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Intense Superoxide Dismutase-1 Immunoreactivity in Intracytoplasmic Hyaline Inclusions of Familial Amyotrophic Lateral Sclerosis with Posterior Column Involvement

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Abstract. This report concerns retrospective immunohistochemical and immunoelectron microscopic studies on superoxide dismutase-1 (SOD1) in intracytoplasmic hyaline inclusions (IHIs) of the anterior horn cells of three patients with familial amyotrophic lateral sclerosis (ALS) with posterior column involvement. All of the patients were members of the American "C" family. Almost all of the IHIs, present in the soma and cordlike swollen neurites of some affected neurons of the three patients, were intensely stained by an antibody to human SOD1. By contrast, the cytoplasm of anterior horn cells of the ALS patients and of ten control individuals reacted only weakly with the antibody or not at all. Immunoelectron microscopy revealed that the granule-associated thick linear structures that composed the IHIs were intensely labeled by the antibody to SOD1. The IHIs were also positively stained by antibodies to ubiquitin and phosphorylated neurofilament protein, with the distribution of immunoreactivity resembling that seen with the anti-SOD1 antibody. The DNA analysis disclosed a single-site GCC to GTC substitution at codon 4 ($Ala^4 \rightarrow Val$) in the SOD1 gene from the brain samples of the patients and from the peripheral blood of their family members. Our results suggest that SOD1 is a component of IHIs and may interact with ubiquitin and neurofilament protein, and point to the possibility that the presence of intense SOD1 immunoreactivity in the IHIs may be of relevance in processes involving structurally altered SOD1 molecules encoded by the mutated gene.

Key Words: Amyotrophic lateral sclerosis; Anterior horn cells; Gene analysis; Immunoelectron microscopy; Immunohistochemistry; Intracytoplasmic hyaline inclusions; Superoxide dismutase.

INTRODUCTION

Superoxide dismutase (SOD; EC 1.15.1.1) is an antioxidant metalloenzyme that catalyzes the detoxification of superoxide radical to form hydrogen peroxide and molecular oxygen (1). Three isoforms of the enzyme have been identified in humans: the cytosolic Cu/Zn-binding form (SOD1) (2), the mitochondrial Mn-dependent form (SOD2) (3) and the extracellular Cu/Zn-binding form (SOD3) (4). Recent studies have shown that mutations in the SOD1 gene, located on chromosome 21q22.1 (5), can be detected in certain cases of familial amyotrophic lateral sclerosis (ALS) (6–21). Moreover, in a preliminary study on the spinal cords of three ALS patients (22, 23) of the American "C" family (24) we demonstrated that an antibody to SOD1 reacted strongly with intracytoplasmic hyaline inclusions (IHIs), whereas the reactive astrocytes were intensely stained by an antibody to SOD2 (25). Earlier investigation had indicated that IHI is a characteristic neuropathological feature (26) of familial ALS with posterior column involvement (27), and several reports (28–30) have shown that the inclusion is positively immunostained by antibodies to ubiquitin (UBQ) (31, 32) and neurofilament protein (NFP) (33, 34). We have now extended the study (25) of the "C" family, and in this report we present the results of applying immunohistochemical and immunoelectron microscopic procedures for determining the precise localization of SOD1 in the anterior horn cells, focusing mainly on IHIs. In addition, the results of a molecular analysis of the SOD1 gene of the patients and their family members will also be presented.

MATERIALS AND METHODS

Materials

The immunohistochemical portion of this study was performed on archival, formalin-fixed, paraffin-embedded spinal cords, obtained at autopsy of 3 patients with familial ALS and of 10 control individuals (ages: 45 to 90 years [yr]) who died of non-neurological disorders. The three ALS patients were males, and their ages at death were 39 (Case 1), 46 (Case 2), and 66 years (Case 3). Cases 1 and 2 were brothers, and Case 3 was their cousin (Fig. 1). The clinicopathological findings of these patients were described before (22, 23), and they were consistent with familial ALS with posterior column involvement (26, 27). SOD1 gene analysis was carried out on archival brain samples of the three ALS patients and on 13 peripheral blood samples of their offsprings, siblings and spouses (Fig. 1).

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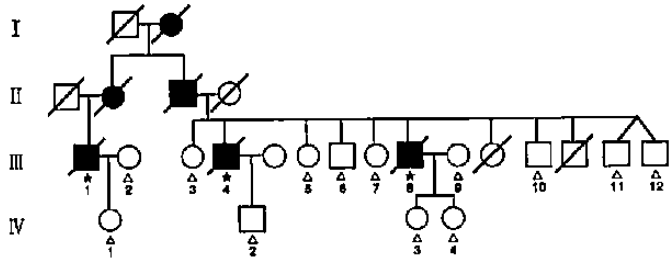

Fig. 1. Pedigree of the "C" family (#585). Cases 1, 2 and 3 are III-8, III-4 and III-1, respectively. Of the 13 individuals whose blood samples tested for SOD1 gene mutation, 11 were at risk, and two (III-2, 9) were not. Abbreviations: circles = female; squares = male; filled symbols = affected; bars = deceased; triangles = blood-tested; stars = brain-tested.

Primary Antibodies

The primary antibodies were a rabbit antiserum raised against human SOD1 (35, 36), an affinity-purified rabbit antibody to cow UBQ (Dako, Glostrup, Denmark), and a mouse monoclonal antibody to rat phosphorylated NFP (SMI-31; Sternberger Monoclonals, Baltimore, MD). The specificity of the anti-SOD1 antibody for human SOD1 was established previously by Western blotting and radioimmunoassays (36).

Immunoblotting

These tests were performed to rule out the possibility that the antibody to SOD1 cross-reacted with UBQ and NFP, which had been shown to colocalize in IHIs (28–30), the abnormal structures in which we demonstrated the presence of SOD1 (25). Western blotting and dot blotting were used. For Western blotting, the mixture of purified SOD1 (Sigma Chemical, St Louis, MO), SOD2 (Sigma), and UBQ (Sigma) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (37). After blocking nonspecific binding sites with 5% skim milk in 50 mM Tris-buffered saline, pH 7.6 (TBS) containing 0.1% Tween 20, the membranes were rinsed with Tween 20 (0.1%)-containing TBS and incubated for 3 hours (h) at room temperature with the antibodies to SOD1 (diluted 1:5,000) and UBQ (diluted 1:1,000). Immunoreactive bands were visualized by treatment with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and streptavidin-labeled alkaline phosphatase (Amersham International, Buckinghamshire, UK); 5-bromo-4-chloro-3-indoxyl phosphate (Sigma) was the substrate and nitro blue tetrazolium (Sigma), the chromogen. Prior to dot blotting onto polyvinyliden fluoride membranes, SOD1 and NFP (Sigma) were dissolved separately in 10 mM sodium phosphate buffer solution (pH 7.5) containing 6 M urea, 5 mM EDTA, and 1% 2-mercaptoethanol. Subsequently, nonspecific binding was blocked with 3% skim milk in 10 mM phosphate-buffered saline, pH 7.6 (PBS), and the membranes were rinsed with PBS, and then incubated for 2 h at 37°C with the antibodies to SOD1 (diluted 1:5,000) and phosphorylated NFP (diluted 1:5,000). After incubation, the membranes were treated with Triton X-100 (0.05%)-containing PBS. Immunoreactive spots were detected by the avidin-biotin-immunoperoxidase complex (ABC) method using the respective Vectastain ABC kit (Vector), following the manufacturer's protocol; hydrogen peroxide (Mitsubishi Gas Chemical, Tokyo, Japan) was the substrate and 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Dojin Chemicals, Kumamoto, Japan), the chromogen. Replicate membranes were used for protein staining with Coomassie brilliant blue.

Immunohistochemical Assays

Multiple 6-μm-thick sections were cut at different levels of the formalin (10%)-fixed, paraffin-embedded spinal cord of each ALS patient and control individual. Sections were deparaffinized, stained with eosin or hematoxylin-eosin (H&E), and examined with a light microscope, and special features, including IHIs were photographed. After decolorization of H&E with 70% ethanol containing 1% hydrochloric acid, the sections were rehydrated, quenched for 30 min with 0.3% hydrogen peroxide, rinsed in PBS, and then incubated overnight at 4°C with the antibodies to SOD1 (diluted 1:10,000), UBQ (diluted 1:1,000), and phosphorylated NFP (diluted 1:10,000). Sections from which the primary antibodies were omitted served as reaction controls. Selected sections were incubated with the anti-SOD1 antibody which had been preabsorbed with 0.5 mg/ml of SOD1, UBQ or NFP. Antibody binding was visualized by using the appropriate Vectastain ABC kit, following the manufacturer's instructions; DAB was the chromogen and methyl green, the counterstain. The location of immunoreactivity was verified by comparison with the initial photographs of a given eosin- or H&E-stained section.

The distribution of reaction product deposits obtained with one primary antibody was compared with that with another by reimmunostaining of the same section. For this purpose, consecutive sections were incubated overnight at 4°C with the anti-SOD1 antibody (diluted 1:10,000) and with the anti-UBQ antibody (diluted 1:1,000). Antibody binding was detected with the Vecatstain ABC kit using 3-amino-9-ethylcarbazole (AEC) (Nakarai Chemicals, Kyoto, Japan) as the chromogen. Following examination, the immunoreactive structures were photographed. After decolorization of AEC with 100% ethanol, the immunoreaction was eluted by twice incubating the sections for 60 min at room temperature with glycine-hydrochloric acid buffer solution (pH 2.2). The completeness of the elution step was verified by obtaining a negative reaction after re-application of AEC. Subsequently, sections already immunostained with the antibody to SOD1 were incubated overnight at 4°C with the anti-UBQ antibody (diluted 1:500) and with SMI-31 (diluted 1:5,000). Conversely, sections already immunostained for UBQ were incubated overnight at 4°C with the anti-SOD1 antibody (diluted 1:5,000). Immunoreactivity was detected with the peroxidase-antiperoxidase complex (Dako) procedure using DAB as the chromogen. The immunoreactive IHI structures were examined individually and compared with the inclusions photographed initially in a given immunostained section. The results of the consecutive immunostaining procedure were verified by comparing the corresponding SOD1-positive and UBQ-positive IHIs in serial sections stained individually with the respective antibodies. By this approach we controlled and ruled out any false positives among the reimmunostained sections.

Immunoelectron Microscopy

Paraffin sections on glass slides were immunostained with the anti-SOD1 antibody using DAB as the chromogen, post-fixed for 60 min at room temperature in 2% osmium tetroxide, rinsed in PBS, dehydrated with 100% ethanol, embedded in epoxy resin (Epon 812), and subsequently detached from the glass slides. The presence of SOD1-positive structures was confirmed by light microscopy. The Epon blocks were trimmed and cut with a diamond knife into ultrathin sections. These were mounted on bronze meshes and examined with the Hitachi HU-12A electron microscope.

DNA Analysis

Genomic DNA of the ALS patients and the family members were extracted from their peripheral blood or autopsied samples (38) (Fig. 1). Primer sequences for polymerase chain reaction (PCR) amplification of exons 2 and 4 have been reported in (6) as set b, those of exons 1 and 5 have been described in (7), and exon 3 primer sequences have been reported in (21). The PCR products were analyzed with single-stranded conformation polymorphism (SSCP) in 0.5 × Mutation Detection Enhancement (MDE; J. T. Baker, Phillipsburg, NJ), 5% glycerol gels. The PCR products were also purified in a 6% acrylamide gel and sequenced with PCR cycle sequencing independently using two amplification primers.

RESULTS

Immunoblot Analysis

Western blot analysis (Fig. 2a) showed that the anti-SOD1 antibody reacted only with SOD1, but not with UBQ or SOD2. Conversely, the anti-UBQ antibody reacted only with UBQ and not with SOD1 or SOD2. Similarly, dot blot analysis (Fig. 2b) indicated that the anti-SOD1 antibody did not react with NFP, and that SMI-31 did not recognize SOD1.

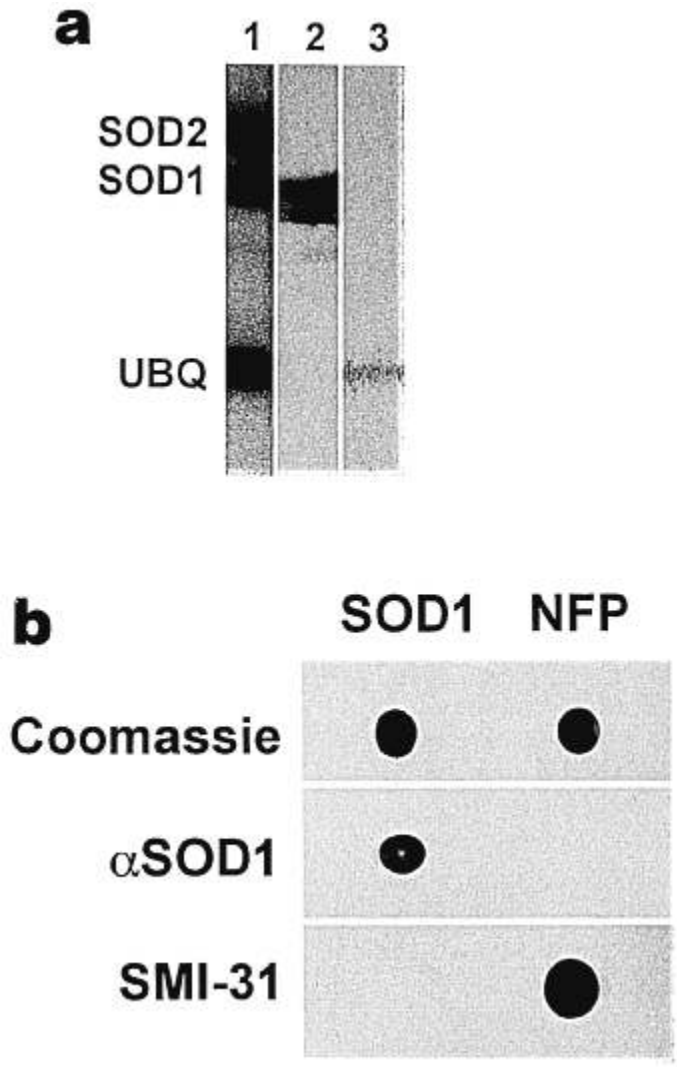

Fig. 2. Immunoblot analysis. (a) Western blotting of UBQ, SOD1 and SOD2. Protein stain with Coomassie brilliant blue showed the bands of UBQ, SOD1 and SOD2 (Lane 1). SOD1 was labeled by the antibody to SOD1, but not by that to UBQ (Lane 2), and vice versa (Lane 3). Neither antibody reacted with SOD2. (b) Dot blotting of SOD1 and NFP. SOD1 reacted with its respective antibody (αSOD1), but not with SMI-31. Conversely, NFP was labeled by SMI-31, but not by αSOD1.

Neuropathological and Immunohistochemical Observations

The neuropathological examination of the spinal cords of the 3 ALS patients revealed a decreased number of neurons in the anterior horns and Clarke's nuclei, degeneration of the pyramidal tracts, posterior columns and posterior spinocerebellar tracts, and reactive astrocytosis in the degenerated areas (22, 23). IHIs were seen in the soma (Fig. 3a, c) and the proximal (Fig. 3c) and distal cordlike swollen neurites of some affected anterior horn cells of each patient. The IHIs were composed of pale and eosinophilic areas, and displayed round, sausagelike, ring-shaped, or ill-defined profiles (Fig. 3a, c). No abnormal intracellular inclusions were observed in anterior horn cells of the 10 control individuals.

No reaction product deposits were seen in sections incubated with all reagents except the primary antibodies. The erythrocytes, present in the specimens of each case, served as positive internal controls for SOD1 immunoreactivity (Fig. 3g). Almost all of the IHIs identified in the soma (Fig. 3b, d) and cordlike swollen neurites (Fig. 3d, e) of the patients' anterior horn cells were intensely immunolabeled by the anti-SOD1 antibody. Immunoreactivity was evident in almost the entire IHI, but staining intensity varied from one IHI portion to another. A marked reduction in immunostaining of IHIs was seen when sections were incubated with the anti-SOD1 antibody preabsorbed with an excess of SOD1. Pretreatment of the antibody with UBQ or NFP

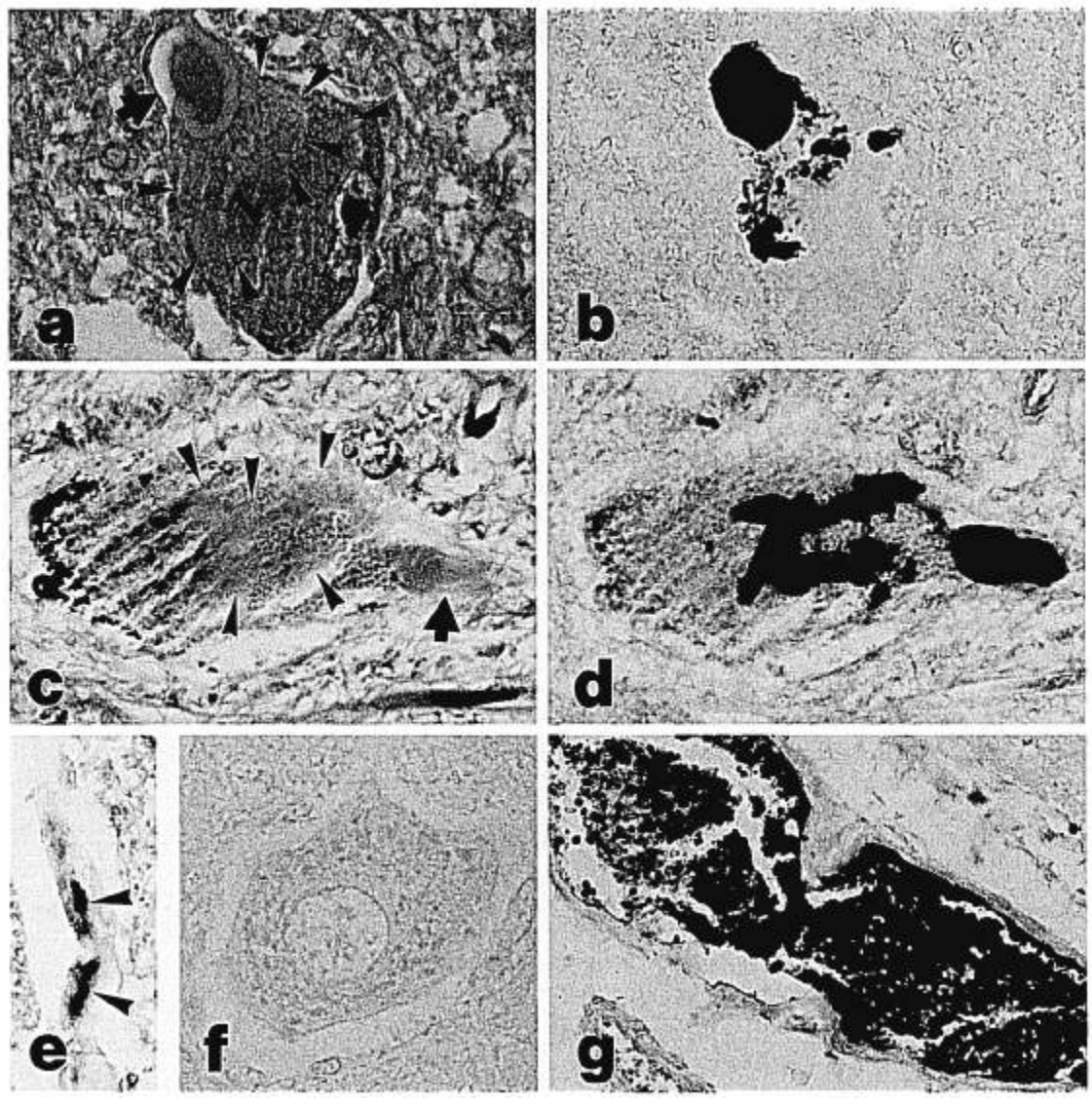

Fig. 3. Spinal cord sections stained with eosin (a) and hematoxylin-eosin (c), and immunostained with the anti-SOD1 antibody (b, d–g); (a, c) are the same sections as (b, d), respectively. a–f: ×660, g: ×165. (a) Round (arrow) and ill-defined (arrowheads) intracytoplasmic hyaline inclusions (IHIs) were seen in the soma of a chromatolytic anterior horn cell (Case 1). The round IHI consisted of an eosinophilic inner portion and a surrounding pale zone. Other IHIs were composed of obscure pale materials. (b) The same neuron as in (a) showed that each inclusion was intensely labeled by the antibody to SOD1. Immunostaining of the central portion of the round IHI was less intense. (c) Ill-defined IHIs (arrowheads) were seen in the soma of an affected anterior horn cell (Case 3). The proximal cordlike swollen neurite had a sausagelike IHI (arrow). These inclusions were composed of pale materials. (d) Each inclusion of the neuron shown in (c) was intensely labeled by the antibody to SOD1. (e) A distal cordlike swollen neurite (Case 2) contained immunoreactive spots (arrowheads) corresponding to IHIs. (f) A morphologically intact anterior horn cell of a control individual displayed no significant SOD1 immunoreactivity. (g) SOD1-positive erythrocytes were seen in subarachnoid vessels of a control individual.

did not affect SOD1 immunostaining intensity. The cytoplasm of morphologically normal-appearing (Fig. 3f) and affected (Fig. 3b, d) anterior horn cells of the three ALS patients, and of normal neurons of the ten control subjects was labeled faintly or not at all. The reactive astrocytes of the ALS patients exhibited weak to moderate immunostaining with the antibody to SOD1, while no positive reaction was seen in other cells or cell components.

The distribution of UBQ immunoreactivity in IHIs resembled that of SOD1 rather very closely (Fig. 4a–c). By contrast, in relation to the SOD1-positive structures of the IHIs, two distinct immunostaining patterns were seen with SMI-31, the antibody to phosphorylated NFP. In

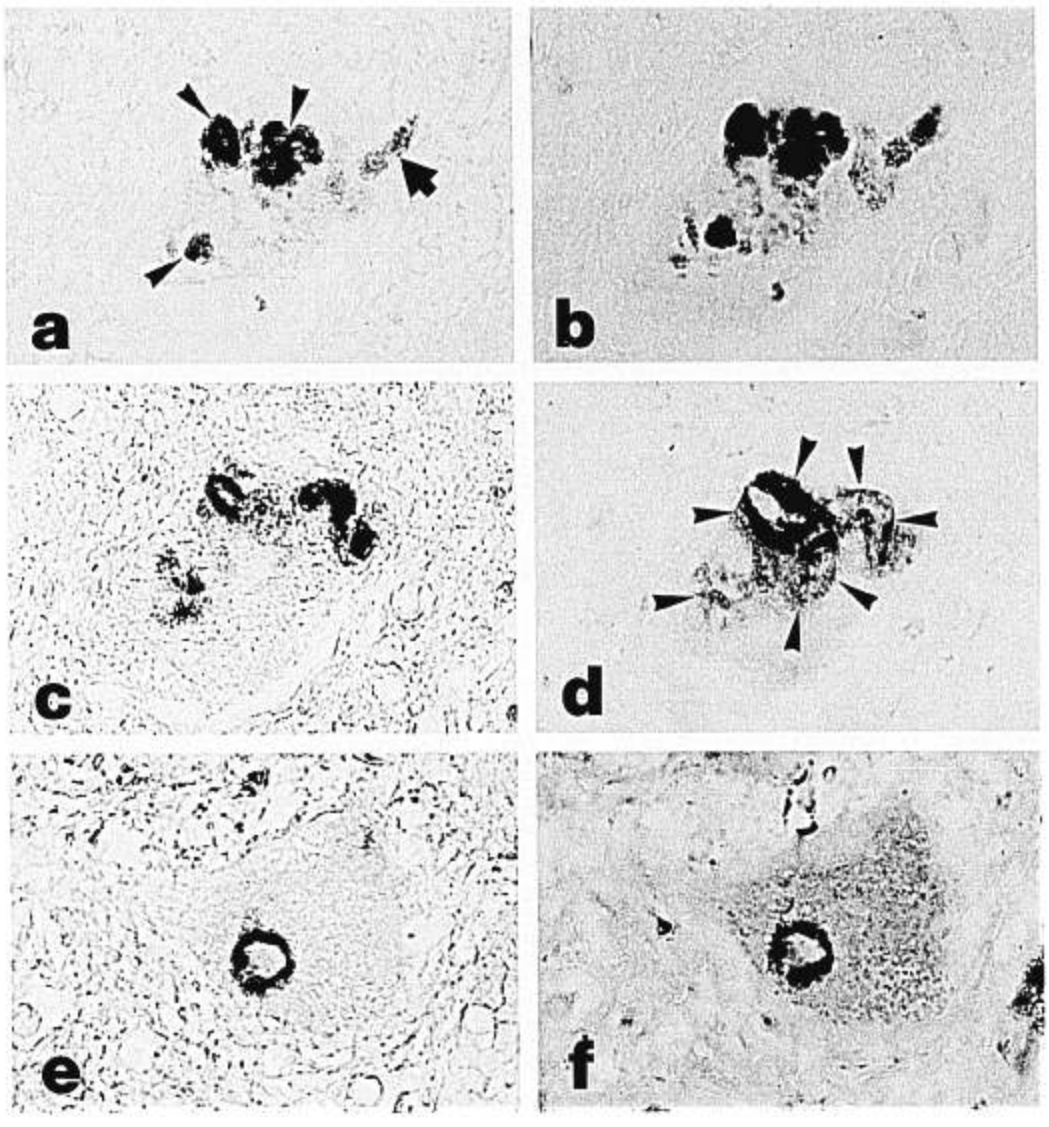

Fig. 4. Intracytoplasmic hyaline inclusions (IHIs) in anterior horn cells of the ALS patients immunostained with antibodies to UBQ (a), SOD1 (b, c, e) and phosphorylated NFP (d, f); (a, c, e) are the same sections as (b, d, f), respectively, a–f: ×660. (a) An abnormal neuron (Case 1) had UBQ-positive structures corresponding to IHIs in the soma (arrowheads) and the neurite (arrow). (b) The same neuron as in (a) showed that the distribution of SOD1 immunoreactivity of the inclusions was closely parallel with that of UBQ immunoreactivity (a). (c) The same abnormal neuron in a section consecutive to that depicted in (a and b) indicated that each inclusion had SOD1-positive structures similar to those stained by the antibody to UBQ (a). (d) In the same neuron as in (c), the immunoreactive products with the anti-phosphorylated NFP antibody (SMI-31) were deposited in the marginal and surrounding areas (arrowheads) of the SOD1-positive structures (c). (e) Intense SOD1 immunoreactivity of a ring-shaped IHI was observed in the soma of a neuron (Case 3). (f) In the same neuron as in (e), the SMI-31 immunoreactive structure overlapped the SOD1-positive structure (e). The surrounding cytoplasm was only weakly stained by SMI-31.

one, staining was observed in the margins and around the SOD1-positive structures (Fig. 4c, d), and in the other, staining was superimposed (Fig. 4e, f). The cytoplasm of some abnormal neurons of the ALS patients was weakly immunoreactive with SMI-31 (Fig. 4f), but not with the anti-UBQ antibody. No significant cytoplasmic staining was observed in intact anterior horn neurons incubated with either antibody.

Immunoelectron Microscopic Observations

Intense labeling with the anti-SOD1 antibody was evident as an osmiophilic meshwork of IHIs in the soma (Fig. 5a) and cordlike swollen neurites (Fig. 5d) of anterior horn cells of the ALS patients. The meshwork was composed of ill-defined granules and coarse linear structures, approximately 30 to 60 nm in diameter (Fig. 5b, c,

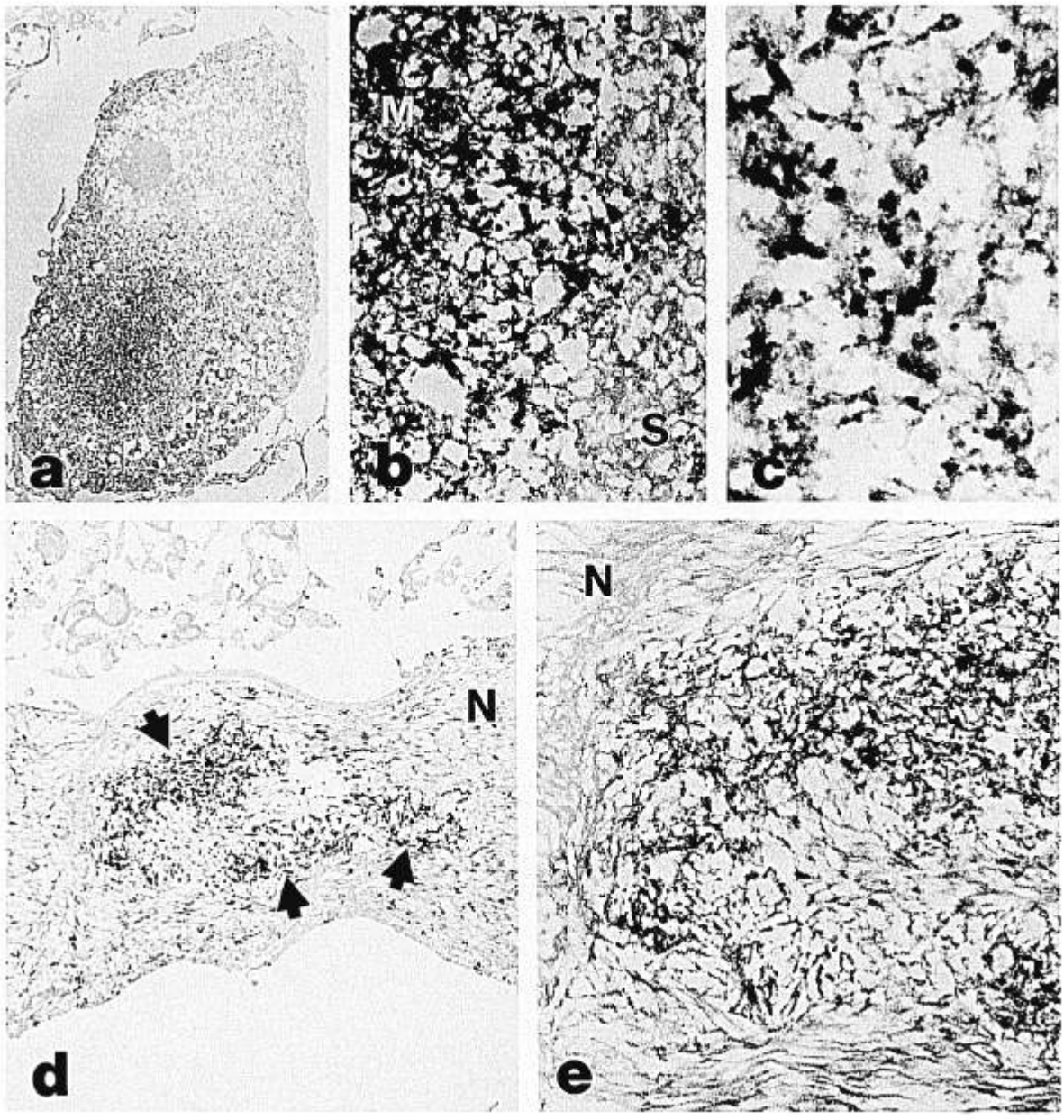

Fig. 5. Immunoelectron microscopic examination of intracytoplasmic hyaline inclusions (IHIs) in anterior horn cells (Case 3) with the antibody to SOD1. (a) A round IHI in the soma of an anterior horn cell was intensely labeled by the antibody, whereas the cytoplasm and organelles are not labeled. ×1,500. (b) A higher magnification of (a) showed a positively stained meshwork (M) in the IHI and the nonstained surrounding cytoplasm (S). ×12,000. (c) At a higher magnification of (b), the immunoreactive meshwork proved to consist of the granule-associated thick linear structures. ×30,000. (d) IHIs in a cordlike swollen neurite appeared as SOD1-positive islands (arrows) within a stream of neurofilaments. The surrounding neurofilaments (N) were not labeled by the antibody. ×3,000. (e) A higher magnification of (d) indicated that the SOD1-positive islands were composed of a meshwork of the granule-associated thick linear structures (M). The immunoreactive structures were intermingled with irregularly arranged, nonlabeled neurofilaments (N). ×6,000.

e), that corresponded to the granule-associated thick linear structures described before (39). The cytoplasm, neurofilaments, and organelles, including the nucleus, were not immunolabeled (Fig. 5a, b, d, e), and no significant immunoreactivity was found in other cells, except in the erythrocytes and some reactive astrocytes.

SOD1 Gene Analysis

Based on SSCP analysis, a single mutant allele of exon 1 was found in two of the 3 ALS patients and seven of the 11 individuals at risk (Figs. 1, 6a). No PCR-mediated DNA amplification was obtained with the genomic DNA

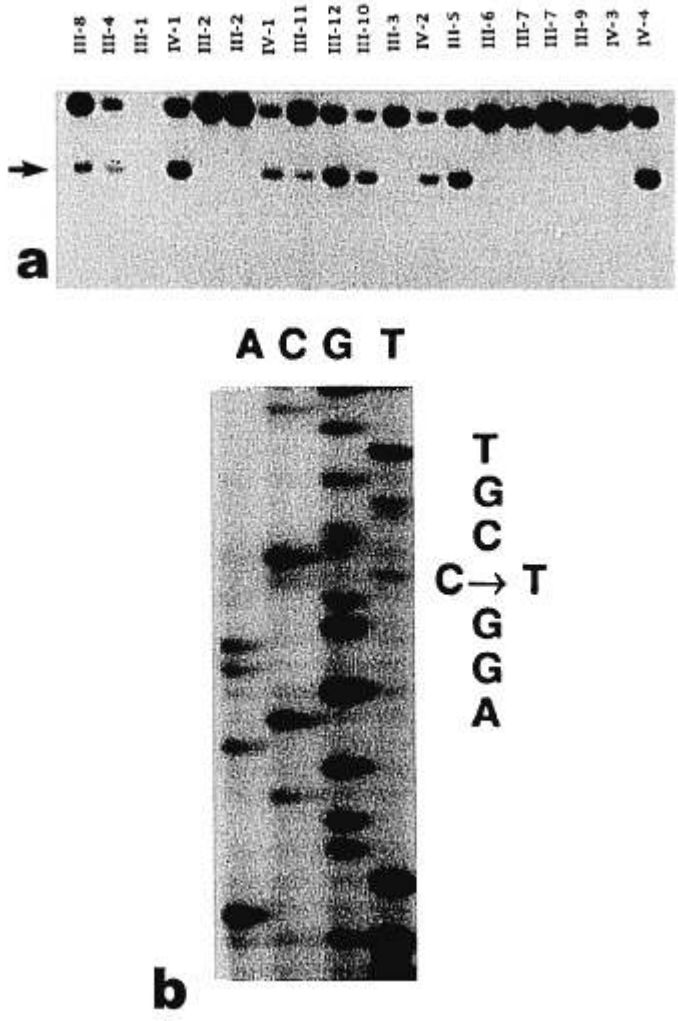

Fig. 6. SOD1 gene analysis. (a) SSCP analysis of PCR-mediated SOD1 gene exon 1. The PCR products with the defective allele appeared as the normal and variant (arrow) bands (III-4, 5, 8, 10–12; IV-1, 2, 4). The genomic DNA of patient III-1 could not be amplified after repeated attempts. No mutation was detected in four individuals at risk (III-3, 6, 7; IV-3) and two of the patients' spouses (III-2, 9). (b) Direct nucleotide sequencing of the SOD1 gene exon 1 of one individual with the defective allele. A heterozygous single-site substitution of GCC to GTC (alanine to valine) was detected at codon 4.

extracted from the archival material of Case 3 (III-1). The mutation, verified by sequencing of the amplified DNA fragment, consisted in a C to T substitution at the second position of codon 4 ($Ala^4 \rightarrow Val$) in exon 1 of the SOD1 gene (Fig. 6b). No mutation was detected in other exons.

DISCUSSION

The DNA analysis disclosed an $Ala^4 \rightarrow Val$ mutation in the SOD1 gene from members of the "C" family. No PCR-mediated amplification from tissue aliquots of Case 3 resulted, probably from the degradation of the genomic DNA by prolonged formalin fixation. However, since the clinical and histopathologic findings of this patient (III-1) resembled those of Cases 1 and 2, and the defective allele was detected in his daughter (IV-1) but not in his spouse (III-2), it is most likely that he might have had the same mutation as his cousins.

The most striking finding in this study with archival, formalin-fixed, paraffin-embedded spinal cord specimens is the presence of intense SOD1 immunoreactivity in almost all IHIs in the soma and cordlike swollen neurites of anterior horn cells of the ALS patients with the SOD1 $Ala^4 \rightarrow Val$ mutation. By contrast, no significant SOD1 immunoreactivity was found in the cytoplasm of both intact and affected anterior horn cells. Immunoelectron microscopy revealed that the intense immunolabeling of the IHIs by the anti-SOD1 antibody was confined to the granule-associated thick linear structures (39), and that the other subcellular components displayed no significant immunoreactivity. These immunohistochemical and immunoelectron microscopic findings imply that in the abnormal anterior horn cells, IHIs have significantly greater SOD1 levels than their cytoplasm, and are particularly relevant since IHIs are considered to be characteristic of familial ALS with posterior column involvement (26). However, this should be considered with the caveat that we have not yet determined whether the anti-SOD1 antibody recognizes not only wild-type SOD1 but also mutant SOD1.

There is precedent for our findings; in one case report (30) a relationship between an $Ala^4 \rightarrow Thr$ mutation in the SOD1 gene and familial ALS with posterior column involvement (26, 27) was pointed out. It is of significance that transgenic mice (40) which express mutant human SOD1 have "hyaline inclusion bodies" (41) that resemble the IHIs of human ALS. These observations would suggest that mutations in the SOD1 gene may be of importance for the formation of the abnormal intracellular structures. Although the origin of the granule-associated thick linear structures that compose the human IHIs is unknown, their SOD1 immunoreactivity implies that SOD1 is at least a component of the IHIs and could be attached to those through a process of IHI formation. Moreover, the immunoreactivity may also reflect a protective mechanism of inclusion-bearing neurons against injury triggered by superoxide radical thought to be potentially generated in association with the degradation of organelles such as endoplasmic reticulum (39), and the disorganization of neuronal cytoskeleton (28), or both.

As our results indicate, IHIs of the 3 ALS patients were stained by the antibodies to SOD1, UBQ, and phosphorylated NFP. However, the distribution of reaction product deposits differed to some extent. Thus, whereas SOD1- and UBQ-positively stained IHI structures appeared to overlap, the reaction product deposits with the antibody to phosphorylated NFP were always

found adjacent to these structures. From these observations and control experiments (immunoblotting and immunoabsorption tests), it is evident that IHIs contain SOD1 as well as UBQ and phosphorylated NFP. This raises the possibility that these three substances may interact with each other and be involved in IHI formation. It is of interest in this context that an earlier immunoelectron microscopic study (28) has shown that UBQ, the small protein inducible in response to oxidative stress (32), as SOD1 is also located in the granule-associated thick linear structures of IHIs. Moreover, because of its participation in the non-lysozomal ATP-dependent proteolytic system (31), it is conceivable that UBQ could have a role in SOD1 degradation. On the other hand, there is ultrastructural evidence (28, 39) that the granule-associated thick linear structures of IHIs are intermingled with randomly oriented neurofilaments, prominently in the margin of IHIs. In the present study, SMI-31 immunoreactive structures corresponding to phosphorylated NFP were seen in the marginal and surrounding areas of IHIs as described before (28–30, 42), although perikaryal neurofilaments have been shown to be a nonphosphorylated form under the physiological conditions (34, 42). The overlap of immunoreactivities for SOD1 and phosphorylated NFP in the ring-shaped IHI may indicate that this thin structure, having the outer and inner surfaces, corresponds itself to the margin. These observations would suggest that both SOD1 and UBQ could be involved in NFP metabolism.

It has been hypothesized that the alteration of proteins essential for normal cellular function, which could result from nitration of tyrosine residues by peroxynitrite generated through the nonenzymatic reaction of nitric oxide and superoxide radical, is closely related to the pathogenesis of ALS (43). It is relevant to this notion that wild-type SOD1 catalyzes the peroxynitrite-mediated nitration of tyrosine residues (44). Moreover, since chemically modified SOD1 reacts more readily with peroxynitrite and scavenges superoxide radical less efficiently than the normal enzyme (45), it is possible that mutant SOD1 might similarly promote the tyrosine nitration (43). NFP could be nitrated as well as other proteins to form neurofilamentous accumulations (43) that are often found in the anterior horn cells of ALS patients (46). Further suggestions that NFP may have a role in motor neuron diseases come from the recent demonstration of ALS-like pathological changes in the spinal cords of transgenic mice that overexpress the human (47) and the mouse (48–50) NFP gene, and from the detection of NFP gene mutations in patients with sporadic ALS (51). Whether our cases of familial ALS with posterior column involvement have nitrated NFPs or a mutated NFP gene remains to be determined.

Many SOD1 gene mutations encode for structurally defective enzyme molecules that have reduced enzyme activity (6, 7, 9, 12, 52, 53), and therefore scavenge superoxide radical with diminished efficiency. The SOD1 $Ala^4 \rightarrow Val$ substitution, the most frequent mutation in familial ALS, interferes with β-barrel fold and dimer contact, consequently affecting normal enzyme function (7, 53–55). Indeed, the marked SOD1 immunoreactivity in IHIs seems to be indicative of a quantitively excessive response compensating for reduced enzyme activity, but it is thought that the reduction in SOD1 enzyme activity, a trigger of injury by free radicals, is not responsible for familial ALS, because SOD1-linked ALS families including the "C" family show autosomal dominant inheritance (56, 57). Since one normal gene encoding enzyme protein is usually essential for normal cellular function, there may be no deficiency of SOD1 activity in patients with both wild-type and mutant form of SOD1 (56, 57). Transgenic mouse models expressing mutant SOD1 suggest that familial ALS results not from enzyme deficiency, but from a novel "gain-of-function" effect such as a toxic influence of mutant protein (40, 56–58).

On the other hand, the presence of SOD1 in the IHIs of our ALS patients is also suggestive of a longer survival of inactive enzyme molecules, reminiscent of the mechanisms associated with aging of rats (59, 60) and human (61, 62), Werner's syndrome (62, 63), Alzheimer's disease (61), and diabetes mellitus (63–65). These pathological conditions could result in the intracellular deposition of enzyme proteins through several modifications including oxidation, glycation, and fragmentation (59–65). One recent study showed that the half-life of mutant SOD1 subunits transiently expressed in primate cells is shorter than that of wild-type subunits, and that this tendency pertains especially to the $Ala^4 \rightarrow Val$ mutation (55). Another study demonstrated that the spinach chloroplast SOD1 is attached to the inner surface of the plasma membrane (66). These observations raise the possibility that mutant SOD1 accumulates in the granule-associated thick linear structures of IHIs in contrast with wild-type SOD1, which is present homogeneously in the cytosol. Finally, the rather very high proportion (100%) of SOD1-immunopositive IHIs in familial ALS that contrasts the much lower percentage (32%) of SOD1-positive inclusions seen in sporadic ALS (67), could be an indication that the mutant enzyme has a greater affinity for the granule-associated thick linear structures than wild-type SOD1. Answers to this and to other questions regarding the ultimate significance of the present results require further investigations.

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