Enhanced expression of manganese-dependent superoxide dismutase in human and sheep *CLN6* tissues

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Neuronal ceroid lipofuscinosis type 6 and its sheep model (OCL6) are lysosomal storage disorders caused by mutations in the *CLN6* gene product of unknown function. It has been proposed that mitochondrial dysfunction, including defects in mitochondrial protein degradation, organelle enlargement and functional changes in oxidative phosphorylation, may contribute to the disease pathology. To further explore the disease mechanisms underlying *CLN6*, protein expression was compared between normal and affected tissues. Using two-dimensional electrophoretic separation of proteins, MS and immunoblotting, MnSOD (manganese-dependent superoxide dismutase) was found to be significantly and specifically increased in fibroblasts and brain extracts of both human and sheep affected with *CLN6*. Both the activity and expression of MnSOD mRNA were enhanced in affected fibroblasts. Confocal

fluorescence microscopy and immunohistochemical studies revealed the presence of MnSOD in mitochondria of *CLN6* fibroblasts and in *CLN6* brain sections within both neurons and hypertrophic astrocytes. These data suggest that oxidative stress and/or the production of pro-inflammatory cytokines are characteristic features of human and sheep *CLN6*, resulting in elevated expression of MnSOD, which may be important for diagnostic purposes.

Key words: fluorescence microscopy, lysosomal storage disorder, manganese-dependent superoxide dismutase (MnSOD), neurodegeneration, neuronal ceroid lipofuscinosis, oxidative stress.

INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs, Batten disease) are a group of fatal autosomal recessive neurodegenerative diseases with infantile, juvenile or adult onset. These diseases are characterized by massive lysosomal storage of aggregated protein in neurons and a wide variety of extraneuronal cells. Clinical features include visual failure, seizures, progressive mental and motor deterioration and behavioural changes. To date, at least seven distinct NCLs are known, each caused by a range of mutations in separate genes [1]. Two of these genes encode soluble lysosomal enzymes, targeted to lysosomes in a mannose-6-phosphate-dependent manner (*CLN1* and *CLN2*) [2,3]. Four other genes encode proteins of unknown function, predicted to be transmembrane proteins (*CLN3*, *CLN5*, *CLN6* and *CLN8*) [4–8]. Defects in *CLN2*, *CLN3*, *CLN5*, *CLN6* and *CLN8* are all associated with the specific lysosomal accumulation of subunit c of mitochondrial ATP synthase [9–12]. Mutations in the lysosomal protease cathepsin D cause another NCL, so far found only in sheep where it causes congenital disease, that is replicated in cathepsin D null mutant mice [13,14].

Recently, the *CLN6* gene was identified as the gene locus FLJ20561, which is localized on human chromosome 15q21-23 [6,7]. It contains 7 exons, encoding a predicted 311-amino-acid transmembrane protein of unknown function. Naturally occurring *CLN6* has also been described in New Zealand South Hampshire sheep and *nclf* mice. Positional cloning studies have localized the sheep gene to a region on sheep chromosome 7 that is syntenic with the 15q21-23 human region, and placed it between

the markers used to localize the human gene [15]. A form in Australian Merino sheep is similar to the New Zealand South Hampshire form and may be caused by the same mutation [16]. An *nclf* mouse disease specific mutation has been identified in the murine homologue of FLJ20561 [6].

Affected South Hampshire and Merino sheep (OCL6) develop behavioural symptoms and visual problems between 10 and 14 months of age, closely resembling the clinical presentation of human *CLN6* [16,17]. Disease progression results in blindness, most likely due to atrophy of the occipital cortex and loss of photoreceptors in the retina [18]. These sheep provide the best described animal model of the NCLs. Specific lysosomal storage of subunit c of mitochondrial ATP synthase was first observed in these sheep, and subsequently in the *CLN2*, *CLN3*, *CLN5* and *CLN8* forms [9,19,20].

In the present study, detergent soluble proteins extracted from normal and affected sheep brains, and human and sheep fibroblasts, have been compared by two-dimensional electrophoresis and MS. Up-regulation of MnSOD (manganese-dependent superoxide dismutase; EC 1.15.1.1) in affected tissues was the most striking finding, and was confirmed by immunohistochemical studies of *CLN6* tissues. MnSOD is normally located in the mitochondrial matrix and is considered to be one of the most important radical scavenger proteins [21], regulated in response to ROS (reactive oxygen species) [22] and a variety of pro-inflammatory mediators [23,24]. These data suggest that oxidative stress and/or inflammatory processes are characteristic features of human and sheep *CLN6* and form part of the pathogenetic mechanisms that occur in this disorder.

Abbreviations used: CNS, central nervous system; COX, cytochrome c oxidase; 2-D, two-dimensional; GPDH, glycerol-3-phosphate dehydrogenase; IEF, isoelectric focusing; lamp1, lysosomal associated membrane protein; ML fraction, mitochondrial/lysosomal fraction; MnSOD, manganese-dependent superoxide dismutase; NCL, neuronal ceroid lipofuscinosis; PDI, protein disulphide isomerase; ROS, reactive oxygen species; RT-PCR, reverse transcriptase PCR.

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EXPERIMENTAL

Materials

IEF (isoelectric focusing) stripes, Dry Strip cover Fluid and Rainbow™-coloured protein molecular-mass marker were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Dulbecco's modified Eagle's medium and antibiotics (penicillin/streptomycin) were purchased from Gibco Life Technologies (Karlsruhe, Germany). Protease inhibitor cocktail (P-2714) was obtained from Sigma; Bradford reagent and Trans-Blot nitrocellulose membrane (0.2 µm) were obtained from Bio-Rad (Munich, Germany). First Strand cDNA Synthesis Kit for RT (reverse transcriptase)-PCR was purchased from PerkinElmer (Branchburg, NJ, U.S.A.), LightCycler-FastStart cDNA Master SYBR Green I from Roche (Mannheim, Germany) and Vectastain ABC kit from Vector (Burlingame, CA, U.S.A.).

Cells and tissues

Fibroblasts from human CLN6 patients (numbers 1, 2 and 3) and brain autopsy material from a CLN5 (aged 22 years) and four CLN6 cases (for Western blotting, the brain material was obtained from a 8-year-old CLN6 patient) had been obtained previously for diagnostic purposes, with informed consent and with prior approval of the institutional review board at the Institute of Inherited Metabolic Disorders, Charles University, Prague. The diagnoses were based on the clinical phenotype, electrophysiology and on ultrastructural criteria [25]. Brain tissues were obtained at autopsies of 6-, 9-, 20- and 23-month-old affected South Hampshire sheep and age-matched controls. Fibroblast cultures were prepared from the skin of 12- and 27-month-old control and affected sheep. A second series of sheep CNS (central nervous system) autopsy material was obtained from affected Australian Merino sheep aged between 13 and 17 months, and a group of age matched clinically normal Australian Merinos [16]. Mice deficient for cathepsin D (CtsD^{-/-}) [14] and control animals at postnatal day 23 were kindly provided by Dr Paul Saftig (Institute of Biochemistry, University of Kiel, Germany). The tissues were immediately frozen and kept at -80 °C until used or fixed and processed for histology as described below. Human and sheep fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum and penicillin/streptomycin in a humidified atmosphere containing 5 % CO₂/95 % O₂ at 37 °C.

Antisera

The polyclonal antibody against human MnSOD was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.), and the monoclonal antibody against COX (cytochrome *c* oxidase; subunit I) was from Molecular Probes (Leiden, The Netherlands). The monoclonal anti-lamp1 (lysosome-associated membrane protein 1) antibody H4A3, developed by J. T. August and J. E. K. Hildreth, was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by Department of Biological Sciences, University of Iowa, Iowa City, IA, U.S.A. The antiserum against PDI (protein disulphide isomerase) was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Goat anti-rabbit and anti-mouse secondary antibodies conjugated to FITC and Cy3 respectively were obtained from Sigma. The antibody against the mitochondrial GPDH (glycerol-3-phosphate dehydrogenase) was kindly provided by Dr J. M. Weitzel (Institute of Medical Biochemistry and Molecular Biology, University of Hamburg, Germany).

Preparation of tissue extracts

Fractions enriched in mitochondria and lysosomes (ML fraction) were prepared from eight plates (10-cm diameter) of confluent human or sheep fibroblasts, as described previously [26]. Brain extracts were prepared from the grey matter dissected from human and sheep cerebral cortices, and then homogenized in water containing a protease inhibitor cocktail using a Tissue Tearer (Dremel, Model 985-370; Biospec Products), followed by sonification (Bandelin Sonoplus HD60, Berlin, Germany) for 1 min at maximum power on ice. Protein concentration of the samples were determined by the Bradford protein assay with BSA as the standard.

IEF

Brain extracts or ML fractions (0.15 mg protein) were diluted 1:1 in buffer A (8 M urea/4 % Triton X-100/40 mM Tris/HCl, pH 8.6) and incubated for 30 min at 22 °C. After centrifugation the samples were processed and loaded on to IEF stripes, pH 3–10, according to manufacturer's instructions [27], using Multiphore II (Amersham Pharmacia Biotech). IEF was stopped once it reached 20 kVh.

SDS/PAGE and Western-blotting analysis

ML fractions and brain extracts were separated by SDS/PAGE (10–15 % acrylamide as indicated), and either stained by silver or transferred on to nitrocellulose membrane for 90 min at 900 mA (Hoefer Pharmacia Biotech Inc., San Francisco, CA, U.S.A.). Following blocking (5 % milk powder in 10 mM PBS, pH 7.4, 0.05 % Tween), the membrane was incubated with anti-MnSOD antibody (1:500) in blocking solution for 1 h. After washing, the immunoreactive bands were visualized after incubation with goat anti-rabbit IgG-conjugated to horseradish peroxidase (1:10 000) and enhanced chemiluminescence (Pierce). Band intensities were quantified by densitometric scanning (Hewlett-Packard Scan Jet 4c/T using Advance Image Data Analyzer software; Raytest, Straubenhardt, Germany).

MS

Excised silver-stained polypeptide spots were digested with trypsin and the tryptic peptides were analysed by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS (Megamedics, Wedel, Germany). The polypeptides were identified by comparing the peptide maps with the Swiss-Prot.010501 database using MS-Fit/ProteinProspector software (<http://prospector.ucsf.edu>).

MnSOD activity

The activity of MnSOD in ML fractions of human and sheep fibroblasts was measured photometrically, as described previously [28]. For the in-gel assays, 30 µg of the different ML fractions were analysed by SDS/PAGE under non-reducing conditions without prior boiling of the samples. The gels were shaken in 2.5 % Triton X-100 for renaturation of the proteins and further processed, as described previously [29]. In both cases, 0.33 M sodium cyanide was added to inhibit the copper/zinc-dependent SOD and the MnSOD activity was determined as a function of Nitro Blue Tetrazolium inhibition.

Immunofluorescence microscopy

Human fibroblasts were plated on to poly(L-lysine)-coated 12-mm glass cover slips. After 24 h, the cells were fixed with 3 % paraformaldehyde for 30 min at 22 °C and washed with 10 mM

PBS, pH 7.4. The cells were permeabilized by adding ice-cold methanol for 6 min on ice. After incubation for 15 min at 22 °C with PBS containing 1 % BSA (blocking solution), cells were co-incubated with rabbit anti-MnSOD (1:100) antibody and either mouse anti-COX (mitochondria; 1:5) antibody, mouse anti-lamp 1 (lysosomes; 1:200) antibody, or mouse anti-PDI (endoplasmic reticulum; 1:300) antibody in blocking solution at 22 °C for 2 h. After washes, the anti-rabbit and anti-mouse fluorescence-conjugated IgG antibody (1:2000) in blocking solution were added and incubated for 1 h in the dark. After further washing steps, the cover slips were mounted on to object glasses with glycerol gelatin. The extent of co-localization of MnSOD with characteristic markers of organelle phenotype was determined by dual channel laser confocal microscopy (LSM 510; Zeiss, Jena, Germany) set at excitation wavelengths 488 nm (FITC) and 552 nm (Cy3), and emission at 575 nm (FITC) and 570 nm (Cy3).

Real-time RT-PCR

Total RNA was isolated from cultured human fibroblasts by NucleoSpin RNAII from Macherey-Nagel (Düren, Germany), according to the manufacturer's instructions. RNA (1 µg) was reverse transcribed in a total volume of 20 µl using the Perkin-Elmer RNA PCR Kit (Branchburg, NJ, U.S.A.). For amplification of β -actin and MnSOD, parts of the cDNA products (2 µl) were subjected to real-time PCR analysis on a Light Cycler Instrument (Roche, Mannheim, Germany). MnSOD cDNA was amplified with primers MnSOD_{for} (5'-AGCCTCCCCGACCTGCCCTAC-3') and MnSOD_{rev} (5'-TGAAACCAAGCCAACCCCAACCTGAG-3'). PCR was performed in 50 cycles of 10 s at 95 °C (denaturation), 10 s at 65 °C (annealing) and 20 s at 72 °C (extension), with fluorescence detection at 89 °C after each cycle by LightCycler Instrument and software package. β -Actin, which was used as internal control, was amplified with primers β -actin_{for} (5'-GCGGGAAATCGTGCGTGACATT-3') and β -actin_{rev} (5'-GATGGAGTTGAAGGTAGTTTCGTG-3'). The comparative threshold method was used to determine the relative expression level of MnSOD mRNA.

MnSOD immunohistochemistry

Sections of control and affected sheep brains were fixed in 4 % paraformaldehyde or 10 % neutral buffered formalin, embedded in paraffin and sectioned at 4 µm or 10 µm. For MnSOD staining, the paraffin was removed from the sections, and the sections were heated in a microwave 4 times for 5 min in 10 mM citrate buffer, pH 6. The endogenous peroxidase activity was blocked by incubation in methanol containing 1.6 % hydrogen peroxide at 22 °C for 30 min. The sections were then incubated with 5 % BSA in PBS at 22 °C for 30 min, followed by incubation with rabbit anti-(human MnSOD) antibody (1:200 in PBS containing 1 % BSA) overnight at 4 °C. After rinsing, sections were incubated for 1 h in biotinylated goat anti-rabbit (1:200 in PBS containing 1 % BSA) antibody, rinsed again and incubated in Vectastain ABC reagent according to manufacturer's instructions (Vector Laboratories). Immunoreactivity was subsequently visualized using either 3-amino-9-ethylcarbazole or diaminobenzidine in the presence of H₂O₂. The slides were rinsed again and counterstained with haematoxylin, prior to coverslipping in the normal manner. Pre-immune serum or PBS alone were used instead of primary antibody to verify the specificity of the antisera. The slides were counterstained with haematoxylin and were viewed on a Zeiss Axioskop2 MOT. Images were processed by Zeiss AxioVision 3.0 software.

Figure 1 2-D separation of proteins of fibroblasts from control and CLN6 patient

Extracts of ML fractions from control (Co) and affected (CLN6) human fibroblasts (150 µg protein) were separated by 2-D electrophoresis on a 12.5 % polyacrylamide gel under reducing conditions, and the gel was silver stained. The arrows indicate the position of proteins altered in their expression. The protein spot (number 1) was excised and analysed by MS. The positions of the molecular-mass markers (in kDa) are given. The preparation of extracts and the 2-D separation were carried out in parallel under identical conditions and repeated several times with similar results.

RESULTS

MnSOD protein expression in CLN6 and OCL6

Separation of proteins from fractions enriched in ML fractions from control and fibroblasts of a CLN6 patient by 2-D (two-dimensional) gel electrophoresis followed by silver staining, revealed several differently expressed proteins (Figure 1). A polypeptide spot of approx. 24.5 kDa (pI 7.7) in cell extracts of the CLN6 patient, which was not found in extracts from normal control cells, was excised and identified as mitochondrial MnSOD by MS. Its identity was confirmed by Western blotting using antibodies against human MnSOD (Figure 2A). Increased amounts of immunoreactive MnSOD (5- to 6-fold increase as shown by densitometry) were found in three fibroblast cell lines of non-related CLN6 patients (Figure 2B). When extracts from brain material of another human CLN6 patient were analysed by Western blotting and densitometry, the MnSOD immunoreactive material was 7-fold more than was found in a neurologically normal control brain extract of similar age (Figure 3A). In both samples equal amounts of another mitochondrial protein, GPDH were observed (Figure 3A).

To examine the specificity of MnSOD accumulation in CLN6, extracts from fibroblasts of CLN2 and CLN3 patients were tested by immunoblotting. Similar amounts of MnSOD immunoreactive material were detected in these cells compared with control fibroblasts (Figures 3B and 3C). The amounts of MnSOD in brain extracts of cathepsin D-deficient mice, an animal model for another NCL form, were also not altered compared with control mouse brain (Figure 3D). MnSOD immunoreactive material was strongly increased in extracts of ML fractions of fibroblasts of OCL6-affected sheep (Figure 4A). When extracts prepared from brain cortex of control and OCL6 sheep of 6, 9 and 20 months of age were analysed by Western blotting, the MnSOD immunoreactive material was constantly increased in the affected OCL6 brain samples (Figure 4B). The 2-D immunoblot of OCL6 brain extracts also showed a second MnSOD isoform which has a pI of approx. 7.5 (Figure 4C). Densitometric evaluation of one-dimensional and 2-D immunoblots revealed about 5- to 21-fold more immunoreactive material in OCL6 brain and fibroblast subcellular fractions respectively compared with control levels.

Figure 2 MnSOD immunoblot analysis in extracts of fibroblasts of CLN6 patients

(A) Proteins (150 μ g) of ML fractions prepared from control (Co) and CLN6 fibroblasts were separated by 2-D gel electrophoresis (12.5 % acrylamide), transferred on to nitrocellulose membranes and analysed with an antibody against MnSOD. Arrows indicate the position of MnSOD (absent in the control). (B) Proteins of ML fractions (30 μ g) of control (patient number 4) and three non-related CLN6 patients (numbers 1, 2 and 3) were separated by SDS/PAGE (10 % acrylamide) and analysed by Western blotting with antibodies against MnSOD or lamp1. Immunoreactive bands were visualized by peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

Figure 3 CLN6-specific expression of MnSOD

Protein (30 μ g) of control (Co) and CLN6 brain homogenate (A), or of ML fractions prepared from fibroblasts of control, CLN2 and CLN6 patients (B), control, CLN3 and CLN6 patients (C) or of control (+/+) and cathepsin D (CtsD)-deficient (-/-) mouse brain homogenates (D) were separated by SDS/PAGE (10 % acrylamide) and analysed by Western blotting with antibodies against MnSOD or GPDH.

Figure 4 MnSOD expression in fibroblasts and brain cortex of OCL6 sheep

(A) Extracts from ML fractions of control (Co) and affected (OCL6) sheep fibroblasts (30 μ g protein) or (B) brain cortex extracts (30 μ g protein) prepared from control (Co) and OCL6 sheep at the age of 6, 9, and 20 months were separated by SDS/PAGE (10 % acrylamide) and analysed by immunoblotting with antibodies against MnSOD. (C) Extracts (150 μ g protein) prepared from brain cortex of control and OCL6 sheep at the age of 23 months, were separated by 2-D gel electrophoresis (12.5 % acrylamide), transferred on to nitrocellulose membranes and analysed with an antibody against MnSOD.

Figure 5 MnSOD activity in human and sheep cultured fibroblasts

Protein (30 μ g) of ML fractions from control and CLN6 patients, and control and OCL6 sheep fibroblasts, were separated by SDS/PAGE under non-denaturing conditions. Following electrophoresis, the gel was incubated in Nitro Blue Tetrazolium, as described in the Experimental section, and illuminated for 10 min. A representative experiment of one out of three, with similar results, is shown.

MnSOD activity

When extracts of fibroblasts from CLN6 patients or OCL6 sheep were tested for MnSOD activity using an in-gel Nitro Blue Tetrazolium staining assay, a prominent band was observed in both affected cell lines, which was weakly detectable in control cell extracts (Figure 5). Measurements of MnSOD activity by a photometric assay revealed 2- to 3.8-fold increased activities in CLN6 and OCL6 cell material respectively (results not shown).

MnSOD mRNA expression

MnSOD mRNA levels were estimated by real-time PCR to examine whether these increases in MnSOD protein and enzymic activities are due to increased transcription. Measurements from

Figure 6 Real-time RT-PCR measurements of MnSOD

Total RNA was isolated from control (Co; patient number 4) and from fibroblasts of three CLN6 patients (1, 2 and 3). Quantitative PCR was performed as described in the Experimental section. The amounts of MnSOD mRNA were expressed in relation to the β -actin mRNA level which was estimated to be 479 ± 7 (4), 583 ± 7 (1), 852 ± 17 (2) and 500 ± 80 (3) fg/100 ng of total RNA. The results are expressed as the means $\pm$ S.D. from three independent experiments performed in triplicate. ** Statistically significant differences ($P < 0.001$) between Co (4) and CLN6 patients (1 and 2); * $P < 0.01$ between Co and CLN6 patient 3.

fibroblasts from three different CLN6 patients showed that the amount of MnSOD mRNA was 3- to 7-fold more than in control cells, using β -actin mRNA levels as an internal standard (Figure 6). These data indicate that the elevated amounts of MnSOD in CLN6 fibroblasts are accompanied by increased transcription.

Intracellular localization of MnSOD

Lysosomal accumulation of subunit c of mitochondrial ATP synthase has been described in many forms of NCL [9–12]. In order to examine whether MnSOD also accumulates in lysosomes, the intracellular localization of MnSOD was studied by dual channel confocal microscopy. As shown in Figure 7, MnSOD co-localizes with mitochondrial COX, indicated by the yellow colour in merged images (Figures 7C and 7F). In contrast, MnSOD did not co-localize with either the lysosomal membrane marker lamp1 (Figures 7I and 7L) or with the endoplasmic reticulum marker, PDI (results not shown). There were no differences in the subcellular localization of MnSOD in CLN6 and control cells.

MnSOD immunoreactivity in brain

Evaluation of MnSOD immunoreactivity in control frontal cortex revealed granular cytoplasmic staining compatible with a mitochondrial localization of the enzyme (Figures 8A and 8C). MnSOD immunoreactivity was most prominent in neurons, especially in large neurons in the deep cortical layers, although MnSOD positive glial cells were also observed, albeit less frequently. Within white matter, glial staining, as well as punctate staining of the neuropil, was observed. MnSOD staining was much more pronounced in sections from CLN6 patients than from controls (Figures 8B and 8D). Hypertrophic astrocytes in the cerebral cortex stained very intensely. In contrast, the remaining neurons that contained abundant storage material showed relatively little MnSOD staining. Storage material itself exhibited no immunoreactivity. In many instances the MnSOD immunoreactive granules were pushed to the margins of individual neurons by the accumulated storage material. At the pontine level, the reactive astrocytes of corticospinal tracts showed most prominent staining in the affected brains (Figure 8F), whereas no similar staining of astrocytes was observed in the pons of normal controls or in a CLN5 patient (Figure 8E).

The differences in MnSOD staining between the control and affected sheep brains were less dramatic. Neurons were prominently

stained in both normal and affected sheep, particularly those in the deep layers of the cortex, and in a variety of subcortical nuclei (Figures 8G–8J). The distribution of immunoreactivity appeared essentially the same in brains from both the affected South Hampshire (Figures 8G and 8H) and Merino sheep (Figures 8I and 8J). The marginalization of positive punctate staining within neurons was pronounced in both affected sheep models, which also exhibited more intense punctate staining distributed through the neuropil than controls. Individual neurons exhibited a variety of staining intensities in different regions, with immunopositive granules in the large neurons in the deep layers of the cerebral cortex, as well as in pons, appearing more prominent in the affected sheep than in the controls. Occasional MnSOD positive cells with astrocyte morphology were observed in affected sheep, but at a far lower frequency than in CLN6 human tissue.

DISCUSSION

The present study shows that one of the proteins altered in their expression, the mitochondrial enzyme, MnSOD, is up-regulated in brain and fibroblasts of CLN6 patients, and in the sheep models of this disease. The altered amounts of MnSOD were initially detected by 2-D electrophoresis and MS. Up-regulation was confirmed by immunologic techniques and elevated MnSOD activity. The results of real-time RT-PCR suggest that this up-regulation of MnSOD is most likely due to an increase in transcription.

Human MnSOD (or SOD2) is a nuclear-encoded antioxidant enzyme containing four identical subunits and one Mn^{2+} per subunit. MnSOD is a member of the primary antioxidant enzyme family that plays an important role in protecting cells from oxidative stress. Other enzymes in this family are a homodimeric copper zinc SOD (CuZnSOD or SOD1), found primarily in the cytosol, and a homotetrameric, glycosylated extracellular CuZnSOD (SOD3) [30]. The known function of SOD is to catalyse the dismutation of superoxide ($O_2^{\cdot-}$) radicals into H_2O_2 , which is then converted into water by catalase or glutathione peroxidases. Superoxide anions as well as other ROS, such as hydroxyl radicals ($OH^{\cdot}$) and peroxynitrite ($ONOO^-$), have been implicated in a variety of neurodegenerative disorders including Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease [31,32]. The mitochondrial production of ROS may result in oxidation of mitochondrial lipids, proteins and DNA [33], and lead to the activation of the mitochondrial permeability transition pore and the release of cytochrome c, pro-caspases and apoptosis-initiating factors [34,35]. Leakage of electrons in the mitochondrial electron transport chain is a major source of ROS [36]. The expression of MnSOD is selectively inducible by oxidative stress provoked by H_2O_2 and by pro-inflammatory mediators, such as interleukin-1, tumour necrosis factor α and γ -interferon [22–24].

The accumulation of MnSOD in fibroblasts and brain tissue of OCL6 sheep and CLN6 patients suggests that the induction of MnSOD is an adaptive response to oxidative stress and/or inflammation in CLN6. An inflammatory process in the CNS is believed to play an important role in apoptotic cell death in a number of neurodegenerative diseases [37,38]. This inflammatory response is mediated by microglia, and has been found to be activated in the CNS of patients and animal models with lysosomal storage disorders, such as Sandhoff disease or cathepsin D deficiency [39,40], triggering acute neurodegeneration through the expression of neurotoxic cytokines.

Immunohistochemical staining of CLN6 tissues showed increased MnSOD immunoreactivity in agreement with the results

Figure 7 Immunofluorescence localization of MnSOD in fibroblasts of CLN6 patients

Fibroblasts from control (Co) and CLN6 patient were fixed, permeabilized and immunostained either with rabbit anti-MnSOD antibody, followed by goat anti-rabbit secondary antibody conjugated to FITC (green) or mouse anti-COX antibody, mouse anti-lamp1 antibody, then followed by goat anti-mouse secondary antibodies conjugated to Cy3 (red). The red and green channels were merged after adjustment of both fluorescence signals to similar levels.

of 2-D electrophoresis, Western blotting and mRNA quantification. Increased staining was particularly prominent in the affected human brain, where the numerous reactive astrocytes were strongly stained for MnSOD. Reactive changes in populations of astrocytes are a feature of many degenerative disorders and may serve as early indicator of damage or protective responses within affected areas. Our data suggest that these responses also include increased expression of MnSOD, which could already be detected in brain samples of 6-month-old OCL6 South Hampshire sheep. Whereas gross alterations are not observed within the first 4 months of age in OCL6 sheep, early loss of neurons affects most layers of the cortex accompanied by reactive astrocytosis between 2.5 and 5 months of age [41]. Thus at present it is unknown

whether the elevated MnSOD expression reflects an increased oxidative burden or an activation of glial cytokine production as a result of disease. The more mild immunohistochemical staining of MnSOD seen in the sheep sections, suggests the sheep disease as being less advanced clinically than the end-point disease for the human samples. Alternatively, it could be a consequence of a species difference or of different mutations in the *CLN6* (FLJ20561) gene in the human and sheep diseases.

The up-regulation of MnSOD observed in the present study appears to occur at the transcriptional level, as shown by the elevated MnSOD mRNA expression level in CLN6 fibroblasts. However, the regulation may also occur at the post-transcriptional level modulating the stability of the MnSOD mRNA [42].

Figure 8 Immunohistochemical staining of MnSOD in human and OCL6 sheep brain tissue

In human control frontal cortex (A), large neurons (marked by arrows) in the deep cortical layers contained prominent MnSOD immunoreactivity and showed uniformly spread immunopositive granules in the cell soma, better seen at higher magnification (C). Occasionally, darkly stained neurons (asterisks) were observed in control tissues. In the CLN6 frontal cortex (B), the most prominent staining was observed in the reactive astrocytes (arrowheads) throughout the cortex. The remaining neurons (arrows) in the deep cortical layers were loaded with storage material, which was not stained by MnSOD antiserum (D). Additionally, punctate staining of the neuropil was always observed in the affected individuals. In the cerebrospinal tracts at the pontine level, faint immunostaining of small cells with typical astrocytic morphology was observed in one CLN5 case studied (E). In contrast, very intense staining of astrocytes was observed in the cerebrospinal tracts of the one CLN6 case studied (F). Immunohistochemical staining of OCL6 brain tissue reveals elevated MnSOD expression in the cortex of affected South Hampshire (H) and affected Merino sheep (J) versus unaffected control sheep of either breed (G and I respectively). In South Hampshire sheep large neurons (arrows) in the deep layers of the frontal cortex exhibited modest granular staining (G). In contrast, in age-matched affected South Hampshire sheep, staining of the large neurons in the deep cortical layers was more intense (H). In the entorhinal cortex of affected Merino sheep, large neurons of lamina V also exhibited more intense MnSOD immunoreactivity (J) compared with age-matched control Merino sheep (I). In both breeds, within neurons with pronounced accumulation of storage material (arrowheads), MnSOD immunoreactive granular material was found more peripherally within the cytoplasm appearing pushed aside by the accumulated negatively stained storage material (H and J). In both breeds, a higher level of MnSOD immunoreactivity was also present within the neuropil of affected sheep (H and J) than in age-matched control sheep (G and I). The 3-amino-9-ethylcarbazole chromogen used to reveal that MnSOD immunoreactivity in South Hampshire tissue produces a less intense red–pink reaction product than the darker brown diaminobenzidine reaction product used to reveal MnSOD immunoreactivity in Merino tissue. Scale bars, 10 μ m.

Furthermore, post-translational modifications may be involved in the regulation of MnSOD. It has been reported that peroxynitrite (ONOO^-) is able to nitrate critical tyrosine residues in MnSOD, resulting in enzymic inactivation [43] and in a subsequent compensatory positive feedback mechanism [21]. It is possible that portions of cellular MnSOD are inactivated by nitration in CLN6 and OCL6 tissues. This would explain the two forms of MnSOD, of different pI, observed in the MnSOD immunoblot analysis of proteins from OCL6 brain, and differences between MnSOD activity and relative amounts of immunoreactive protein.

Subunit c of mitochondrial ATP synthase is the dominant component of lysosomal storage material in CLN2–8, OCL6 and in cathepsin D-deficient mice [9–12,14,44]. It is thought that the lysosomal accumulation found in different forms of NCL is the consequence of more general defects in lysosomal protein degradation. In contrast, the mitochondrial matrix protein MnSOD does not accumulate in lysosomes, but co-localizes with the mitochondrial marker cytochrome c oxidase, as shown in the present study by double immunofluorescence microscopy. Furthermore, MnSOD was not detected in isolated brain storage material from OCL6 (C. Heine and J. Tynnelä, unpublished work). In contrast with accumulation of subunit c of ATP synthase, accumulation of MnSOD appears to be specific to CLN6/OCL6. It was not detected in fibroblasts from CLN2 and CLN3 patients, in brain from a CLN5 patient, or in the brains of cathepsin D-deficient mice. Therefore, MnSOD might be used as a new diagnostic marker of this disease. Thus Western-blot analysis of skin fibroblasts could render time-consuming electron microscopy less necessary. However, further studies to clarify the function and the subcellular localization of the CLN6 protein are needed to explain the molecular link between increased MnSOD activity, lysosomal dysfunction, damage in mitochondria and the mutant CLN6 gene product.

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