FEBS Letters 400 (1997) 108–112 FEBS 17968

Instability of expressed Cu/Zn superoxide dismutase with 2 bp deletion found in familial amyotrophic lateral sclerosis

Yasuhiro Watanabe[a,*], Yasuhisa Kono[b], Eiji Nanba[c], Eisaku Ohama[d], Kenji Nakashima[a]

[a]*Division of Neurology, Institute of Neurological Sciences, Faculty of Medicine, Tottori University, 36-1 Nishimachi, Yonago 683, Japan*
[b]*Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue 690, Japan*
[c]*Gene Research Center, Tottori University, 36-1 Nishimachi, Yonago 683, Japan*
[d]*Division of Neuropathology, Institute of Neurological Sciences, Faculty of Medicine, Tottori University, 36-1 Nishimachi, Yonago 683, Japan*

Received 7 October 1996; revised version received 11 November 1996

Abstract The mutant Cu/Zn superoxide dismutase (SOD1) associated with familial amyotrophic lateral sclerosis (FALS) with a 2 bp deletion was produced in two protein expression systems. The mutant SOD1, expressed as a fusion protein in *E. coli*, had immunoreactivity to an anti-human SOD1 antibody but no SOD activity. It was more susceptible to proteolysis and its immunoreactivity decreased more rapidly than the wild type. The mutant SOD1, expressed in Cos1 cells, was not detected by either SOD activity staining or Western blot analysis, although expression of its mRNA was confirmed. These results suggest that the mutant SOD1 is seriously unstable in mammalian cells.

Key words: Cu/Zn superoxide dismutase; Familial amyotrophic lateral sclerosis; Two basepair deletion; Expression system; Copper ion; Hydroxyl radical

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fetal neurodegenerative disorder that primarily involves motor neurons [1]. The familial form of ALS (FALS) is known to consist of 10–15% total ALS [2]. About 20–25% of all FALS families have mutations in the gene coding Cu/Zn superoxide dismutase (SOD1) [3]. We previously reported a pedigree of FALS with 2 bp deletion in the 126th codon of the SOD1 gene [4], which has its origin in the small Japanese islands of Oki [5]. This mutation is predicted to cause a frameshift and to generate premature termination in the 130th codon of the SOD1 gene [6]. In this family, the patients and clinically non-affected members with the mutation showed a reduction in SOD1 activity in red blood cells (RBCs) by 70% in the patients and 30% in the non-affected members with the mutation [7,8]. We failed to find evidence of the presence of the mutant SOD1 protein in postmortem brain tissue and RBCs from one patient belonging to this FALS pedigree, while reverse transcription (RT)-polymerase chain reaction (PCR)-single strand conformational polymorphism (SSCP) analysis showed an additional mRNA from the mutant SOD1 gene [8]. These results led us to investigate the stability of the mutant SOD1, because the mutant SOD1 in this family is proposed to be seriously unstable [4]. We constructed two kinds of expression systems in *Escherichia coli* and mammalian cells to produce the recombinant SOD1 protein which was compatible with the frameshift mutation. In this paper, several items of evidence concerned with the instability of the mutant SOD1 protein are presented.

*Corresponding author. Fax: (81) (859) 34-8083.
E-mail: yawatana@grape.med.tottori-u.ac.jp

Abbreviations: FALS, familial amyotrophic lateral sclerosis; SOD1, Cu/Zn superoxide dismutase; RT, reverse transcription; PCR, polymerase chain reaction; SSCP, single strand conformational polymorphism; MBP, maltose binding protein; PAGE, polyacrylamide gel electrophoresis

2. Materials and methods

2.1. Materials

The human SOD1 cDNA and mammalian expression vector, pYN3215, were kindly provided by Prof. K. Sato (Molecular Biology, Tottori University). Materials used in the present study were pGEM-7Zf(+) (Promega, Madison, USA); Gene Taq, ISOGEN and deoxyribonuclease (DNase) (Nippon Gene, Toyama, Japan); pMAL-cRI, amylose resin and factor Xa (New England BioLabs, Beverly, MA, USA); exonuclease III and mung bean nuclease (MBI Fermentas, Vilnius, Lithuania); human SOD1 (Sigma, St. Louis, MO, USA); a sheep anti-human SOD1 polyclonal antibody (The Binding Site, Birmingham, UK); an anti-sheep IgG (H+L) antibody labeled with horseradish peroxidase (HRP) (ZYMED Laboratories, San Francisco, CA, USA); ECL (Amersham, Buckinghamshire, UK); and GenAMP (Perkin-Elmer, New Jersey, USA). All other chemicals were of reagent grade.

2.2. Site directed PCR mutagenesis

The mutant cDNA of the SOD1 was obtained by two subsequent PCR amplifications using one specific mutagenic primer and two universal primers [9]. The entire coding region of the human SOD1 was cloned into pUC19 (SOD1-pUC19) and pGEM-7Zf(+) (SOD1-pGEM7Zf). The first PCR primers were the mutagenic primer: 5′-AAAGCAGATGACGGGCAAAGGTGG-3′ (the wild-type sequence: TGACTTGGGC [4]) and T7 primer: 5′-TTGTAATACGACTCACTATAG-3′. The first PCR reaction (20 μl) was performed in 2 μl of appended 10×PCR buffer, 50 ng SOD1-pGEM7Zf vector as a template, 200 μM dNTPs, 200 pM mutagenic primer, 200 pM T7 primer, and 2.5 units Taq polymerase with 30 amplification cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. Then the first PCR products were hybridized with SOD1-pUC19 and extended by Taq polymerase for 2 min at 72°C to obtain single strand mutant cDNA. The second PCR reaction (40 μl) was performed using T7 primer and reverse M13/pUC primer, 5′-TCACACAGGAAACAGCTATGAC-3′. The open reading frame of the mutant cDNA was confirmed to have no misreading by sequencing.

2.3. Construction of the expression vectors

For the expression in *E. coli*, a commercial expression vector, pMAL-cRI, was used. In order to avert the incorporation of excess amino acids, the non-coding region ahead of the start codon in the SOD1 gene was deleted by exonuclease III and mung bean nuclease [10]. The wild-type and mutant SOD1 genes were inserted downstream of the *malE* gene which encoded the maltose binding protein (MBP) at 42 kDa (wt SOD1-pMALcRI and del SOD1-pMALcRI), and resulted in the expression of MBP-SOD1 fusion proteins.

The wild-type and mutant SOD1s were also cloned into the mammalian expression vector pYN3215 carrying the *SRa* promoter [11] (wt SOD1-pYN3215 and del SOD1-pYN3215). In this system, recombinant SOD1s were produced in the same manner as in human tissue.

PII S0014-5793(96)01362-2

2.4. Purification of expressed SOD1s in E. coli

The host *E. coli*, XL1-Blue, was transformed with wt SOD1-pMALcRI or del SOD1-pMALcRI, and the transformed *E. coli* was treated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h to induce the formation of the fusion proteins. The *E. coli* were lysed by sonication for 1 min on ice in lysis buffer containing 10 mM sodium phosphate, 30 mM NaCl, 0.25% (w/w) Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml soybean trypsin inhibitor at pH 7.0. Purification of the fusion proteins was performed by the following procedure: the lysates were treated with affinity chromatography with amylose resin; then the resin was washed 2 times with 3 bed volumes of the column buffer (10 mM sodium phosphate, 30 mM NaCl, 1 mM sodium azide, 10 mM β-mercaptoethanol, and 1 mM EGTA at pH 7.0) with 0.25% Tween 20, and 2 times with 5 bed volumes of the column buffer without Tween 20; after that the fusion proteins were eluted with column buffer containing 10 mM maltose. To separate the SOD1s from the MBP, the fusion proteins were cleaved with factor Xa with a 4-h incubation in the cleavage mixture (20 μl) containing 2 μg proteins, 50 μg/ml factor Xa, 20 mM Tris-HCl, 100 mM NaCl, 1 mM sodium azide, 10 mM β-mercaptoethanol, and 1 mM EGTA at pH 8.0.

2.5. Expression in Cos1 cells

Cos1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The cells were transfected with wt SOD1-pYN3215 and/or del SOD1-pYN3215 according to the procedure of Chen and Okayama [12]. Then they were incubated at 37°C for 24 h. After renewal of the medium, the cells were further incubated for 24 h. Then they were washed three times with phosphate buffer saline (PBS) and collected.

2.6. Western blot analyses

Western blot analysis of the extracts of *E. coli* was performed with the samples after the affinity chromatography with or without cleavage by factor Xa.

The transfected Cos1 cells were homogenized with lysis buffer: 50 mM Tris, 5 mM EDTA, 125 mM NaCl, 1 mM PMSF, and 10 μg/ml soybean trypsin inhibitor at pH 7.4. The homogenates were sonicated for 1 min on ice.

The samples from the *E. coli* and Cos1 cells were fractionated on native or sodium dodecyl sulfate (SDS) linear gradient 10–20% polyacrylamide gel electrophoresis (PAGE). Electrotransfer to the nitrocellulose membrane was performed by the method of Bjerrum and Shafer-Nielsen [13]. The antigen bound membrane was treated with PBS containing 0.5% Tween 20 and 5% skim milk (pH 7.4), prior to the addition of a sheep anti-human SOD1 polyclonal antibody as a primary antibody. An anti-sheep IgG (H+L) antibody labeled with HRP was used as the second antibody, and the SOD1s binding to these antibodies were detected by the HRP detection system, ECL.

2.7. Activity staining

Cell-free extracts were electrophoretically separated on native linear gradient polyacrylamide gel (10–20%). Staining for SOD activity was performed according to the method described by Beauchamp and Fridovich [14].

2.8. RT-PCR-SSCP analysis for the mRNA of Cos1 cells

Total RNA was extracted from the transfected Cos1 cells using ISOGEN and was treated with DNase to avoid contamination by plasmid DNA. Then reverse transcription to DNA using the reverse transcriptase, GenAMP, was performed. Reverse transcript products (10 μl) were added to 50 μl PCR reaction mixture containing Taq polymerase. PCR primers were 5′-GATGGTGTGGCCGATGTGTC-TA-3′ and 5′-TTCTACAGCTAGCAGGATAACA-3′. These primers hybridize a 252 bp part in the human SOD1 sequence [15] in which the segment of 2 bp deletion is contained. Conformational polymorphism of the PCR products, which were electrophoresed on 12% polyacrylamide gel containing 5% glycerol at 4°C and 3 W overnight, were detected by silver staining [16].

3. Results

3.1. Purification of expressed SOD1s in E. coli

Because of premature termination due to the amber mutation that was located on the 5′ terminal of the start codon of the SOD1 gene, two protein bands, MBP (42 kDa) and MBP-SOD1 fusion proteins (the wild type: 60 kDa or the mutant: 57 kDa), were observed (Fig. 1, lanes 1–4). However, a single band was seen in Western blot analysis (lanes 6, 8). The estimated molecular weight of the human erythrocyte SOD1 was 16 kDa (lane 5). After cleavage by factor Xa, the wild-type SOD1 (18 kDa, lane 7) and the mutant SOD1 (15 kDa, lane 9) were separated from the MBP. The larger molecular sizes of the recombinant SOD1s are accounted for by the fact that these SOD1s have an additional 17 amino acid residues at the N terminal of the proteins.

In activity staining, the wild-type fusion SOD1 (lane 2) and the wild-type separated SOD1 (lane 3) showed SOD activity (Fig. 2). The wild-type separated SOD1 (lane 3) migrated to almost the same degree as the human erythrocyte SOD1 (lane 1). On the other hand, the mutant fusion SOD1 (lane 4) and

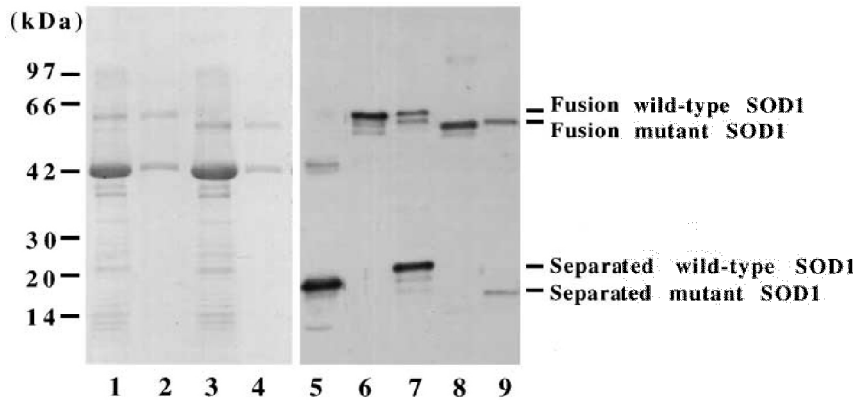

Fig. 1. Purification of recombinant SOD1s expressed in *E. coli*. Fusion SOD1s were purified from crude extracts of *E. coli* using affinity chromatography, which were then cleaved with factor Xa. Each sample was separated on SDS-PAGE and the gels were stained with Coomassie blue (lanes 1–4) or detected with Western blot analysis (lanes 5–9). The amount of proteins applied were 30 μg (lanes 1, 3), 0.2 μg (lane 5) and 1 μg (other lanes). Lanes: 1, crude extract of *E. coli* transformed by wt SOD1-pMALcRI; 2, purified MBP-SOD1 (wild-type) fusion protein; 3, crude extract of *E. coli* transformed by del SOD1-pMALcRI; 4, purified MBP-SOD1 (mutant) fusion protein; 5, human erythrocyte SOD1; 6, purified MBP-SOD1 (wild-type) fusion protein; 7, separated wild-type SOD1; 8, purified MBP-SOD1 (mutant) fusion protein; 9, separated mutant SOD1. The immunoreactivity of the mutant SOD1 (lanes 8, 9) was about 3 times weaker than the wild-type SOD1 (lanes 6, 7). The 42 kDa band in lane 5 was due to an impurity.

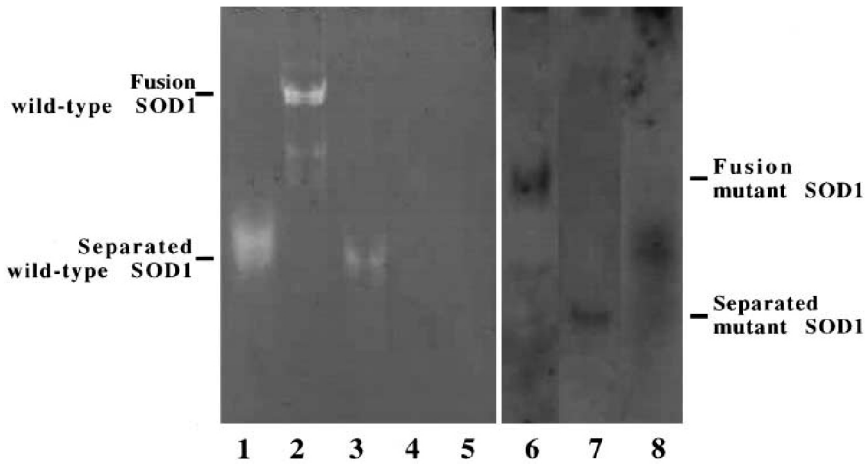

Fig. 2. Recombinant SOD1s separated by native PAGE. Recombinant SOD1s expressed in *E. coli* were separated on native PAGE and the gels were stained with SOD1 activity (lanes 1–5) or detected with Western blot (lanes 6–8). The amounts of proteins applied were 0.2 μg (lanes 1, 8) and 1 μg (other lanes). Lanes: 1, human erythrocyte SOD1; 2, fusion wild-type SOD1; 3, separated wild-type SOD1; 4, fusion mutant SOD1; 5, separated mutant SOD1; 6, fusion mutant SOD1; 7, separated mutant SOD1; 8, human erythrocyte SOD1. The mutant SOD1s had no SOD activity (lanes 4, 5). Approximate molecular weight of each protein was fusion wild-type SOD1: 120 kDa, fusion mutant SOD1: 60 kDa, separated wild-type SOD1: 30 kDa, and separated mutant SOD1: 15 kDa. The fusion mutant SOD1 and the separated mutant SOD1 seem to be monomeric proteins.

the mutant separated SOD1 (lane 5) revealed no SOD activity, though reactivity against the anti-human SOD1 polyclonal antibody was observed (lanes 6, 7). The molecular weight of the separated mutant SOD1 was less than expected.

3.2. Susceptibility of the SOD1s to proteolysis

As shown in Fig. 3A, the degradation of the wild-type SOD1 was observed after the treatment with proteinase K at more than 100 ng/ml. On the other hand, the degradation of the mutant SOD1 was confirmed with 1 ng/ml proteinase K.

3.3. Stability of the SOD1s with or without copper ions

The wild-type SOD1 was stable during incubation at 22°C for 24 h, and the addition of $CuSO_4$ had no significant effect on the immunoreactivity of SOD1 (Fig. 3B). In contrast, the immunoreactivity of the mutant SOD1 decreased during the 24-h incubation. The addition of $CuSO_4$ partly prevented this decrease.

3.4. Expression in Cos1 cells

While the wild-type SOD1 was expressed in Cos1 cells, judged from the results of activity staining and Western blot analysis, Cos1 cells transfected with the mutant cDNA were revealed to have neither SOD activity nor immunoreactivity (Fig. 4A,B). We co-transfected with wt SOD1-pYN3215 and del SOD1-pYN3215, to yield heterodimeric SOD1 between the wild-type and mutant subunits. No activity band on the gels was observed except for the homodimer of the wild-type SOD1 and native Cos1 SOD1, and the heterodimer between the wild-type and Cos1 SOD1 subunits (Fig. 4A). RT-PCR-SSCP analysis revealed that there was mRNA due to the mutant SOD1 gene (Fig. 4C).

4. Discussion

Transgenic mice overexpressing the mutant SOD1 gene associated with FALS manifested clinically and pathologically motor neuron disease-like features [17,18]. This fact suggests that 'gain of function' results in motor neuron degeneration in FALS. It has been reported, however, that a high level of SOD1 activity was sometimes toxic, as in the case of the reoxygenated heart [19] or mouse epidermal cells [20], as well as a low level of SOD1 activity [21]. On the other hand, SOD1-deficient mice revealed no manifestations of motor neuron disease [22]. We previously reported the absence of the mutant SOD1 protein in postmortem tissues from a FALS patient, despite the existence of mRNA due to the mutant SOD1 gene [8]. We referred to the possibility that the truncated protein was immediately degraded [8]. Indeed, this mutation of a 2 bp deletion was predicted to be a sole null mutation [2]. However, two possibilities still remain: the first is that the mutant SOD1 protein is seldom translated from mRNA, and the second is that the mutant SOD1 lacks immunoreactivity to the anti-human SOD1 polyclonal antibody used in the experiment.

In the present study, the existence of the mutant SOD1 protein was confirmed when it was expressed as a MBP-SOD1 fusion protein in *E. coli*, although it was more fragile than the wild-type SOD1 and probably lacked dimer forming ability. The fact that the presence of copper ions prevented the decrease of the mutant SOD1 in a proteinase free condition suggests the possibility that copper ions are concerned with the structural stability of the mutant SOD1. We think perhaps that the mutant SOD1, which binds less tightly to copper ions, alters its molecular structure after the release of copper ions, and that the structural alteration results in the reduction of SOD1 immunoreactivity. The monomer condition and structural alteration of the mutant SOD1 probably induce susceptibility to attack by various proteinases. Cos1 cells transfected with del SOD1-pYN3215 showed neither immunoreactivity nor SOD activity. The presence of mRNA transcribed from the mutant SOD1 was confirmed by RT-PCR-SSCP analysis. We conclude that the mutant SOD1 hardly exists in mammalian cells, although there is a slight possibility that the mutant SOD1 protein is not translated from the abnormal mRNA in mammalian cells.

We previously reported that the level of copper ions of the

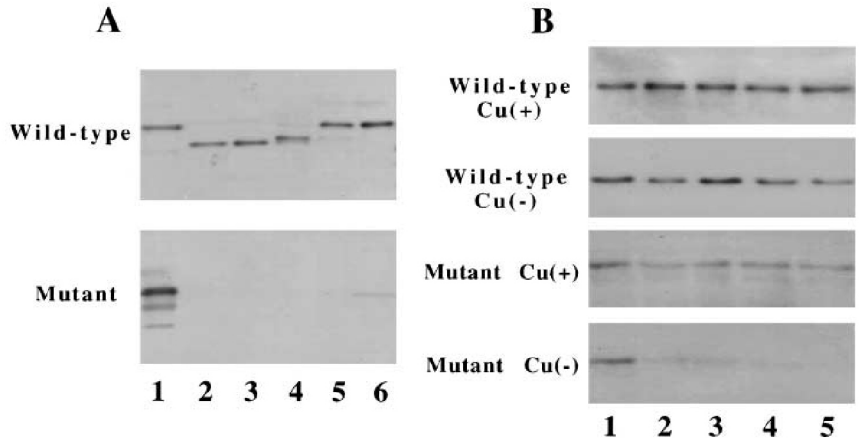

Fig. 3. Characterization of the mutant SOD1. A: Susceptibility of the SOD1s to proteolysis. The purified fusion SOD1s were cleaved with factor Xa with proteinase K at final concentrations of 10 μg/ml, 1 μg/ml, 100 ng/ml, 10 ng/ml, or 1 ng/ml at 22°C for 4 h. The reaction mixtures were separated on SDS-PAGE. In the mutant SOD1, each sample was applied on the gel at three-fold the wild-type SOD1 (wild type: 1 μg proteins and mutant: 3 μg proteins). The SOD1s were detected by Western blot analysis. Lanes: 1, proteinase K (none) as control; 2, 10 μg/ml; 3, 1 μg/ml; 4, 100 ng/ml; 5, 10 ng/ml; 6, 1 ng/ml of proteinase K. B: Stability of the SOD1s with or without copper ions. The separated SOD1s were incubated with or without 1 mM $CuSO_4$ at 22°C. Once in 6 h, mixtures were partly held and treated with sample buffer for SDS-PAGE. In the mutant SOD1, each sample was applied on the gel at three-fold the wild-type SOD1 as in A (wild type: 1 μg proteins and mutant: 3 μg proteins). Then each sample was separated on SDS-PAGE. Detection of SOD1s was performed by Western blot analysis. All experiments were performed at least three times and the same results were confirmed. Lanes: 1, 0 h (after factor Xa cleavage for 4 h); 2, 6 h; 3, 12 h; 4, 18 h; 5, 24 h incubation. The immunoreactivity of the mutant SOD1 disappeared during 24-h incubation and the presence of copper ions partly prevented this decrease. Because the SOD1s were purified using affinity chromatography, this experiment was carried out under proteinase free condition. This decrease of the SOD1 immunoreactivity may come from spontaneous conformational alteration of the mutant SOD1.

brain from one patient belonging to this FALS pedigree was about 1.9 times higher than in the control subjects [23]. Copper ions have been reported to promote lipid peroxidation [24] and to catalyze formation of the highly reactive hydroxyl radical (·OH) from H_2O_2 [25]. The mixture of copper ions with H_2O_2 promotes site specific DNA damage [26,27]. Cytotoxic activity of copper ions was also reported in cell cultures [28–30]. It has been reported that the SOD1 plays a role in intracellular copper buffering: overexpression of SOD1 enhanced copper resistance, whereas a deletion of SOD1 conferred an increased sensitivity toward copper ions in *Saccharomyces cerevisiae*. [28]. Our results seem to suggest that the mutant SOD1 itself can not be harmful to motor neurons. The metabolites of the mutant protein, including copper

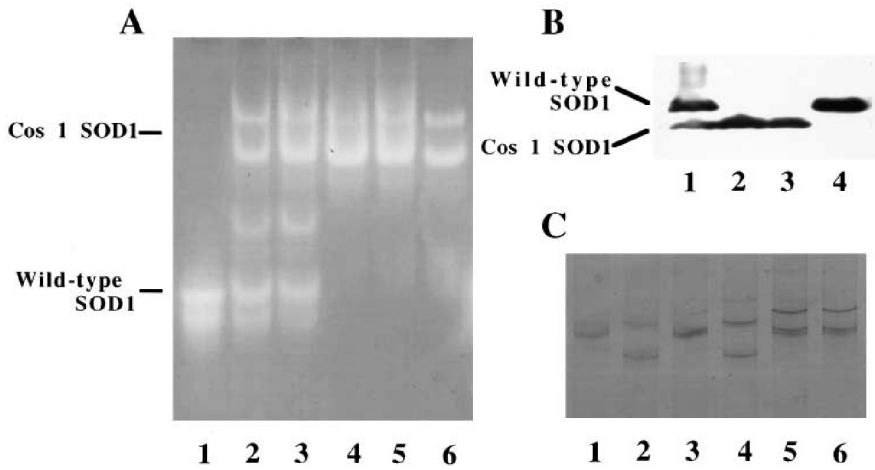

Fig. 4. Expression in Cos1 cells. A: Activity staining. The wild-type and the mutant SOD1s were expressed in Cos1 cells as described in Section 2. Samples were separated on native PAGE. 20 μg protein extracts were applied in each lane. Lanes: 1, human erythrocyte SOD1; 2–4, cells transfected with the SOD1 cDNA as follows: 2, wild type; 3, wild type and mutant; 4, mutant; 5, cells transfected with vector without SOD1; 6, untransfected cells. No activities were identified in Cos1 cells transfected with del SOD1-pYN3215, except for Cos1 SOD1 (lane 4). While the heterodimeric SOD1s between the wild-type and the Cos1 SOD1 subunits (lane 2) were seen, no activity suggesting a heterodimer between either the wild-type and mutant subunits or the mutant and the Cos1 SOD1 subunits were observed (lanes 3, 4). B: Western blot analysis. Each sample (30 μg protein extracts) was electrophoretically separated on SDS-PAGE. Lanes: 1, cells transfected with the wild-type cDNA; 2, cells transfected with the mutant cDNA; 3, untransfected cells; 4, human erythrocyte SOD1. C: RT-PCR-SSCP analysis. The analysis was performed as described in Section 2. Lanes: 1, PCR products from the wild-type cDNA; 2, PCR products from the mutant cDNA; 3–5, RT-PCR products from cells transfected with the cDNA as follows: 3, the wild type; 4, the mutant; 5, vector without SOD1; 6, RT-PCR products from untransfected cells. In lanes 5 and 6, RT-PCR products were applied at five-fold those of lanes 1–4, because these signals of PCR products were weaker than those of lanes 1–4.

ions, may play an important role in the neuronal death in FALS. At least it appeared so in this pedigree.

Acknowledgements: We thank Prof. K. Sato for providing human SOD1 cDNA and pYN3215, and for kind suggestions. This work was partially supported by the Research Committee of CNS Degenerative Diseases, the Ministry of Health and Welfare of Japan, and by the Nakabayashi Trust for ALS Research.

References

[1] Oppenheimer, D.R. and Esiri, M.M. (1992) in: Greenfield's Neuropathology (Adams, J.H. and Duchen, L.W., Eds.) pp. 988–1045, Oxford University Press, New York.
[2] Brown, R.H., Jr. (1995) Cell 80, 687–692.
[3] Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S.M., Berger, R., Tanzi, R.E., Halperin, J.J., Herzfeldt, B., Van den Bergh, R., Hung, W.Y., Bird, T., Deng, G., Mulder, D.W., Smyth, C., Laing, N.G., Soriano, E., Pericak-Vance, M.A., Haines, J., Rouleau, G.A., Gusella, J.S., Horvitz, H.R. and Brown, R.H., Jr. (1993) Nature 362, 59–62.
[4] Pramatarova, A., Goto, J., Nanba, E., Nakashima, K., Takahashi, K., Takagi, A., Kanazawa, I., Figlewicz, D.A. and Rouleau, G.A. (1994) Hum. Mol. Genet. 3, 2061–2062.
[5] Takahashi, K., Nakamura, H. and Okada, E. (1972) Arch. Neurol. 27, 292–299.
[6] Deng, H.X., Hentati, A., Tainer, J.A., Iqbal, Z., Cayabyab, A., Hung, W.Y., Getzoff, E.D., Hu, P., Herzfeldt, B., Roos, R.P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H.E., Ahmed, A., Roses, A.D., Hallewell, R.A., Pericak-Vance, M.A. and Siddique, T. (1993) Science 261, 1047–1051.
[7] Nakashima, K., Watanabe, Y., Kuno, N., Nanba, E. and Takahashi, K. (1995) Neurology 45, 1019–1020.
[8] Watanabe, Y., Kono, Y., Nanba, E., Nakashima, K. and Takahashi, K. (1996) in: Free Radicals in Brain Physiology and Disorders (Packer, L., Hiramatsu, M. and Yoshikawa, T., Eds.) pp. 371–376, Academic Press, Orlando, FL.
[9] Barettino, D., Feigenbutz, M., Valcárcel, R. and Stunnenberg, H.G. (1994) Nucleic Acids Res. 22, 541–542.
[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Melecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[11] Nakabeppu, Y., Oda, S. and Sekiguchi, M. (1993) Mol. Cell. Biol. 13, 4157–4166.
[12] Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752.
[13] Bjerrum, O.J. and Schafer-Nielsen, C. (1986) in: Electrophoresis '86: Proceedings of the Fifth Meeting of the International Electrophoresis Society, London, 1986 (Dunn, M.J., Ed.) pp. 315–327, Verlag Chemie, Weinheim.
[14] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
[15] Levanon, D., Lieman-Hurwitz, J., Dafni, N., Wigderson, M., Sherman, L., Bernstein, Y., Laver-Rudich, Z., Danciger, E., Stein, O. and Groner, Y. (1985) EMBO J. 4, 77–84.
[16] Yuasa, I., Kofler, A., Braun, A., Umetsu, K., Bichlmaier, R., Kammerer, S. and Cleve, H. (1995) Hum. Genet. 95, 507–512.
[17] Gurney, M.E., Pu, H., Chiu, A.Y., Chiu, D.C.M., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y.W., Deng, H.X., Chen, W., Zhai, P., Sufit, R.L. and Siddique, T. (1994) Science 264, 1772–1775.
[18] Dal Canto, M.C. and Gurney, M.E. (1995) Brain Res. 676, 25–40.
[19] Omar, B.A., Gad, N.M., Jordan, M.C., Striplin, S.P., Russel, W.J., Downey, J.M. and McCord, J.M. (1990) Free Radical Biol. Med. 9, 465–471.
[20] Amstad, P., Peskin, A., Shah, G., Mirault, M.E., Moret, R., Zbinden, I. and Cerutti, P. (1991) Biochemistry 30, 9305–9313.
[21] Troy, C.M. and Shelanski, M.L. (1994) Proc. Natl. Acad. Sci. USA 91, 6384–6387.
[22] Reaume, A.G., Elliott, J.L., Hoffman, E.K., Kowall, N.W., Ferrante, R.J., Siwek, D.F., Wilcox, H.M., Flood, D.G., Flint Beal, M., Brown, R.H., Jr., Scott, R.W. and Snider, W.D. (1996) Nature Genet. 13, 43–47.
[23] Nakashima, K., Watanabe, Y., Kono, Y. and Nanba, E. (1995) in: Annual Report of the Research Committee of CNS Degenerative Disease, The Ministry of Health and Welfare of Japan (in press).
[24] Hochstein, P., Kumar, K.S. and Forman, S.J. (1980) Ann. NY Acad. Sci. 355, 240–248.
[25] Aruoma, O.I., Halliwell, B., Gajewski, E. and Dizdaroglu, M. (1991) Biochem. J. 273, 601–604.
[26] Tachon, P. (1990) Free Radical Res. Commun. 9, 39–47.
[27] Spencer, J.P.E., Jenner, A., Aruoma, O.I., Evans, P.J., Kaur, H., Dexter, D.T., Jenner, P., Lees, A.J., Marsden, D.C. and Halliwell, B. (1994) FEBS Lett. 353, 246–250.
[28] Reiners, J., Jr. and Colby, A.B. (1988) Carcinogenesis 9, 629–632.
[29] Bumgardner, J.D., Lucas, L.C. and Tilden, A.B. (1989) J. Biomed. Mater. Res. 23, 1103–1114.
[30] Cullotta, V.C., Joh, H.D., Lin, S.J., Slekar, K.H. and Strain, J. (1995) J. Biol. Chem. 270, 29991–29997.