

A mutation in the ovine cathepsin D gene causes a congenital lysosomal storage disease with profound neurodegeneration

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The neuronal ceroid lipofuscinoses (NCLs) constitute a group of neurodegenerative storage diseases characterized by progressive psychomotor retardation, blindness and premature death. Pathologically, there is accumulation of autofluorescent material in lysosome-derived organelles in a variety of cell types, but neurons in the central nervous system appear to be selectively affected and undergo progressive death. In this report we show that a novel form of NCL, congenital ovine NCL, is caused by a deficiency in the lysosomal aspartyl proteinase cathepsin D. A single nucleotide mutation in the cathepsin D gene results in conversion of an active site aspartate to asparagine, leading to production of an enzymatically inactive but stable protein. This results in severe cerebrocortical atrophy and early death, providing strong evidence for an important role of cathepsin D in neuronal development and/or homeostasis.

Keywords: aspartyl proteinase/Batten's disease/
cathepsin D/ceroid lipofuscinosis/neurodegeneration

Introduction

Neuronal ceroid lipofuscinoses (NCLs) are a group of inherited neurodegenerative storage diseases characterized by progressive psychomotor retardation, blindness and premature death (Rapola, 1993; Goebel and Sharp, 1998). Pathologically, there is accumulation of autofluorescent material in lysosome-derived organelles in a variety of cell types, but neurons in the central nervous system are most severely affected and undergo progressive death (Rapola, 1993; Goebel and Sharp, 1998) by an apoptotic pathway (Lane *et al.*, 1996). In early-onset forms of NCL, neuronal loss is accompanied by secondary loss of myelin and results in severe cerebral atrophy.

The human NCLs are classified according to the age of onset, clinical course, morphological findings and genetic basis. Five of the NCL genes have been identified and

sequenced including *CLN1* (OMIM 256730) (Vesa *et al.*, 1995), *CLN2* (OMIM 204500) (Sleat *et al.*, 1997), *CLN3* (OMIM 204200) (International Batten Disease Consortium, 1995), *CLN5* (OMIM 256731) (Savukoski *et al.*, 1998) and *CLN8* (OMIM 600143) (Ranta *et al.*, 1999). *CLN3*, *CLN5* and *CLN8* encode putative membrane proteins of unknown functions (Järvelä *et al.*, 1998; Savukoski *et al.*, 1998; Ranta *et al.*, 1999), while *CLN1* and *CLN2* encode the soluble lysosomal enzymes palmitoyl protein thioesterase 1 (Camp and Hofmann, 1993; Verkruse and Hofmann, 1996) and pepstatin-insensitive proteinase/tripeptidyl peptidase 1 (Sleat *et al.*, 1997; Vines and Warburton, 1999), respectively.

In this report, we show that a novel form of NCL, congenital ovine NCL (CONCL), is caused by a deficiency in the lysosomal aspartyl proteinase cathepsin D. A single nucleotide mutation in the cathepsin D gene leads to production of an inactive but stable protein. This results in extreme cerebrocortical atrophy and early death, providing strong evidence for an important role of cathepsin D in neuronal development and/or homeostasis.

Results

CONCL was observed in a flock of White Swedish Landrace sheep maintained on an experimental farm in Northern Sweden (Järplid and Haltia, 1993). Transmission was consistent with an autosomal recessive mode of inheritance: of 25 sheep studied, six (born from apparently normal parents) were affected and 19 were apparently normal. The newborn affected lambs were weak, trembling and unable to rise and support their body. However, they were able to vocalize, support their head and suckle if bottle-fed.

At autopsy, the brains of the affected lambs were strikingly small (about half the weight of brains of normal littermates), the thickness of the cerebral cortex was severely reduced and the white matter was largely devoid of myelin in both cerebrum and cerebellum (Figure 1). The deep layers of the cerebral cortex showed pronounced neuronal loss, reactive astrocytosis and infiltration of macrophages (Figure 2A). There was severe degeneration of hippocampal pyramidal neurons. The cerebellum was less affected: in addition to a slight, patchy loss of Purkinje cells, there was some neuronal loss in the internal granule cell layer, whereas the external granule cell layer was thicker than that in controls. This may suggest a delayed maturation of the cerebellum. The basal ganglia, thalamus and brain stem were relatively spared. Visceral tissues examined, including heart, kidney, liver, gut, spleen and thymus, appeared macroscopically unaffected (data not shown).



Fig. 1. Coronal sections of the left cerebral hemisphere of a normal and CONCL lamb. Note the striking reduction in size, lack of distinction between gray and white matter, and the enlarged lateral ventricle in the CONCL brain (scale in millimeters).

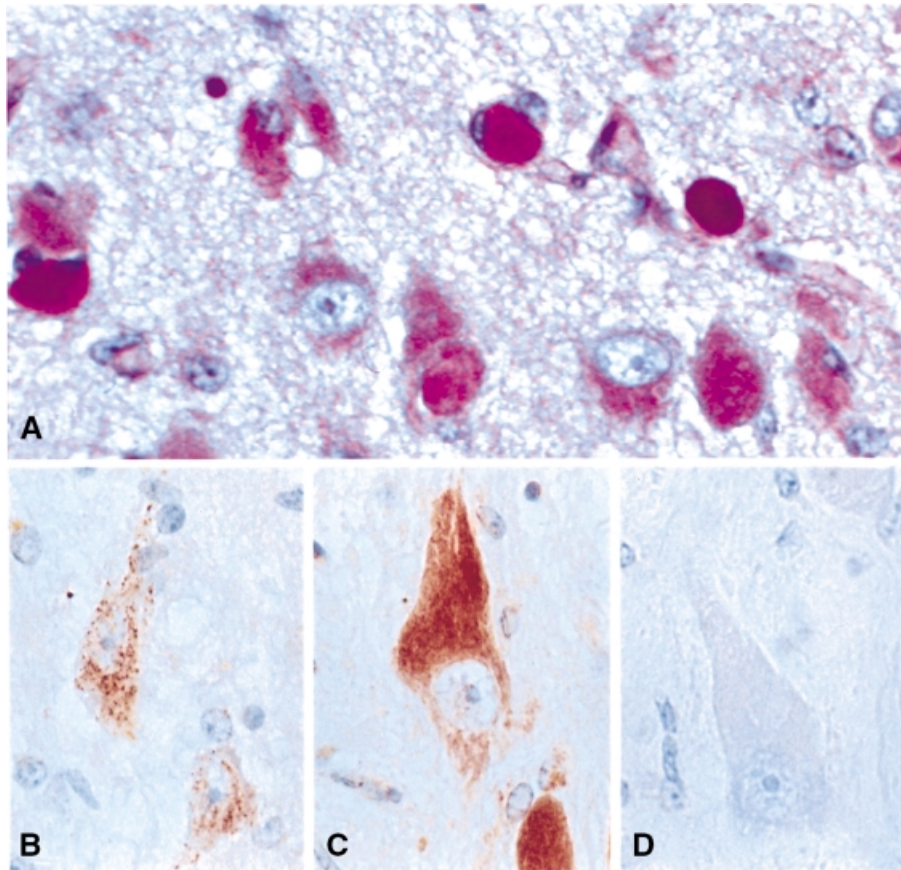


Fig. 2. Histochemical and immunocytochemical stainings of paraffin-embedded sections of CONCL and control brain. The storage material had histochemical characteristics typical of NCL (Rapola, 1993), showing positive staining with PAS (A). There is marked loss of neurons and infiltration of macrophages (seen as dark pink in PAS staining) in the cerebral cortex of the affected lambs (A). In the large neurons of basal ganglia of normal sheep, SAP staining shows a punctuate lysosomal pattern (B), whereas intense staining of the storage material filling the whole neuronal cytoplasm is seen in affected sheep (C). The storage material is negative for subunit c of mitochondrial ATP synthase (D). Preimmune sera and PBS were used to verify the specificity of the antisera (not shown). Magnification, $\times 450$.

Table I. Lysosomal enzyme activities in control and CONCL sheep^a

Enzyme	Specific activity		CONCL:control ratio
	Control	CONCL	
Brain			
cathepsin D	1180 ± 252 (n = 5)	-41 ± 44 (n = 4)	0.0*
α-fucosidase	78 ± 15 (n = 5)	232 ± 33 (n = 2)	3.0
α-glucosidase	6.91 ± 1.01 (n = 5)	8.76 ± 2.97 (n = 2)	1.3
α-iduronidase	10.7 ± 4.1 (n = 5)	15.9 ± 7.8 (n = 2)	1.5
acid phosphatase	578 ± 71 (n = 5)	1073 ± 137 (n = 2)	1.9
β-galactosidase	20 ± 6.3 (n = 5)	43.4 ± 5.1 (n = 2)	2.2*
β-glucuronidase	20.2 ± 6.1 (n = 5)	52.6 ± 0.4 (n = 2)	2.6*
cathepsin A	206 ± 54 (n = 5)	506 ± 71 (n = 2)	2.5
cathepsin B	123 ± 41 (n = 5)	403 ± 53 (n = 2)	3.3*
cathepsin C	317 ± 84 (n = 5)	1536 ± 65 (n = 2)	4.8*
cathepsin H	179 ± 30 (n = 5)	168 ± 21 (n = 2)	0.9
cathepsin L	227 ± 70 (n = 5)	565 ± 60 (n = 2)	2.5*
tripeptidyl peptidase I	77 ± 27 (n = 3)	461 ± 85 (n = 4)	6.0*
Liver			
cathepsin D	1484 ± 95 (n = 3)	75 ± 56 (n = 5)	0.1*
β-galactosidase	77 ± 39 (n = 5)	102 ± 9 (n = 4)	1.3
tripeptidyl peptidase I	149 ± 33 (n = 3)	335 ± 52 (n = 5)	2.2*

^aEnzyme activities are given as mean ± SD and were measured as described previously. Specific activities are expressed as nanomoles of substrate hydrolyzed per hour per milligram of protein in extract for all enzymes except cathepsin D, which is expressed as picomoles of substrate hydrolyzed per hour per milligram of protein.

*Significant at $p < 0.05$.

Generalized neuronal storage of autofluorescent granules was observed in the cerebral cortex of CONCL lambs. There appeared to be some, but not absolute, correlation between accumulation of autofluorescent storage material and cell death. For instance, neurons with pronounced storage and axonal enlargements were particularly common in the deeper cortical layers where the neuronal loss was most intense. However, there was also marked storage in the relatively spared large neurons of the basal ganglia (Figure 2B–D). Moderate storage was observed in the brainstem nuclei and spinal cord. A variety of visceral tissues, including heart and liver, showed slight storage, which was not associated with cellular destruction. No changes were observed in the thymus, spleen or gut.

Previous electron microscopic examination of CONCL neuronal storage bodies revealed granular osmiophilic deposits (GRODs) (Järplid and Haltia, 1993). This ultrastructure resembled that found in human infantile NCL (Haltia *et al.*, 1973), a disease caused by a deficiency in palmitoyl protein thioesterase 1 (Vesa *et al.*, 1995). In human infantile NCL, the storage material contains high levels of sphingolipid activator proteins (SAPs) and, in contrast to most other NCLs, there is no accumulation of mitochondrial ATP synthase subunit c (Tyynelä *et al.*, 1993). Our immunohistochemical studies revealed that amounts of SAPs A and D, which are normal lysosomal constituents (Figure 2B), were greatly elevated in CONCL neurons (Figure 2C). There was no lysosomal accumulation of mitochondrial ATP synthase subunit c (Figure 2D). Despite the ultrastructural and immunochemical similarities to human infantile NCL, enzyme assays have shown that the CONCL sheep do not have a palmitoyl protein thioesterase deficiency (L.A.Lee and S.L.Hoffman, University of Texas, personal communication), indicating that the molecular bases of human infantile NCL and CONCL are distinct.

The pathology of CONCL would suggest that it is a lysosomal storage disease. To address this possibility, we measured a range of lysosomal enzyme activities (Table I). As in certain human forms of NCL (Prasad and Pullarkat, 1996; Sleat *et al.*, 1998), many lysosomal enzyme activities, including that of tripeptidyl peptidase I (the defect of which causes human late-infantile NCL), were elevated in CONCL brain (Table I). This probably represents a cellular response to a primary defect that disrupts lysosomal function. However, the key observation was a striking deficiency of cathepsin D activity in the CONCL brain and liver compared with controls (Table I). Cathepsin D activity was also essentially undetectable in other CONCL tissues (spleen, kidney, heart and pancreas; data not shown). In cultured fibroblasts of CONCL lambs the activity was <5% of normal, and ~40% of normal in the fibroblasts of a heterozygous lamb.

Western blot analysis revealed that the steady-state level of cathepsin D protein was markedly higher in CONCL brain than in controls, while the processed single-chain form (48 kDa) and the heavy chain (32 kDa) of the two-chain form appeared to be of similar size to controls (Figure 3). Thus, an enzymatically inactive, yet stable, and apparently normally processed cathepsin D is present in CONCL. This finding provides further support for earlier studies showing that catalytically inactive mutants of cathepsin D can be proteolytically processed by other cellular proteases (Wittlin *et al.*, 1999) and that conversion of procathepsin D to both the processed single-chain protein and the two-chain form depends on cysteine proteases (Gieselmann *et al.*, 1985; Samarel *et al.*, 1989).

To identify the molecular defect in CONCL, we sequenced overlapping RT-PCR products of both control and CONCL sheep cathepsin D, in the process deducing the complete amino acid sequence of the mature, enzymatically active cathepsin D and most of the propeptide (DDBJ/EMBL/GenBank accession No.

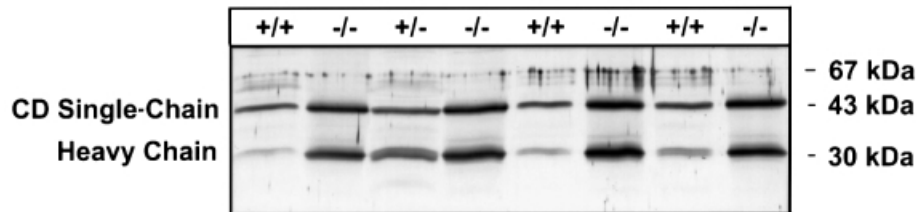


Fig. 3. Western blot analysis of cathepsin D in four CONCL ($-/-$), one heterozygous ($+/-$) and three control ($+/+$) sheep. Brain homogenates (30 μ g protein) were fractionated by SDS-PAGE and transferred to nitrocellulose. The processed single-chain form (48 kDa) and the heavy chain (32 kDa) of the two-chain form of cathepsin D were detected using rabbit antisera and chromogenic substrates. Molecular weight markers are indicated on the right.

AF164143). Sheep cathepsin D was found to have considerable homology with its human (85% identity) and mouse (79% identity) equivalents (Figure 4).

The only difference found between CONCL and control animals was at the residue corresponding to G934 of the human cathepsin D cDNA, where a homozygous G \rightarrow A mutation was found in all CONCL animals. We conducted a genetic analysis of 25 sheep; six affected animals were homozygous for the G \rightarrow A mutation, while the remaining 19 unaffected sheep were either heterozygous for the mutation ($n = 9$) or homozygous wild type ($n = 10$). The mutation associated with CONCL results in substitution of an asparagine for the aspartate that corresponds to Asp295 of human cathepsin D (Figure 4) and Asp215 of pepsin. This residue is conserved amongst all aspartyl proteinases and represents one of the two aspartate residues that are essential for catalytic function of these proteins (Davies, 1990).

Discussion

The abrogation of enzymatic activity resulting from the CONCL cathepsin D missense mutation is entirely consistent with the known properties of the enzyme. Cathepsin D is classified in family A1 of aspartyl proteinases, which includes the prototype enzyme pepsin and structurally related enzymes from animals, plants, fungi and protozoa (Barrett *et al.*, 1998). The structures of bovine and human cathepsin D, different pepsins and different aspartyl proteinase-inhibitor complexes have provided detailed insights into the catalytic mechanisms of these enzymes (Davies, 1990; Barrett *et al.*, 1998). The aspartyl proteinases consist of two lobes, each of which contains a key aspartate (Asp32 and Asp215 in pepsin numbering); together these are thought to position and activate a water molecule, which hydrolyzes the substrate peptide bond. Consistent with this, structure-function studies have shown that mutation of an active site aspartate is sufficient to eliminate enzymatic activity without affecting the processing of the enzyme (Lin *et al.*, 1989; Wittlin *et al.*, 1999). The direct causal relationship of the mutation and missing enzyme activity is further supported by finding 40% of normal activity in the fibroblasts carrying one wild-type and one disease allele of the gene.

In addition to its role as a protease, cathepsin D may have non-enzymatic functions. For instance, it has been reported that the inactive procathepsin D zymogen functions as a mitogen in some cancer cell lines and that this is due to interaction of the propeptide with a cell surface receptor (Fusek and Vetvicka, 1994; Vetvicka *et al.*,

1998). It may also be relevant that, in ungulates, there is a series of pregnancy-associated glycoproteins that have clear sequence homology to aspartyl proteinases but lack protease activity (Xie *et al.*, 1997). These proteins are produced by the placenta and may participate in fetal-maternal interactions. If non-enzymatic cathepsin D functions are biologically significant, these may well be preserved in CONCL given that the animals produce high steady-state concentrations of the mutant cathepsin D protein.

CONCL is the first reported disease arising from a naturally occurring cathepsin D mutation. The phenotype of the affected animals is markedly different from that of a genetically engineered mutant mouse strain produced by introduction of a gross lesion into the cathepsin D gene (Saftig *et al.*, 1995). Unlike CONCL lambs, these mutant mice are apparently normal at birth. The first reported symptom is atrophy of the ileal mucosa, occurring at day 14. Subsequently, massive loss of lymphoid cells in spleen and thymus was observed. In contrast, histological examination of newborn CONCL lambs did not reveal any pathological changes in the lymphoid organs or gut, although we cannot exclude the possibility that such changes would occur in older animals. The most striking feature of CONCL is the dramatic brain pathology. No overt brain pathology was noted in the initial report of the cathepsin D-deficient mice (Saftig *et al.*, 1995), although more extensive examination indicates that the mice do exhibit some brain abnormalities (Y.Uchiyama, Osaka University Medical School, Japan, personal communication).

There are a number of possible explanations for the marked differences between the cathepsin D-deficient mice and sheep. In terms of brain atrophy, it is likely that neurodegeneration in CONCL is due to a metabolic insult, which increases over time and crosses a threshold before manifesting toxic effects. Thus, the observed differences between the mice and sheep may relate to their gestation periods (3 weeks for mice and 21 weeks for sheep) and to species-specific differences in the maturity of the central nervous system at birth. If so, conditional inactivation of cathepsin D in mouse neurons should result in neurodegeneration over time. The lack of visceral symptoms in the CONCL sheep may be due to inherent differences between the two species or it may be obscured by the early death of CONCL lambs. However, the visceral pathology in the mice may also arise from a non-enzymatic deficiency in cathepsin D function. This intriguing possibility could be investigated by producing specific targeted mutations in the mouse cathepsin D gene.

to determine whether congenital human NCL represents a cathepsin D deficiency.

In Alzheimer's disease, changes in the expression of cathepsin D occur very early in disease progression (Cataldo *et al.*, 1995) and genetic variation in cathepsin D is associated with increased risk for disease (Papassotiropoulos *et al.*, 1999, 2000). The amyloid precursor protein-cleaving enzymes (β - and γ -secretases) have been suggested to be aspartyl proteinases and potential therapeutic targets in Alzheimer's disease (Haass and De Strooper, 1999; Vassar *et al.*, 1999). In light of the results presented here, it will be important to ensure that therapeutic approaches directed at such amyloidogenic aspartyl proteinases do not affect neuronal cathepsin D activity.

Materials and methods

Tissue specimens

Tissue specimens for biochemical analyses were obtained by autopsies of affected newborn lambs and their normal age-matched littermates; blood samples from affected and normal animals of different age were also collected. Diagnoses based on physical examination were verified by electron microscopy and genotyping (see below). The tissues were immediately frozen and kept at -70°C until used for biochemical analyses, or fixed in 2.5% phosphate-buffered formaldehyde and embedded in paraffin for histochemical and immunohistochemical analyses.

Histochemistry and immunocytochemistry

Paraffin sections (5 μm) were stained by the periodic acid-Schiff (PAS) method and counterstained with hematoxylin. For immunocytochemistry, paraffin sections were pretreated with 4% (w/v) pepsin in 0.37% (w/v) HCl (20 min at 37°C) and stained for SAPs A and D (Tyynele *et al.*, 1995) or subunit c of mitochondrial ATP synthase (a kind gift of Eiki Kominami, Juntendo University, Tokyo, Japan) using the Vectastain Elite kit (Vector Laboratories) according to the manufacturer's instructions.

Western blot analysis

Samples of sheep brain homogenates (30 μg protein) were fractionated by 12% SDS-PAGE and transferred to nitrocellulose. Cathepsin D was detected with polyclonal rabbit antisera (Bioscience International) using chromogenic substrates (Bio-Rad Laboratories).

Enzyme assays

Enzyme activities were measured as described previously: α -fucosidase, α -iduronidase, acid phosphatase, β -galactosidase, β -glucuronidase and cathepsins B and L according to Sleat *et al.* (1996), α -glucosidase and cathepsin C according to Sohar *et al.* (1998), cathepsin A according to Galjart *et al.* (1991), tripeptidyl peptidase I activity according to Sohar *et al.* (1999) and Vines and Warburton (1999), and cathepsin H according to Barrett and Kirschke (1981). Cathepsin D activity was obtained by measuring hydrolysis of fluorescein-labeled hemoglobin at pH 3.5 in the presence and absence of 3.5 μM pepstatin A as described previously (Sohar *et al.*, 1999).

Genotype analysis

Initial analysis of cathepsin D was achieved by automated dye-terminator sequencing of overlapping RT-PCR products derived from total RNA purified from sheep autopsy brain samples. As the nucleotide sequence of the ovine cathepsin D cDNA was not known, PCR was conducted using four degenerate oligonucleotide primer pairs, which were designed from an alignment of the human (DDBJ/EMBL/GenBank accession No. M11233), mouse (X52886), rat (X54467) and partial bovine (P.Metcalf, personal communication) cathepsin D nucleotide sequences. Degenerate primer pairs for RT-PCR contained either M13-21 or M13 reverse tails and were otherwise as follows (forward/reverse): ATCCCBCTGCRC-AAGTTCAC/ACAGTGTCTGGCTCAGGTACCC, GACAAGTCCAGCACCTAYGTGAA/GGGTCCCTGYTCAGGWAGAAGG, GACAA-CCTGATGMARCAAG/CACCTCNTCCACMGSCCCAC; CTG-ACCSTGTGCAAGGRRGGCT/GTCAAACACRGTGTAGTAG. The

partial sequence of the ovine cathepsin D cDNA is deposited in DDBJ/EMBL/GenBank (accession No. AF164143); in comparison with the other species, it encodes residues 26-412 of the 412 residue preprocathepsin D. Subsequent genotype analysis from tissue or blood samples was performed by dye-labeled primer sequencing of a single PCR product from genomic DNA that encompassed the site of the Asp \rightarrow Asn mutation. The flanking primers contained M13-21 or M13 reverse tails and were otherwise as follows: GCTGGACGTGGGCAG-CAGTCTGACC/TCGCCTGGATCAGCGGCACGGCCC.

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