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APPARENT CATHEPSIN B DEFICIENCY IN NEURONAL CEROID LIPOFUSCINOSIS CAN BE EXPLAINED BY PEROXIDE INHIBITION[1]

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Fibroblasts from patients with various forms of neuronal ceroid lipofuscinosis (NCL or Batten's disease) showed decreasing cathepsin B activity with increasing passage number and time in culture. In contrast, other lysosomal hydrolase activities were largely unaffected. Cathepsin B activity, was found to be associated with the lysosome-enriched fraction following cell disruption and Percoll gradient fractionation. Exposure of fibroblasts to low concentrations (<0.1 mM) of hydrogen peroxide either *in vivo* or *in vitro* resulted in a dose-dependent loss of cathepsin B activity with no concomitant loss in cathepsin H activity or lysosomal hydrolase activity. These results suggest that a primary defect resulting in accumulation of abnormal peroxides could produce a secondary cathepsin B inhibition in lysosomes and lead to observed peptide and dolichol accumulation in NCL.

Neuronal ceroid lipofuscinosis (NCL; Batten's Disease) is an inherited storage disorder characterized by the accumulation of autofluorescent pigment (lipofuscin) primarily in neurons and retinal pigmentary epithelial cells (1,2). The storage material is not a discrete entity but consists of peptides, polyisoprenoids (dolichols), lipid derivatives and metal ions such as ferrous and zinc (3-9). One long-held controversial hypothesis was that the neurodegenerative disease was the result of a peroxidase deficiency (10) but many laboratories have failed to confirm this observation. Evidence was also presented that tissues from NCL patients were enriched in peroxides (e.g. hydrogen peroxide in leukocytes) (10,11)) but the basic defect in NCL is unknown. The association of cathepsin B deficiency with NCL arose from two

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unconnected observations. Firstly, intracerebral injection of leupeptin (a cathepsin B (thiol protease) inhibitor) into rats resulted in the accumulation of pigment resembling lipofuscin and the accumulation of dolichols (12). Secondly, isolation and characterization of lipofuscin from tissues of the ovine form of NCL (OCL) showed that lipids were normal (apart from increased amounts of dolichols, ubiquinone and cholesterol) but that several small (3.5-15 kDa) peptides accumulated in all OCL tissues (9). From this it was inferred that a lysosomal protease deficiency could be the basic defect in NCL. We therefore initiated a study of lysosomal cathepsin B activity in fibroblasts from a number of patients with different forms of childhood NCL.

MATERIALS AND METHODS

Fibroblasts were grown from upper arm skin biopsies as described previously (13) and maintained up to a density of $5x10^6$/100 mm petri dish in modified Eagle's medium supplemented with 10% fetal calf serum. Affected (NCL), normal and pathological control lines were tested at intervals to ensure that they were free from mycoplasma contamination.

Most lysosomal hydrolase assays were carried out on sonicated cell extracts in pH 6.0 buffer. The incubation buffer was 0.1M sodium citrate/sodium phosphate buffer pH 4.4 (pH 4.9 for α-fucosidase) with 4-methylumbelliferyl glycoside substrates as described previously (14). Cathepsin B assays were carried out at pH 6.0 in 0.2M phosphate, 0.002M EDTA, 0.004M cysteine buffer using either Nα-benzoyl-DL-Arg-β-naphthylamide (BANA; Sigma Chemical Co.) or Z-Arg-Arg-methylcoumarin Peninsula Labs. Inc., CA. (15). Both the cathepsin B substrate (Z-Arg-Arg-MCA) and cathepsin H substrate (Arg-MCA) were dissolved in dimethylsulfoxide to make stock solutions which could be stored in the dark at 4°. To initiate the reaction, the substrates were diluted into 0.1% Nonidet P-40 and mixed with equal volumes of buffer and cell extract. 7-Amino-4 methylcoumarin fluorescence was determined by excitation at 370nm and emission at 460nm.

A specific [^{125}I] affinity substrate was used to bind cathepsin B in cell homogenates so that it could be electrophoresed in a 12.5% SDS-polyacrylamide gel and identified and quantitated by autoradiography (16). The cathepsin B affinity ligand [^{125}I]TALACK (Tyr-Ala-Lys-Arg-CH_2Cl) was a generous gift from Dr. D.F. Steiner, Univ. of Chicago. Binding to cathepsin B in fibroblast homogenates was carried out at pH 6 for 1h in MES/Dithiothreitol buffer. Under these conditions, a major 31-kDa species is labelled, together with a 39-kDa precursor and a 5-kDa autocatylytic product. Inhibitors of cathepsin B such as Ep 475 were a generous gift from Dr. Kazunori Hanada, Taisho Pharmaceutical Co. Ltd. Protein concentrations were determined by the method of Lowry *et al.* (17).

RESULTS AND DISCUSSION

Lysosomal hydrolase and thiol protease activity in NCL. As described previously (18), all lysosomal hydrolase activites (*N*-acetyl-β-hexosaminidase,

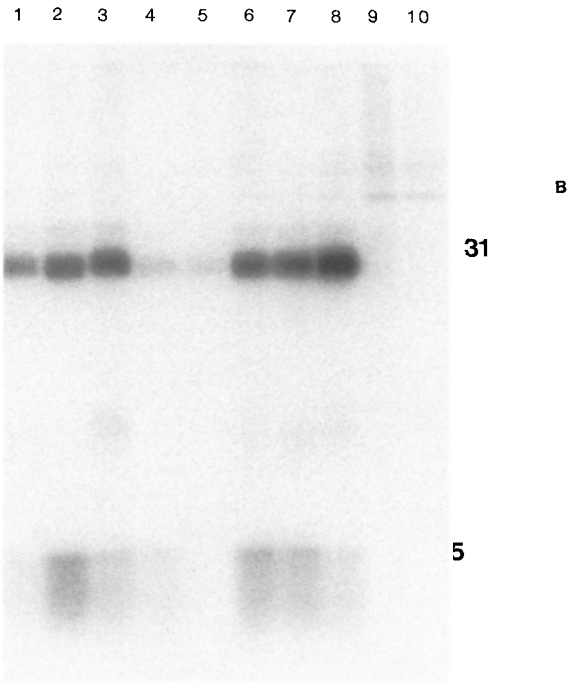

Fig. 1. Autoradiography of a 12.5% of SDS-polyacrylamide gel of [^{125}I] Tyr-Ala-Lys-Arg-CH_2Cl affinity-labelled proteins. Fibroblast homogenates were incubated with [^{125}I] TALACK for 1h in a pH 4.0 MES/DTT buffer (to amplify the 5KDa autocatalytic fragment) as described previously (16). Lane 1, adult Tay-Sachs disease (passage 10); lane 2, Tay-Sachs passage 7; lane 3, Sandhoff's disease passage 8; Lane 4, late infantile Batten's disease (DW), passage 15; lane 5, infantile Batten's disease (EC), passage 12; lane 6, juvenile Batten's disease, passage 4, (SB); lane 7, late infantile Batten's disease, passage 4 (CB); lane 8, fucosidosis, passage 5; lane 9, fucosidosis plus (Ep-475; lane 10, fucosidosis plus leupeptin. The amount of protein applied was the same for each sample and levels of α-L-fucosidase (typical lysosomal hydrolase) were comparable for all 10 samples (except lanes 8-10 which are totally deficient in α-L-fucosidase activity. A cell passage is defined as trypsinization of a cell monolayer and subsequent replating at a 1 to 4 dilution.

acid phosphatase, α-fucosidase etc.) in NCL fibroblasts were within the normal range for human skin fibroblasts. Cathepsin H (Arg-MCA) activity was also normal but cathepsin B activity showed some initially puzzling variations which appeared to be dependent on the length of time the cells had been in culture. Figure 1 shows active (normal) cathepsin B (31-kDa) activity in fibroblasts from patients with lysosomal storage diseases (lanes 1, 2, 3 and 8) which can be blocked with either EP475 (lane 9) or leupeptin (lane 10). High passage late infantile NCL (lane 4) or infantile NCL (lane 5) showed very low activity, whereas low passage juvenile NCL (lane 6) or infantile NCL (lane

7) showed normal activity. Binding was carried out at pH 4, to enhance the 5-kDa autocatalytic fragment as well as the mature (31-kDa) form. All lanes contained the same amount of protein (each lane showed the same coomassie blue-stained protein profile) and aliquots had approximately the same level of activity of other lysosomal hydrolases. Good correlation between the [^{125}I] TALACK and Z-Arg-Arg-MCA assays for cathepsin B activity was observed but the same cathepsin B variability was observed, preventing the use of cathepsin B activity levels as a diagnostic test for NCL.

Subcellular distribution of cathepsin activity in human skin fibroblasts. Because of this apparent cathepsin B deficiency and preliminary reports of abnormal organelles which could resemble lysosomes in NCL we undertook a study of cathepsin B subcellular localization in NCL. Cells were disrupted and fractionated on an 18% Percoll gradient according to the method of Hildreth et al. (19), using N-acetyl-β-hexosaminidase as the lysosomal marker (A,C,E). Cathepsin B activity was found to associate with the lysosomal-enriched fraction both in normal skin fibroblasts (a dense fraction which pellets at the bottom of the tube) (B) and in fibroblasts from a patient with sialic acid storage disease (a buoyant fraction which floats near the top of the gradient) (D) (Fig. 2F). In some NCL patients there was evidence of lysosomal hydrolase activity in both dense and buoyant fraction (C) but this was not consistent for all NCL strains studies. However, when a buoyant fraction was seen in NCL (Fig. 2, E,F), β-Hex activity was seen in both dense and buoyant whereas cathepsin B activity was only seen in the dense fraction. This suggested that cathepsin B was either absent or inactivated in the storage lysosomes.

Effect of peroxides on cathepsin B and other lysosomal hydrolases. We searched for toxic agents which could be accumulating in NCL fibroblasts with age. Peroxides are known to be toxic to cells both in the form of hydrogen peroxide (H_2O_2) (e.g., bacterial destruction by myeloid cells or) as the result of spontaneous oxidation of unsaturated fatty acids in membranes by

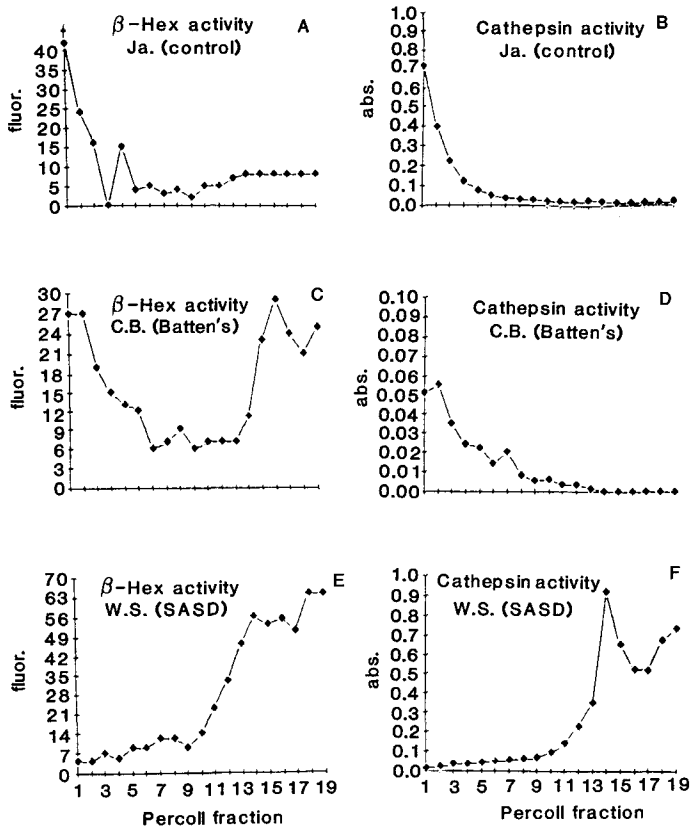

Fig. 2. N-Acetyl-β-hexosaminidase (A,C,E) and cathepsin B (B,D,F) activities of Percoll gradient fractions (collected from bottom of tube (Fraction 1) upwards to fraction 19) derived from normal (Ja) (A,B), sialic acid storage disease (WS) (E,F) and Batten's (CB) (C,D). Samples placed on Percoll gradients were post-nuclear supernatants of fibroblasts prepared as described previously (19). β-Hex was measured by hydrolysis of 4-methylumbelliferyl-β-N-acetylglucosamine, cathepsin B activity was measured colorimetrically following hydrolysis of BANA (15). Density marker beads showed fraction 2 = 1.070g/ml; fraction 10 = 1.050g/ml and fraction 14 = 1.035g/ml. Other subcellular organelles are found in different fractions as described previously (19).

molecular oxygen to yield toxic lipid hydroperoxides (20). These hydroperoxides are normally detoxified by reduction to hydroxy-derivatives by both selenium-dependent and independent glutathione peroxidases, or by carotenoids and tocopherols. Recently, a more prominent role for phospholipase A_2 in preventing lipid peroxidation has been proposed (20). Phospholipase A_2 has been shown to be activated by lipid peroxidation in lysosomes and microsomes, preferentially removing oxidized fatty acids from membrane phospholipids. A phospholipase A_2 deficiency in NCL could lead to accumulation of peroxidized phospholipid, subsequent chemical generation of

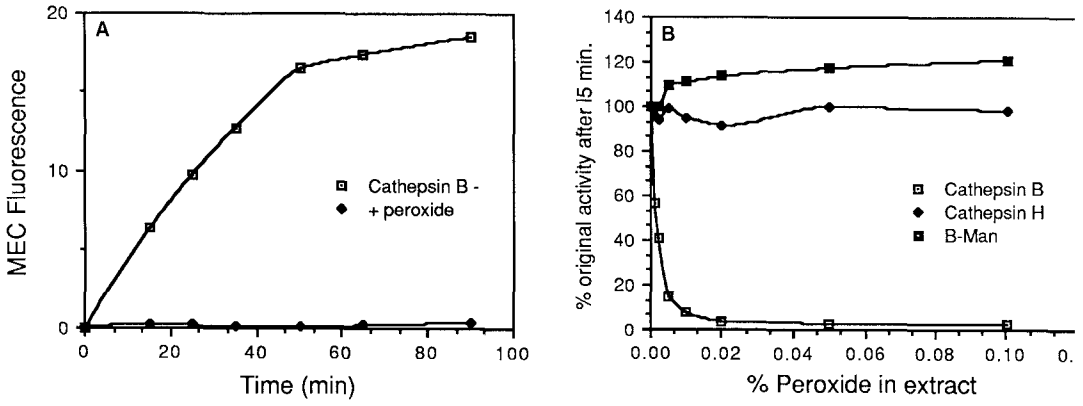

Fig. 3. A. Effect of peroxide pre-treatment (0.01%) of skin fibroblast monolayer cultures on release of 7-amino-4-methylcoumarin from Z-Arg-Arg-MCA by cell homogenates in pH 6.0 buffer.

B. Specificity of the inhibitory effect of increasing peroxide concentrations on activity of cathepsin B (measured by MCA release from Z-Arg-Arg-MCA) compared to cathepsin H (Arg-MCA) and lysosomal hydrolases (acid phosphatase (4MU phosphate) and β-mannosidase (4MUβ-Man)). 0.01% H_2O_2 (3 mM) gave 95% inhibition.

toxic aldehydes (such as 4-hydroxynonenal, $CH_3(CH_2)_4CH(OH)CH=CH-CHO$) and H_2O_2 from peroxidized polyunsaturated fatty acids and subsequent cross-linking of lipids and proteins to form the observed lipofuscin.

When normal human skin fibroblasts were cultured with 0.1mM (0.003%) H_2O_2 for up to 60 min. there was a complete loss of cathepsin B activity without any loss of other lysosomal hydrolase activity (Fig. 3A). Similar results were observed in vitro when disrupted cells were exposed to sub-millimolar H_2O_2 (0.0001% - 0.1%) for 5 min and then incubated for a further 30 min with appropriate fluorometric substrates (Fig. 3B). A dose-dependent loss of cathepsin B activity was observed without any concomitant loss of either lysosomal hydrolase activity (acid phosphatase, α-fucosidase or β-mannosidase) or other thiol protease (cathepsin H) activity (Fig. 3B). Recent studies (21) have shown that production of 0.1mM hydrogen peroxide is physiological for many cells such as endothelial cells. We observed inhibition with an IC_{50} of around 0.3 mM which could be physiologically possible in lysosmes.

We therefore conclude that observed deficiencies of cathepsin B activity in NCL fibroblasts could be the result of peroxide-mediated inhibition of cathepsin B and that accumulation of peptides in ovine NCL tissue are probably

a secondary effect of this inhibition. The susceptibility of cathepsin B to peroxide destruction is probably the result of its high histidine (imidazole ring) content, especially in the region of the active site (15) or cysteine residues being oxidized to cysteic acid. However, this latter possibility seems less likely since other thiol proteases (e.g. cathepsin H) were unaffected by low doses of hydrogen peroxide. Leupeptin is a specific cathepsin B inhibitor so its ability to generate lipofuscin *in vivo* is consistent with these findings. It is also worthy of note that the two cell types (neurons and retinal pigment epithelial cells) which contain the highest concentration of polyunsaturated ($C_{20:4}$ and $C_{22:6}$) fatty acids in phospholipids and therefore have the highest potential for generating peroxides, are the two cell types which are most affected in NCL (11,18).

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