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**Noriyuki Shibata · Ryoji Nagai · Satoshi Miyata
Tadashi Jono · Seikoh Horiuchi · Asao Hirano
Shinsuke Kato · Shoichi Sasaki · Kohtaro Asayama
Makio Kobayashi**

Nonoxidative protein glycation is implicated in familial amyotrophic lateral sclerosis with superoxide dismutase-1 mutation

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Abstract To assess a role for oxidative stress in the pathogenesis of amyotrophic lateral sclerosis (ALS), we analyzed the immunohistochemical localization of 8-hydroxy-2′-deoxyguanosine (OHdG) as a nucleic acid oxidation product, acrolein-protein adduct and 4-hydroxy-2-nonenal (HNE)-protein adduct as lipid peroxidation products, N^{ε}-carboxymethyl-lysine (CML) as a lipid peroxidation or protein glycoxidation product, pentosidine as a protein glycoxidation product, and imidazolone and pyrraline as nonoxidative protein glycation products in the spinal cord of three familial ALS patients with superoxide dismutase-1 (SOD1) A4V mutation, six sporadic ALS patients, and six age-matched control individuals. The spinal cord sections of the control cases did not show any distinct immunoreactivities for these examined products. In the familial ALS cases, intense immunoreactivities for pyrraline and CML were confined to the characteristic Lewy body-like hyaline inclusions, and imidazolone immunoreactivity was located in the cytoplasm of the residual motor neurons. No significant immunoreactivities for other examined products were detected in the familial ALS spinal cords. In the sporadic ALS cases, intense immunoreactivities for pentosidine, CML and HNE-protein adduct were seen in the cytoplasm of the degenerated motor neurons, and OHdG immunoreactivity was located in the cell nuclei of the residual neurons and glial cells. The present results indicate that oxidative reactions are involved in the disease processes of sporadic ALS, while there is no evidence for increased oxidative damage except for CML deposition in the familial ALS spinal cords. Furthermore, it is likely that the accumulation of pyrraline and imidazolone supports a nonoxidative mechanism in SOD1-related motor neuron degeneration.

Key words Amyotrophic lateral sclerosis · Imidazolone · N^{ε}-carboxymethyl-lysine · Pyrraline · Superoxide dismutase

N. Shibata (✉) · M. Kobayashi
Department of Pathology, Tokyo Women's Medical University, Kawada-cho 8-1, Shinjuku-ku, Tokyo 162-8666, Japan
Tel.: +81-3-33538111 ext 22233, Fax: +81-3-52697408

R. Nagai · T. Jono · S. Horiuchi
Department of Biochemistry, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860-0811, Japan

S. Miyata
Second Department of Internal Medicine, Kobe University School of Medicine, Kusunoki-cho 7-5-1, Chuo-ku, Kobe 650-0017, Japan

A. Hirano
Division of Neuropathology, Montefiore Medical Center, 111 East 210th Street, Bronx, NY 10467-2490, USA

S. Kato
Division of Neuropathology, Institute of Neurological Sciences, Faculty of Medicine, Tottori University, Nishi-machi 86, Yonago 683-8503, Japan

S. Sasaki
Department of Neurology, Tokyo Women's Medical University, Kawada-cho 8-1, Shinjuku-ku, Tokyo 162-8666, Japan

K. Asayama
Department of Pediatrics, Yamanashi Medical University, Shimokato 1110, Tamaho-cho, Nakakoma-gun, Yamanashi 409-3898, Japan

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder that is clinicopathologically characterized by primary involvement of upper and lower motor neurons and secondary neurogenic amyotrophy of striated muscles, except for extraocular and sphincter muscles [23]. ALS is epidemiologically subclassified into sporadic, familial and endemic forms. Of ALS patients, approximately 10% are familial and inherited by autosomal dominant or recessive transmission. Several molecular genetic studies have obtained evidence that about 20% of the familial ALS cases, which are dominantly inherited, are associated with missense mutations in the gene for superoxide dismutase-1 (SOD1) [8, 61]. SOD1 is a cytosolic Cu/Zn-binding antioxidative enzyme that catalyzes the con-

version of the toxic superoxide radical to hydrogen peroxide and molecular oxygen [17]. Some in vitro studies suggest that motor neuron degeneration is caused by a newly acquired neurotoxicity of mutant SOD1, which catalyzes the peroxynitrite-mediated tyrosine nitration [5], releases reactive Cu ions [11], promotes apoptotic cell death [51], has enhanced peroxidase activity [77], injures the mitochondria to increase cytosolic Ca^{2+} concentration [12], and aggregates in the cytoplasm [8, 10].

We previously demonstrated a focal collection of intense SOD1 immunoreactivity in all of the characteristic Lewy body-like hyaline inclusions (LBHIs) [35] containing the epitopes of ubiquitin and phosphorylated neurofilament protein (pNFP) in the spinal cord anterior horns of ALS patients in a family with SOD1 A4V mutation [56, 57], suggesting a gain-of-function effect of the mutant SOD1. No focal SOD1 accumulation was observed in control spinal cords. Similar findings were subsequently described in other ALS families with different SOD1 mutations (A4T, two base pair deletion at codon 126, L126S, and C146Q) [29, 42, 65, 68]. The LBHIs were also found in the spinal cord of mutant human SOD1 (G85R and G93A)-expressing transgenic mice [9, 59] and of wild-type endogenous murine SOD1-knockout, mutant human SOD1 (G85R)-transgenic mice [10]. In addition, it is of interest that SOD1-containing fragmentous aggregates appear in the cytoplasms of cultured cells transfected with mutant forms (G37R, G93A, I113T, and N139K) of the human SOD1 gene [14]. Thus, SOD1 mutation is considered to be responsible for SOD1 aggregation [8].

We recently reported a marked deposition of N^{ε}-carboxymethyl-lysine (CML) [1] in the LBHIs of the spinal cord of both familial ALS patients with SOD1 mutation (A4V and two base pair deletion at codon 126) [30, 60, 61] and G93A mice [61]. CML is one of the major advanced glycation end products (AGEs) of the Maillard reaction. The AGE structures other than CML that have been identified to date are N^{ε}-carboxyethyl-lysine (CEL) [2], crossline [44], fluorolink [25], imidazolone [33], pentosidine [54], pyrraline [21], vesperlysine [45], and so forth. Protein glycation starts from a nonenzymatic reaction of amino group of proteins with aldehyde or ketone group of reducing sugars to produce a Schiff base, which is in turn converted to an Amadori compound [75]. Subsequently, this compound undergoes further complex reactions to form various AGEs via reactive intermediates such as 3-deoxyglucosone. Although some pathways involve the transition metal-catalyzed oxidative reactions, others such as the 3-deoxyglucosone-related pathway are not associated with oxidative reactions. AGE formation per se alters the structural and functional properties of tissue proteins. For example, collagen increases the resistance towards proteolytic digestion upon modification by AGE, leading to accumulation of AGE-modified collagen in the extracellular matrix [7]. Many reports indicated that AGE-modified proteins interact with various types of cells in some pathological conditions [32, 53, 64, 74, 75, 79]. AGEs have also been detected in human tissue affected with several disorders such as senile cataract [3], arterial atherosclerosis [34], dialysis-related amyloidosis [46], diabetic nephropathy [47], actinic elastosis [41], Alzheimer's disease [63, 67, 73, 78], Pick's disease [31], Parkinson's disease [13], and diffuse Lewy body disease [13]. These observations suggest that AGE-mediated tissue injuries occur in certain disorders.

Since CML is not only a product of protein glycoxidation but also a product of lipid peroxidation [18, 20, 38, 43], it is hypothesized that oxidative reaction-mediated CML formation in the spinal cord of SOD1-mutated ALS patients may be involved in SOD1 aggregation, motor neuron degeneration, or both. Increased oxidative damage in sporadic ALS has also been reported [16, 48]. Oxidative stress may attack nucleic acids, lipids and proteins to form their respective modified products [15, 28, 75]. Based on this background, to determine the involvement of oxidative stress in the pathogenesis of ALS, the present study was performed to demonstrate the immunolocalization of these products in the spinal cord of patients with SOD1-mutated familial ALS in comparison with sporadic ALS using specific antibodies. The products analyzed in this study were 8-hydroxy-2′-deoxyguanosine (OHdG) [28] as a nucleic acid oxidation product, acrolein-protein adduct [71] and 4-hydroxy-2-nonenal (HNE)-protein adduct [15] as lipid peroxidation products, CML as a lipid peroxidation or protein glycoxidation product, pentosidine as a protein glycoxidation product, and imidazolone and pyrraline as nonoxidative protein glycation products.

Materials and methods

Autopsy patients

This investigation was carried out on spinal cords obtained at autopsy from three familial ALS patients (all male; ages 39, 46 and 66 years; mean ± SD 50.33 ± 14.01 years) in the American "C" family associated with SOD1 A4V mutation [57], from six sporadic ALS patients (four male, two female; ages 58–84 years; mean 60.00 ± 12.20 years), and from six control individuals (three male, three female; ages: 43–78 years; mean 58.50 ± 14.87 years) who died of non-neurological disorders without diabetes mellitus. The spinal cords of the familial ALS cases exhibited the neuropathological hallmarks of a certain subgroup with posterior column involvement.

Primary antibodies

The primary antibodies (Table 1) used were a rabbit immunoglobulin (Ig) to ubiquitin (Dako, Glostrup, Denmark), a mouse IgG_1 to pNFP (Sternberger Monoclonals, Baltimore, Md.), a sheep Ig to human SOD1 (Binding Site, Birmingham, UK), a rabbit Ig to human SOD1 [4], a mouse IgG_1 to OHdG (NOF corporation, Tokyo, Japan) [70], a mouse IgG_1 to N^{ε}-3-formyl-3,4-dehydropiperidino (FDP)-lysine of acrolein-protein adduct (NOF) [72], a mouse IgG_1 to Michael form of HNE-protein adduct (NOF) [69], a mouse IgG_1 to AGE [24], a rabbit Ig to pentosidine, a mouse IgG_1 to imidazolone, a mouse IgG_{2a} to pyrraline [40], a mouse IgG_1 to pyrraline, and a rabbit Ig to pyrraline. The rabbit polyclonal anti-SOD1 antibody and the mouse monoclonal anti-OHdG IgG_1 (N45.1), anti-acrolein-protein adduct IgG_1 (5F6), anti-HNE-protein adduct IgG_1 (HNEJ-2), anti-AGE IgG_1 (6D12), and anti-pyrraline IgG_{2a} (pyr-B) antibodies were previously established and characterized elsewhere [4, 24, 40, 69, 70, 72]. The major epitope of the antibody 6D12 has been shown to be CML [26].

Table 1 The primary antibodies (*pNFP* phosphorylated neurofilament protein, *SOD1* superoxide dismutase-1, *OHdG* 8-hydroxy-2′-deoxyguanosine, *ACR-P* acrolein-protein adduct, *HNE-P* 4-hydroxy-2-nonenal-protein adduct, *AGE* advanced glycation end product, *pAb* polyclonal antibody, *mAb* monoclonal antibody, *Ig* immunoglobulin)

Antibody	Animal	Clonality	Fraction	Clone	Dilution
Anti-ubiquitin	Rabbit	pAb	Ig		1:500
Anti-pNFP	Mouse	mAb	IgG_1	SMI-31	1:10,000
Anti-SOD1	Sheep	pAb	Ig		1:800
Anti-SOD1	Rabbit	pAb	Ig		1:10,000
Anti-OHdG	Mouse	mAb	IgG_1	N45.1	0.5 μg/ml
Anti-ACR-P	Mouse	mAb	IgG_1	5F6	0.5 μg/ml
Anti-HNE-P	Mouse	mAb	IgG_1	HNEJ-2	0.5 μg/ml
Anti-AGE	Mouse	mAb	IgG_1	6D12	0.6 μg/ml
Anti-pentosidine	Rabbit	pAb	Ig		1:500
Anti-imidazolone	Mouse	mAb	IgG_1	JNH-27	7.0 μg/ml
Anti-pyrraline	Mouse	mAb	IgG_{2a}	pyr-B	10 μg/ml
Anti-pyrraline	Mouse	mAb	IgG_1	H-12	0.6 μg/ml
Anti-pyrraline	Rabbit	pAb	Ig		1:500

For production of the polyclonal antibodies against pyrraline and pentosidine, caproyl pyrraline and *N,N*-diacetyl-pentosidine were prepared as described previously [21, 54]. To form each immunogen, 6 mg of caproyl pyrraline or *N,N*-diacetyl-pentosidine was incubated for 1 h at room temperature with 6 mg of keyhole limpet hemocyanin (KLH) in the presence of 50 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pierce Chemical Company, Rockford, Ill.) and 2.5 mM *N*-hydroxysulfosuccinimide (Pierce). The reaction was terminated by addition of 20 mM 2-mercaptoethanol, and the mixture was dialyzed against phosphate-buffered saline (PBS) (pH 7.4) for 24 h at 4 °C. To raise the polyclonal antibody, 0.5 mg of caproyl pyrraline-conjugated KLH or *N,N*-diacetyl-pentosidine-conjugated KLH in 50% Freund's complete adjuvant was injected intradermally into a rabbit at 20 skin sites, followed by four booster injections with the same amount of hapten-conjugated KLH in 50% Freund's incomplete adjuvant. The serum was taken 10 days after the final immunization for further affinity purification. The antiserum against hapten-conjugated KLH was passed over 5 ml of protein G-immobilized Sepharose gel (Pharmacia Biotech, Uppsala, Sweden). The column was extensively washed with PBS and eluted with 0.1 M citrate buffer (pH 3.0). The pooled antibody fraction was neutralized, concentrated, dialyzed against PBS, and used as affinity-purified polyclonal antibody in the present study. The titers of these polyclonal antibodies were verified by enzyme-linked immunosorbent assays.

The monoclonal IgG_1 antibody to pyrraline was prepared as described previously [40]. Briefly, splenic lymphocytes from BALB/c mice immunized with caproyl pyrraline-conjugated human serum albumin (HSA) were fused to myeloma P3U1 cells. The hybridoma cells positive for caproyl pyrraline-conjugated KLH but negative for HSA were selected through successive subcloning. One cell line, termed H-12, was obtained from ascitic fluids of BALB/c mice, and further purified on protein G-immobilized Sepharose gel to IgG_1. Likewise, the monoclonal IgG_1 antibody to imidazolone was prepared by immunizing with N^{α}-benzyloxycarbonyl-imidazolone (N^{α}-Z-imidazolone)-conjugated HSA as described previously [47], and the hybridoma cells positive for N^{α}-Z-imidazolone-conjugated KLH but negative for HSA were selected. A cell line (JNH-27) was produced from ascites of BALB/c mice, and then purified using the gel similar to that used above. The titers of these monoclonal antibodies were verified by enzyme-linked immunosorbent assays.

Immunohistochemistry

Formalin (10%)-fixed, paraffin-embedded spinal cord sections of each autopsy patient were deparaffinized, rehydrated, and used for immunohistochemistry. Prior to immunostaining, sections were stained with hematoxylin-eosin (H&E), and several normal and abnormal structures were observed and photographed. The H&E-stained sections were subsequently decolorized with 70% ethanol containing 1% hydrochloric acid, quenched for 20 min at room temperature with 3% hydrogen peroxide for blocking endogenous peroxidase activities, rinsed in PBS (pH 7.6), pretreated with 3% non-immune serum of the same species as secondary antibody-producing animals or bovine serum albumin (BSA) in PBS for blocking nonspecific binding of the antibodies, and incubated overnight at 4 °C with the primary antibodies. Immunoreaction product deposits were visualized by the appropriate avidin-biotin-immunoperoxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.) procedure with PBS containing 0.2 mg/ml 3,3′-diaminobenzidine tetrahydrochloride and 0.002% hydrogen peroxide. Immunostained sections were counterstained with methyl green or hematoxylin, and observed with a light microscope.

For immunostaining with the sheep anti-SOD1 antibody, some sections were pretreated for 30 min at 37 °C with 0.1% trypsin in PBS for antigen retrieval. For immunostaining with the anti-OHdG antibody, some sections were pretreated with 0.05 N NaOH in 40% ethanol for 12 min at room temperature for denaturation of DNA, or pretreated for 1 h at 37 °C with DNase-free RNase (Sigma Chemicals, St. Louis, Mo.) at a concentration of 100 μg/ml in PBS for RNA digestion [66, 70]. Prior to immunostaining with the antibodies to the products of lipid peroxidation, protein glycoxidation and nonoxidative protein glycation, some sections were processed

Table 2 Results of immunostaining in motor neurons (+ distinctly immunostained, +/– faintly immunostained, – negatively immunostained, *F/SALS* familial/sporadic amyotrophic lateral sclerosis, *LBHIs* Lewy body-like hyaline inclusions, *BBs* Bunina bodies, *SIs* skein-like inclusions, *RHIs* round hyaline inclusions, *PYR* pyrraline, *IMI* imidazolone, *PEN* pentosidine, *CML* N^{ε}-carboxymethyllysine, *HNE-P* 4-hydroxy-2-nonenal-protein adduct, *ACR-P* acrolein-protein adduct, *OHdG* 8-hydroxy-2′-deoxyguanosine)

	PYR	IMI	PEN	CML	HNE-P	ACR-P	OHdG
Control							
Nucleus	–	–	–	–	–	–	–
Cytoplasm	–	–	–	–	–	–	–
FALS							
Nucleus	–	–	–	–	–	–	–
Cytoplasm	–	+	–	–	–	–	–
LBHIs	+	–	+/–	+	–	–	–
SALS							
Nucleus	–	–	–	–	–	–	+
Cytoplasm	–	–	+	+	+	–	+/–
BBs	–	–	–	–	–	–	–
SIs	–	–	–	–	–	–	–
RHIs	–	–	–	–	–	–	–

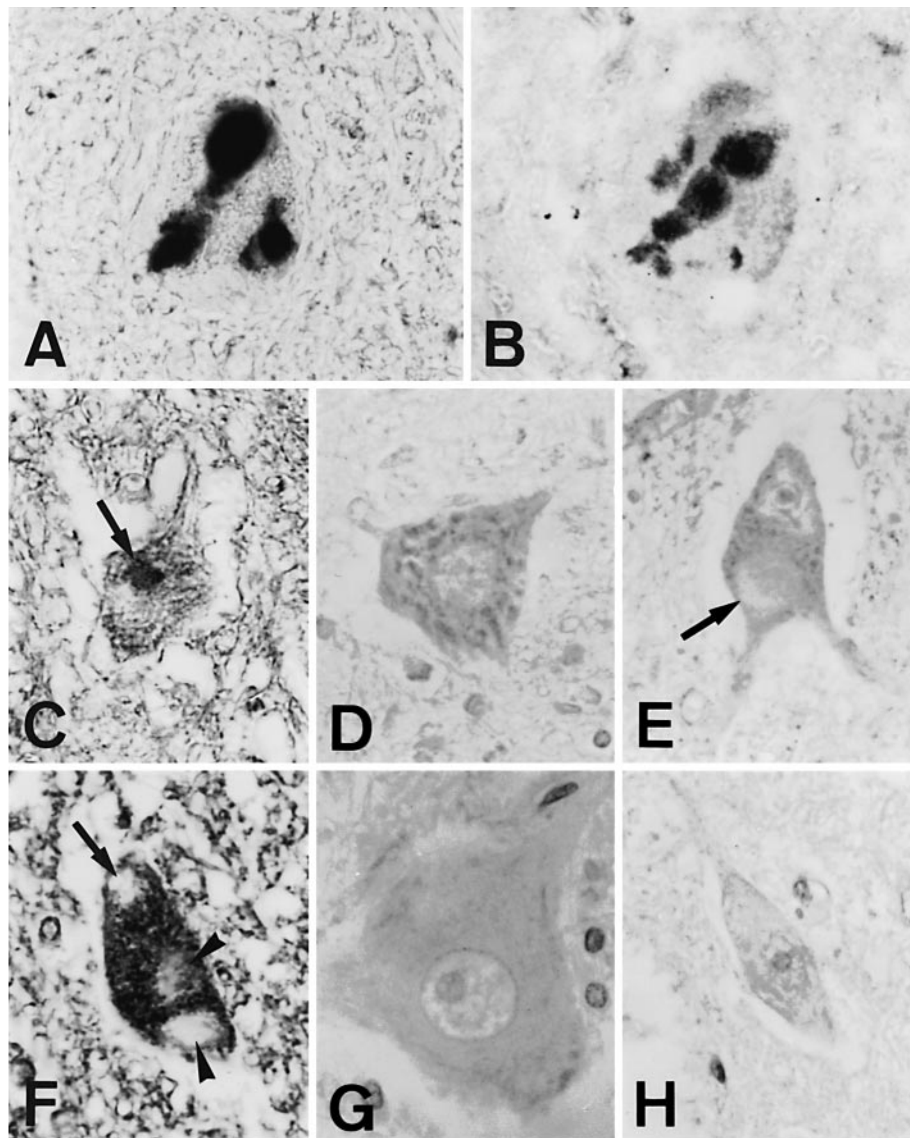

Fig. 1 Photomicrographs of immunohistochemistry for SOD1, pyrraline and imidazolone in the spinal cord of control individuals (**D, G**), familial ALS patients with SOD1 A4V mutation (**A–C, F**), and sporadic ALS patients (**E, H**). **A** A lower motor neuron bearing LBHIs in a section immunostained with a rabbit polyclonal antibody to ubiquitin. The antibody reacts selectively with the LBHIs. **B** A section consecutive to that shown in **A** immunostained with a sheep polyclonal antibody to SOD1. SOD1 immunoreactivity similar to ubiquitin immunoreactivity (**A**) is restricted to the LBHIs. **C** A lower motor neuron bearing an LBHI (*arrow*) in a section immunostained with a mouse monoclonal antibody to pyrraline. The antibody recognizes the LBHI (*arrow*), but not the surrounding cytoplasm or neuropil. **D** A morphologically intact anterior horn cell in a section immunostained with a rabbit polyclonal antibody to pyrraline. The neuron and neuropil are only weakly stained for pyrraline or not at all. **E** An affected motor neuron containing a round hyaline inclusion (*arrow*) in a section immunostained for pyrraline. The inclusion, cytoplasm and nucleus display no significant pyrraline immunoreactivity. **F** A motor neuron bearing LBHIs (*arrowheads*) in a section immunostained with a mouse monoclonal antibody to imidazolone. The cytoplasm is positively stained, while the LBHIs and nucleus (*arrow*) are negatively stained. **G** A normal-appearing anterior horn cell in a section immunostained with the anti-imidazolone antibody. No imidazolone immunoreactivity is found in the section. **H** A small neuron in a section immunostained for imidazolone. The antibody does not react with the neuron or neuropil (*SOD1* superoxide dismutase-1, *ALS* amyotrophic lateral sclerosis, *LBHIs* Lewy body-like hyaline inclusions). **A–H** × 600

for 20 min at 95 °C (500 W) in a microwave oven (MWF-2; Nisshin-EM, Tokyo, Japan) for antigen retrieval on paraffin-embedded sections. Selected sections were incubated with the antibodies against OHdG, acrolein-protein adduct, HNE-protein adduct, CML, pentosidine, imidazolone and pyrraline that had been preabsorbed with excess amounts of OHdG (Japan Institute for the Control of Aging, Fukuroi, Japan; diluted 0.5 µg/ml), FDP-lysine (NOF; diluted 5.0 µmol/ml), HNE-HSA (NOF; diluted 0.2 µmol/ml), CML-BSA (diluted 1.0 mg/ml), pentosidine-BSA (diluted 0.7 mg/ml), imidazolone-HSA (diluted 1.0 mg/ml) and free pyrraline (diluted 2.0 µmol/ml), respectively.

Sections from which the primary antibodies were omitted served as negative reaction controls. Skins affected with solar keratosis were used as a positive reaction control for OHdG. Aortas with atherosclerosis and kidneys with diabetic nephropathy and dialysis-related amyloidosis served as positive reaction controls for the prod-

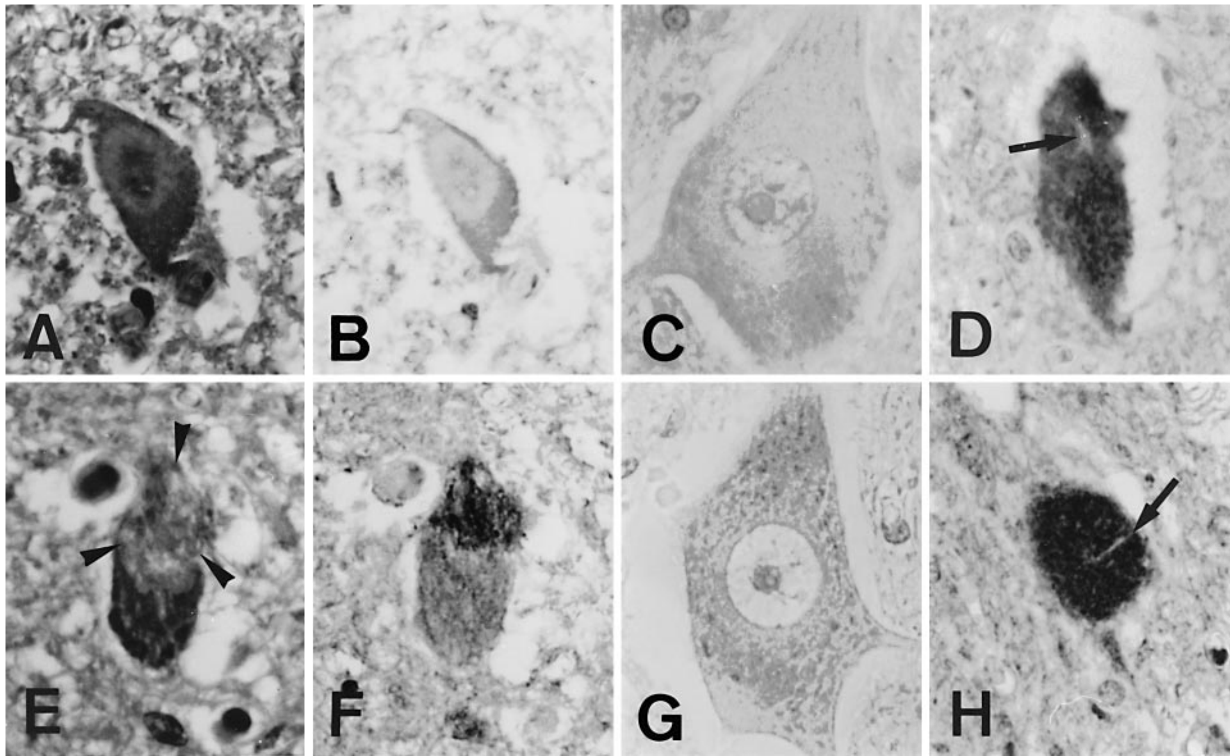

Fig. 2 Photomicrographs of immunohistochemistry for pentosidine and CML in the spinal cord of control individuals (**C**, **G**), familial ALS patients with SOD1 A4V mutation (**A**, **B**, **E**, **F**), and sporadic ALS patients (**D**, **H**). **A** A lower motor neuron bearing a well-demarcated LBHI in a section stained with H&E. **B** The same section as shown in **A** immunostained with a rabbit anti-pentosidine antibody. None of the LBHI, cytoplasm or neuropil display any marked staining for pentosidine. **C** An intact anterior horn cell in a section immunostained with the anti-pentosidine antibody. The neuron and neuropil are negatively stained for pentosidine. **D** A small neuron containing a Bunina body (*arrow*) in a section immunostained for pentosidine. The immunoreactivity is seen in the cytoplasm, but not in the inclusion. **E** An ill-defined LBHI (*arrowheads*)-bearing motor neuron in an H&E-stained section. **F** The same section as in E immunostained with a mouse anti-CML antibody. CML immunoreactivity is restricted to the LBHI. **G** A morphologically intact anterior horn cell in a section immunostained with the anti-CML antibody. The antibody does not detect the neuron or neuropil. **H** A small neuron containing a skein-like inclusion (*arrow*) in a section immunostained for CML. CML immunoreactivity is seen in the cytoplasm, but not in the inclusion (*CML* N^{ε}-carboxymethyl-lysine). **A–H** × 600

ucts of lipid peroxidation, protein glycoxidation, and nonoxidative protein glycation [34, 40, 46, 47, 54, 72]. The location of immunoreactivities for these examined products was confirmed by histological orientation on initially taken photomicrographs of a given H&E-stained section. Ubiquitin, pNFP, and SOD1 were used as the immunohistochemical markers of LBHIs [57].

Results

As described before [57], the neuropathological features of the spinal cord of the three familial ALS patients were characterized by a decrease in number of neurons in the anterior horns and Clarke's nuclei, cord-like swelling of the axons, a slight degeneration of the corticospinal tracts, a marked degeneration of the posterior column middle root zones and posterior spinocerebellar tracts, and reactive astrocytosis of these lesions. The residual neurons in the anterior horns and Clarke's nuclei displayed atrophic or chromatolytic change, and contained the characteristic LBHIs in the cell bodies and cord-like swollen axons (see Fig. 2A, E). The LBHIs were identified on H&E-stained sections or immunostained sections with the antibodies to ubiquitin, pNFP and SOD1 (Figs. 1A, B; 2A, E) [57]. The spinal cords of the six sporadic ALS patients exhibited a marked loss or atrophy of lower motor neurons, appearance of Bunina bodies and skein-like inclusions in some of the neurons, corticospinal tract degeneration, and reactive astrocytosis. Four sporadic ALS patients had round hyaline inclusions (RHIs) [35] in some of the residual motor neurons. The RHIs have been described in a subset of sporadic ALS cases. Although an RHI is histopathologically indistinguishable from an LBHI, the former differs ultrastructurally and immunohistochemically from the latter. Electron microscopy reveals that the RHIs consist of 15- to 20-nm-thick bundles and intermingled neurofilaments, while the LBHIs consist of the granule-coated filaments thicker than intermingled neurofilaments. Immunohistochemical examination shows that the RHIs contain the epitopes of ubiquitin but not pNFP or SOD1 [35], while the LBHIs contain epitopes of ubiquitin, pNFP and SOD1 [57]. In the present study, the immunohistochemical patterns of RHIs and LBHIs for these three substances were consistent with those of previous descriptions.

The results of immunohistochemistry are summarized in Table 2. Neither the sections processed with omission of the primary antibodies nor the sections incubated with

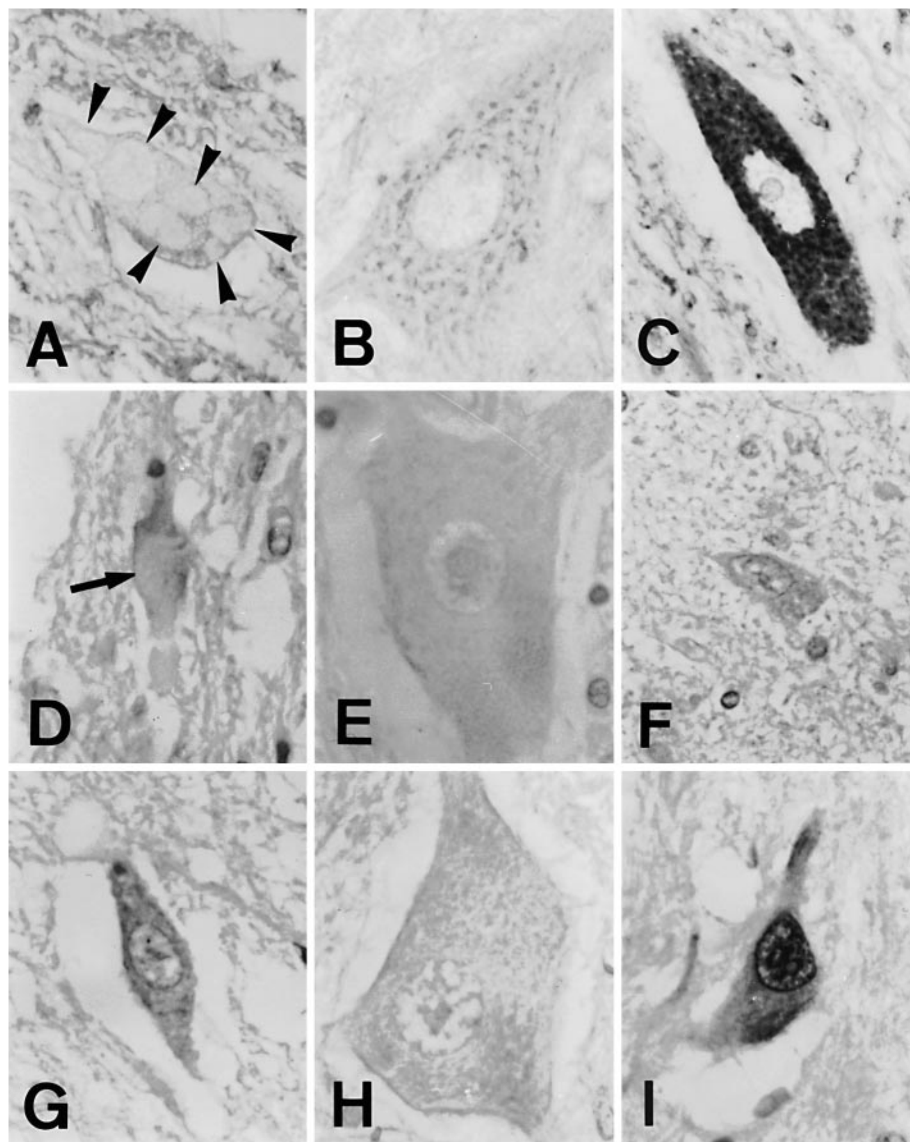

Fig. 3 Photomicrographs of immunohistochemistry for HNE-protein adduct, acrolein-protein adduct and OHdG in the spinal cord of control individuals (**B**, **E**, **H**), familial ALS patients with SOD1 A4V mutation (**A**, **D**, **G**), and sporadic ALS patients (**C**, **F**, **I**). **A** A lower motor neuron bearing LBHIs (*arrowheads*) in a section immunostained with a mouse antibody to HNE-protein adduct. The LBHIs are negatively stained by the antibody, and the cytoplasm and neuropil are only weakly stained or not at all. **B** A morphologically intact anterior horn cell in a section immunostained with the anti-HNE-protein adduct antibody. The antibody does not react with the neuron or neuropil. **C** A small degenerated motor neuron in a section immunostained for HNE-protein adduct. The cytoplasm is selectively immunoreactive. **D** An affected motor neuron bearing an LBHI (*arrow*) in the perikarya in a section immunostained with a mouse antibody to acrolein-protein adduct. The LBHI, nucleus and neuropil are negatively stained. **E** A normal-appearing anterior horn cell in a section immunostained with the anti-acrolein-protein adduct antibody. No obvious immunoreactivity is found in the section. **F** A degenerated motor neuron in a section immunostained for acrolein-protein adduct. The antibody does not detect the neuron or neuropil. **G** A lower motor neuron in a section immunostained with a mouse antibody to OHdG. The neuron and neuropil are negatively stained for OHdG. **H** An intact anterior horn cell in a section immunostained with the anti-OHdG antibody. No OHdG immunoreactivity is seen in the section. **I** An affected small neuron in a section immunostained for OHdG. The neuron exhibits a distinct nucleic staining for OHdG (*HNE* 4-hydroxy-2-nonenal, *OHdG* 8-hydroxy-2′-deoxyguanosine). **A–I** × 600

the preabsorbed antibodies showed any immunoreaction product deposits. SOD1 immunoreactivity with the sheep anti-SOD1 antibody was weak without any pretreatments, and obviously enhanced by trypsin pretreatment, while a pronounced SOD1 immunoreactivity with the rabbit anti-SOD1 antibody was detected even on nonpretreated sections. There was no marked difference of the location of SOD1 immunoreactivities with the sheep and rabbit antibodies. OHdG immunostaining on sections with DNA denaturation and RNA digestion was identical to that without any pretreatments. Immunoreactivities for the products of lipid peroxidation, protein glycoxidation and nonoxidative protein glycation were detectable on nonpretreated sections, and they were enhanced with microwave process-

ing. The location of immunoreactivity with the monoclonal anti-pyrraline antibodies was similar to that with the polyclonal anti-pyrraline antibody.

The spinal cord sections of the control cases exhibited no significant immunoreactivities for the examined products (Figs. 1D, G; 2C, G; 3B, E, H). In the familial ALS cases, some of these products were deposited in certain neuronal components. Almost all of the LBHIs were intensely immunostained for pyrraline (Fig. 1C) and CML (Fig. 2F), and the neuronal cytoplasms were only weakly stained or not at all for pyrraline and CML. The cytoplasms of both of the residual motor neurons and cord-like swollen axons were immunoreactive for imidazolone, and the LBHIs and cell nuclei of the neurons were negatively stained for imidazolone (Fig. 1F). Pentosidine immunoreactivity was only very weak or absent, and rarely seen in the LBHIs (Fig. 2B). On the other hand, no obvious immunoreactivities for HNE-protein adduct, acrolein-protein adduct or OHdG were detected in the familial ALS spinal cords (Fig. 3A, D, G). In the sporadic ALS cases, intense immunoreactivities for pentosidine (Fig. 2D), CML (Fig. 2H) and HNE-protein adduct (Fig. 3C) were seen in the cytoplasms of more than half of the small degenerated neurons, whereas OHdG immunoreactivity (Fig. 3I) was prominent in the cell nuclei of the small neurons and glial cells, and was less intense in the cytoplasms of the large neurons. Bunina bodies, skein-like inclusions and RHIs were negatively stained for all of the examined products (Figs. 1–3).

Discussion

Proteins, lipids and nucleic acids are the major cellular elements, and it is known that chemical modification of these molecules by glycation or oxidation is enhanced under many pathological conditions. Pyrraline is a product of glycation of the protein lysine residue [21]. 3-Deoxyglucosone, a highly reactive intermediate in the post-Amadori reaction, acts as a potent precursor of pyrraline [21]. Similarly, imidazolone is formed by a nonenzymatic reaction of the protein arginine residues with 3-deoxyglucosone [33]. Non-aerobic conditions mediate the formation of pyrraline and imidazolone from 3-deoxyglucosone via the Amadori compound [22]. Pentosidine is a product mainly of oxidative cross-linking of the arginine and lysine residues of pentose-modified proteins [54]. CML is formed either by protein modification with glyoxal derived from glucose autoxidation [76], by oxidation of a Schiff base [20], by Fenton reaction-mediated oxidation of an Amadori compound in the presence of transition metals such as Cu and Fe ions [43], by transition metal-catalyzed oxidation of lipoproteins [18], or by protein modification with glyoxal, glycoaldehyde or other α-hydroxyaldehydes generated during lipid peroxidation [38]. HNE is a toxic α,β-unsaturated aldehyde produced by peroxidation of ω6-unsaturated fatty acids such as linoleic and arachidonic acids, and attacks the protein histidine, lysine or cysteine residues to form a stable Michael form of HNE-protein adduct [15]. Acrolein, which has been identified as a ubiquitous pollutant in the environment, is a toxic β-unsaturated aldehyde produced by lipid peroxidation, and is able to react with protein amino groups to form a stable FDP-lysine of acrolein-protein adduct [71]. Of these products, pentosidine and HNE-protein adduct cross-link protein molecules. OHdG is formed as a result of hydroxylation of the C-8 position of DNA 2′-deoxyguanosine in the presence of oxygen and ascorbic acid [28]. Thus, oxidative reactions may be essential for the formation of these products other than pyrraline and imidazolone.

In the present study we have demonstrated the presence of pyrraline and CML in the LBHIs, the presence of imidazolone in the cytoplasm of the residual motor neurons and cord-like swollen axons, and the absence of pentosidine, HNE-protein adduct, acrolein-protein adduct and OHdG in the residual neurons and glial cells in the spinal cord of patients with familial ALS with SOD1 A4V mutation. These changes do not provide evidence for increased oxidative damage, except for CML deposition in SOD1-mutated familial ALS spinal cords, and suggest that CML formation in the LBHIs is mediated by protein glycation rather than lipid peroxidation, although it remains unclear whether CML can be formed by not only oxidative but also nonoxidative processes. In discussing this accumulation of toxic substances, it is important to consider the antioxidant defense in ALS. However, expression levels of antioxidant enzymes in ALS patients are controversial in the literature. SOD1 enzyme activity in the motor cortex was normal in sporadic ALS cases [50], and was reduced in SOD1-mutated familial ALS cases [6]. SOD1 immunoreactivity in the anterior horn cells was increased in sporadic cases [55], and was decreased in SOD1-mutated familial cases [52]. SOD1 immunoreactivity in the reactive astrocytes was increased in both sporadic and familial cases [58]. SOD2 enzyme activity in the motor cortex of sporadic ALS cases was similar to that of control subjects [50]. SOD2 immunoreactivity in the anterior horn cells was normal [58] or increased [55] in sporadic cases, and was normal or decreased [58] in SOD1-mutated familial cases. SOD2 immunoreactivity in the reactive astrocytes was increased in both sporadic and familial cases [58]. Glutathione peroxidase activity was reduced [50] in the motor cortex, and was normal [19] or increased [27] in the spinal cord of sporadic ALS cases compared to those of control subjects. Catalase activity in the motor cortex of sporadic ALS cases was similar to that of control subjects [50].

Recent investigations have shown HNE neurotoxicity for motor neurons. An in vitro study showed that HNE impaired the glutamate and glucose transport and the choline acetyltransferase activity in cultured motor neurons [49]. A study on autopsy materials indicated that increased levels of HNE were found in the spinal cord of sporadic ALS cases, and also implied that HNE modification of astrocytic glutamate transporter EAAT2 impaired glutamate transport, resulting in excitotoxic motor neuron degeneration in ALS [48]. Another study demonstrated increased levels of both OHdG and protein-bound carbonyl in the motor cortex of sporadic ALS cases [16]. Like these ob-

servations, our data on the deposition of pentosidine, CML, HNE-protein adduct and OHdG in degenerated neurons of the sporadic ALS spinal cords suggest a potential involvement of oxidative stress in the pathomechanisms of this disease. Since OHdG immunoreactivity in the cell nuclei was conserved even after RNA digestion, it is thought that the OHdG determinants originate mainly from the nuclear DNA. In addition, the lack of immunoreactivities for the examined products in the RHIs of sporadic ALS suggest that the nature of the RHIs differs from that of the LBHIs of familial ALS. Furthermore, in the light of the present finding that acrolein-protein adduct was not detectable in the spinal cord of both sporadic and familial ALS patients, acrolein-related cell damage may not occur in ALS.

Unlike the findings in sporadic ALS, it has been shown that there is no increase in OHdG, protein-bound carbonyl or malondialdehyde levels in the motor cortex of either SOD1 A4V-mutated or non-SOD1-linked familial ALS cases [16]. These are consistent with the results obtained from our A4V patients. This may be of relevance to recent evidence that oxidative reactivities of mutant SOD1 are not necessarily higher compared to those of wild-type SOD1 [36, 37, 39, 62]. In fact, both pyrraline and imidazolone are known to be formed via reactive intermediates such as 3-deoxyglucosone without oxidation reactions [22, 33]. Previous studies demonstrated that these compounds have a role in the development of some disorders including diabetes mellitus [40, 47]. Thus, the present results could be an indication that unknown processes without oxidative stress are implicated in motor neuron degeneration of SOD1-mutated familial ALS. A precise mechanism, however, seems to be complicated because of the different intracellular localization of pyrraline and imidazolone. The relationship between the accumulation of specific AGE and SOD1 aggregation occurring in motor neurons needs to be addressed to gain a better understanding of the pathomechanisms of ALS.

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