of intermediate filaments and loss of neurotrophic support for motor neurons such as insulin-like growth factor I (IGF-I), glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF) [25], but none of them substantiated [26]. Although gene analysis, including a superoxide dismutase 1, tau, ApoE and neurofilament heavy chain, has been performed, gene abnormalities were not found in Kii ALS patients [27]. Most likely was the metal-induced calcified degeneration hypothesis proposed by Yase [28] that chronic nutritional deficiencies of calcium and magnesium and relative excess of aluminum resulted in the abnormal accumulation of these elements in neurons and caused motor neuron degeneration. Disappearance of high-incidence ALS and PDC on Guam was accounted for by the changes in dietary habits and local water supplies as a consequence of increased westernization [13]. However, serum calcium and parathyroid hormone levels were within normal limits in the ALS/PDC patients on Guam [29], and the high prevalence rates of Kii ALS/PDC still

continue, despite of the marked changes of food, drinking water, and habits of the residents [30,31].

The present report provides the first quantitative analysis of collagen of skin in Kii ALS patients. The most conspicuous changes observed in Kii ALS patients were a significant decrease in the content of collagen with the duration of illness. None of these findings were observed in controls matched for age and gender. These differences between Kii ALS patients and controls are not due to tissue preparation, since all frozen tissues were processed in the same way at the same time by the same examiner. Differences between Kii ALS and control samples in age, gender, and biopsy sites were not contributory since the former two were statistically identical, and biopsies were taken from the same sites of the body. Five control cases had moderate or marked muscle atrophy. All of our Kii ALS patients were in good nutritional conditions; none showed cachexia. Therefore, muscle, wasting or nutritional status was not associated with the changes in collagen in the skin. It is thought that the decresed level of collagen is not incidental but also attributed to the pathogenesis of Kii ALS.

The most significant findings by electron microscopy of patients with Kii ALS were a small diameter of collagen fibrils, and a markedly large amount of amorphous material separating collagen bundles, which coincided with the previous reports on skin collagen changes in sporadic ALS [32-34]. The chemical data presented in this report substantiate these morphological findings. The present findings are compatible with our previous observations of an unusually low content of collagen in sporadic ALS skin [30] as determined on the basis of 300 residues of Hyp per mole of collagen [22]. These observations suggest that the pathogenesis of Kii ALS is similar to that of sporadic ALS in skin pathology. Ono et al. examined specimens of skin overlying the sacral region, among the most common sites of bedsores, from patients with sporadic ALS, and found that in ALS patients collagen fibrils had a greater density and became more tightly packed with the duration of illness [35]. The results suggest that the increased density of collagen fibrils may protect the skin of sporadic ALS patients from pressure ischemia, a major cause of bedsore formation. However, we have not studied the skin overlying the sacral region from Kii ALS. Additional studies are needed to clarify collagen changes of skin on the sacral region in Kii ALS patients.

So far there have been no biochemical reports concerning skin collagen in kii ALS. Our data suggest that the metabolism of skin collagen might be affected in the disease process of Kii ALS, and that some relationship may exist between collagen metabolism of skin and the form and function of the motor neuron in Kii ALS. Whether the decreased level of collagen is merely a secondary phenomenon, or may represent a more direct biochemical involvement as a result of the primary agent(s) or process(es) that initiate Kii ALS, remains a subject for further study.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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