Structural Changes in Lysosomes from Cultured Human Fibroblasts in Duchenne's Muscular Dystrophy

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ABSTRACT   We have previously reported a decreased activity of the lysosomal enzyme dipeptidyl aminopeptidase-I (DAP-I) in cultured fibroblasts from patients with Duchenne's muscular dystrophy (DMD). Here we report that electron microscope examination of these cells reveals the presence of abundant lamellar bodies, a morphologic abnormality commonly associated with impaired lysosomal function. Morphometric analysis of these cytoplasmic figures in dystrophic cells shows a sevenfold increase relative to normal controls ($P < 0.01$). Analysis of lysosomal density profiles by density gradient centrifugation reveals similar patterns in normal and DMD cells. Treatment of lysosomes with the nonionic detergent Triton X-100 causes an activation of DAP-I. This activation, attributable to structure-linked latency, is markedly diminished in DMD cells which show an optimal activation of only 180% compared to 255% for control fibroblasts ($P < 0.01$). These data suggest an alteration in the properties of the lysosomal membrane in DMD fibroblasts. This suggestion is also supported by studies on the release of DAP-I from lysosomes by osmotic shock which show it to be a membrane-associated enzyme with membrane-binding characteristics intermediate between those of tightly bound β-glucosidase and those of unbound N-acetylgalactosaminidase. The latency characteristics of these other lysosomal enzymes are not altered in the DMD cells, indicating that the effect is specific for DAP-I.

Studies of the biochemical etiology of Duchenne's muscular dystrophy (DMD) are complicated by the presence in the diseased muscle tissue of a variety of nonspecific degenerative changes which serve to obscure the primary error of metabolism (2). To avoid this difficulty, investigations of the properties of certain nonmuscle tissues in these patients have been undertaken. In erythrocytes, phosphorylation of membrane proteins (2) and the activities of the membrane enzymes adenylate cyclase and ($Na^+ + K^+$)ATPase have been reported to be altered in DMD (19, 20), and in lymphocytes a defect in capping has also been reported (23), although many of these changes remain to be confirmed. A small number of studies on the properties of cultured skin fibroblasts from DMD patients have also appeared. Pena et al. (22) have reported that the growth and longevity in culture of these cells are normal, suggesting that study of cultured DMD fibroblasts is not complicated by the presence of extensive degenerative changes. Wyatt and Cox (33) have reported that cultured skin fibroblasts from their DMD patients contain numerous dense inclusion bodies visible in the electron microscope, whereas these bodies

are rarely seen in control cells. Subsequently, Cullen and Parsons (5) reported that they were unable to confirm the presence of excessive amounts of inclusion bodies in their series of fibroblasts from a different set of DMD patients. Ionasescu et al. (11) reported that collagen metabolism was altered in fibroblast cultures from DMD patients, but Stevens et al. (31) have since reported that this parameter was unchanged in their cultured DMD fibroblasts.

We have recently reported that the activity of the lysosomal enzyme dipeptidyl aminopeptidase I (DAP-I) was significantly decreased in DMD cultured skin fibroblasts (8). It was also shown that this decrease in DAP-I activity probably represents a regulatory change in which fewer active DAP-I molecules are present in the DMD cells rather than a change in the structure or catalytic efficiency of the individual enzyme molecules. We have now extended our investigation of the lysosomal characteristics of cultured skin fibroblasts from DMD patients by examining the gross and ultrastructural morphology, lysosomal density profiles, and structure-linked latencies of various lysosomal enzymes. The results of these studies

reported here reveal that there are significant differences in some of these parameters which collectively point to a possible alteration of the lysosomal membrane in DMD fibroblasts.

MATERIALS AND METHODS

Cells

Eight lines of fibroblasts from DMD patients and six age- and sex-matched normal controls were obtained as described previously (8). Briefly, six of the DMD lines and four of the control lines were obtained from the Repository for Mutant Human Cell Strains (Montreal, Canada). Two of the control lines were obtained from the Institute for Medical Research (Camden, N. J.), and two of the DMD lines were developed in our laboratory from skin biopsies obtained from patients at the Neurology Clinic of the University of Cincinnati Medical Center. The maintenance procedures for these cells are described elsewhere (8). All studies were performed on confluent cell cultures, and the passage number for all cell lines was ±2 in any given experiment. Cells were used between passage numbers 5 and 30, which is well before the onset of stage II growth in these cell lines.

Enzyme Assays and Lysosome Preparation

β-glucosidase (EC 3.2.1.21), N-acetylgalactosaminidase (EC 3.2.1.49), and dipeptidyl aminopeptidase I (EC 3.4.14.1) were assayed fluorometrically as previously described (8). Cells were prepared for enzymatic studies by first washing them on the culture dish twice with ice-cold 0.25 M sucrose and then harvesting with a rubber policeman into 3.0 ml of ice-cold 0.25 M sucrose. Cells were passed 20 times through a Kontes no. 23 Teflon-glass tissue grinder (Kontes Glass Co., Vineland, N. J.) with the pestle rotating at 200 rpm. The homogenate was centrifuged at 750 g for 10 min at 4°C, and the postnuclear supernate was centrifuged at 30,000 g for 20 min at 4°C to obtain a crude lysosomal pellet. The supernate was discarded and the crude lysosomal pellet was resuspended in 10 ml of ice-cold 0.25 M sucrose, recentrifuged at 30,000 g for 20 min at 4°C, and this washed pellet was finally resuspended in 2–4 ml of ice-cold 0.25 M sucrose. Protein concentration was assayed by the procedure of Lowry et al. (15), with bovine serum albumin serving as standards.

Acridine Orange Uptake

Cultured fibroblasts from normal and DMD patients were incubated with acridine orange (Eastman Kodak Co., Rochester, N. Y.) by the procedure of Allison and Young (1). The cells were then viewed in a Zeiss fluorescence microscope and photographed with Kodak high-speed ektachrome film. The film was pushed in development to give an ASA rating of 1600: this is necessary to prevent excessive bleaching of the dye molecules during the exposure. The same incubation technique (1) was used to quantitate the amount of fluorescence in whole cells by harvesting the cells with trypsin, exposing them to acridine orange, and measuring the fluorescence at 490 (excitation) and 530 nm (emission) in a Perkin-Elmer spectrofluorimeter (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). The ability of a crude 30,000 g lysosomal pellet prepared as described above to take up acridine orange was measured by the method of Dell'Antone (6).

Ultrastructural Studies

Cultured human fibroblasts were prepared for electron microscopy *in situ* according to the method of Morris et al. (21). Briefly, 100-mm plastic petri dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) containing slightly subconfluent monolayers were washed three times with cold phosphate-buffered saline (PBS), pH 7.4. After a brief incubation in an ice bath, 10 ml of a "fixative" containing 1.3% OsO₄ and 4.2% glutaraldehyde in PBS was added and each dish was incubated at 4°C. After a 90-min fixation period, each sample was washed twice with cold PBS, washed twice additionally with 0.15 M sodium chloride, and postfixed with 0.5% uranyl acetate in 70% ethanol for 30 min at 23°C. The postfixative solution was decanted and tissue dehydration was completed by three subsequent 10-min washes with absolute ethanol. Samples were infiltrated and embedded in Epon 812 (Ernest F. Fullam, Inc., Schenectady, N. Y.) according to the method of Luft (17). The resin was polymerized by an overnight incubation at 40°C followed by 72-h incubation at 80°C. All samples were allowed to remain at 23°C for 24 h, after which the resin containing the intact monolayer was easily separated from the plastic culture dish by peeling the two surfaces apart. Samples were examined microscopically and those areas showing the greatest cell densities were marked and punched out with an electric cork borer. Specimens measuring 0.5 cm in diameter were mounted and sectioned

on a Reichert OM-U3 ultramicrotome with a diamond knife. Sections were viewed unstained with a JEOL-100B electron microscope operating at 60 kV.

Statistics

The statistical comparisons were based on the two-tailed Student's t test (25). The level of significance chosen was $P < 0.05$.

RESULTS

Gross and Ultrastructural Lysosomal Morphology

Ultrastructural examination of numerous profiles of normal human fibroblasts revealed a characteristic spindle shape. The mitochondria were extremely long and thin, the Golgi complexes consisted of flattened vesicles aligned along the long axis of the cell in the perinuclear region, and bundles of microtubules and microfilaments were seen running under the cell surface. These observations are consistent with those reported by Lucky et al. (16). Examination of dystrophic human fibroblasts revealed the same ultrastructural organization except that there was also a pronounced presence of lamellar bodies. These lamellar bodies or "myelin figures" consist of membrane-bounded cytoplasmic vesicles containing a series of concentric, membranelike swirls (Figs. 1 and 2). The membrane surrounding the lamellar body was sometimes found to be discontinuous or disrupted. We do not know the significance of this observation. It was not uncommon to view two or more series of membranous swirls within a single vesicle. Also, frequently viewed within the membrane of a lamellar body was an amorphous, electron-dense substance (Fig. 1). In general, the subcellular location of the lamellar bodies was restricted to the area immediately adjacent to the nucleus.

Subsequent morphometric studies were initiated to determine whether the above observation of an increased number of lamellar bodies in DMD cells was statistically significant. Accordingly, six dystrophic and five normal human fibroblast cell lines were evaluated for the number of lamellar bodies. To avoid ambiguity and to make the study statistically relevant, the following criteria were imposed. First, all cells were viewed randomly: that is, each single grid was viewed until a cell meeting the requirements outlined below was encountered. Once a cell had been scored on a grid, no further cells on that particular grid were scored. Second, a defined area of 7 × 9 cm was viewed at × 20,000. A magnification of 20,000 was chosen for two reasons: (a) the smallest of the lamellar bodies was easily discernible at this magnification, and (b) the area circumscribed typically contained the majority of the lamellar bodies. Third, only the cytoplasmic area immediately adjacent to the nucleus (perinuclear region) was viewed. This reflects an earlier observation that lamellar bodies were generally viewed only in the perinuclear region. In addition to these requirements, all prospective cells had to fulfill the following requirements indicative of good fixation: (a) intact and contiguous plasma membrane, (b) finely granular cytoplasmic ground substance showing no empty spaces, (c) mitochondria showing no signs of distortion, and (d) nucleus present showing no signs of pyknosis.

Using the above criteria, we scored both normal and dystrophic cells for the number of lamellar bodies. Table I shows the results of this study. The average (mean ± SEM) number of lamellar bodies per dystrophic cell was 7.5 ± 2.8, whereas with normal cells only 1.0 ± 0.6 lamellar bodies/cell were seen. This difference is significant at the $P < 0.01$ level. Lamellar bodies were observed in 84% of all dystrophic cells analyzed,
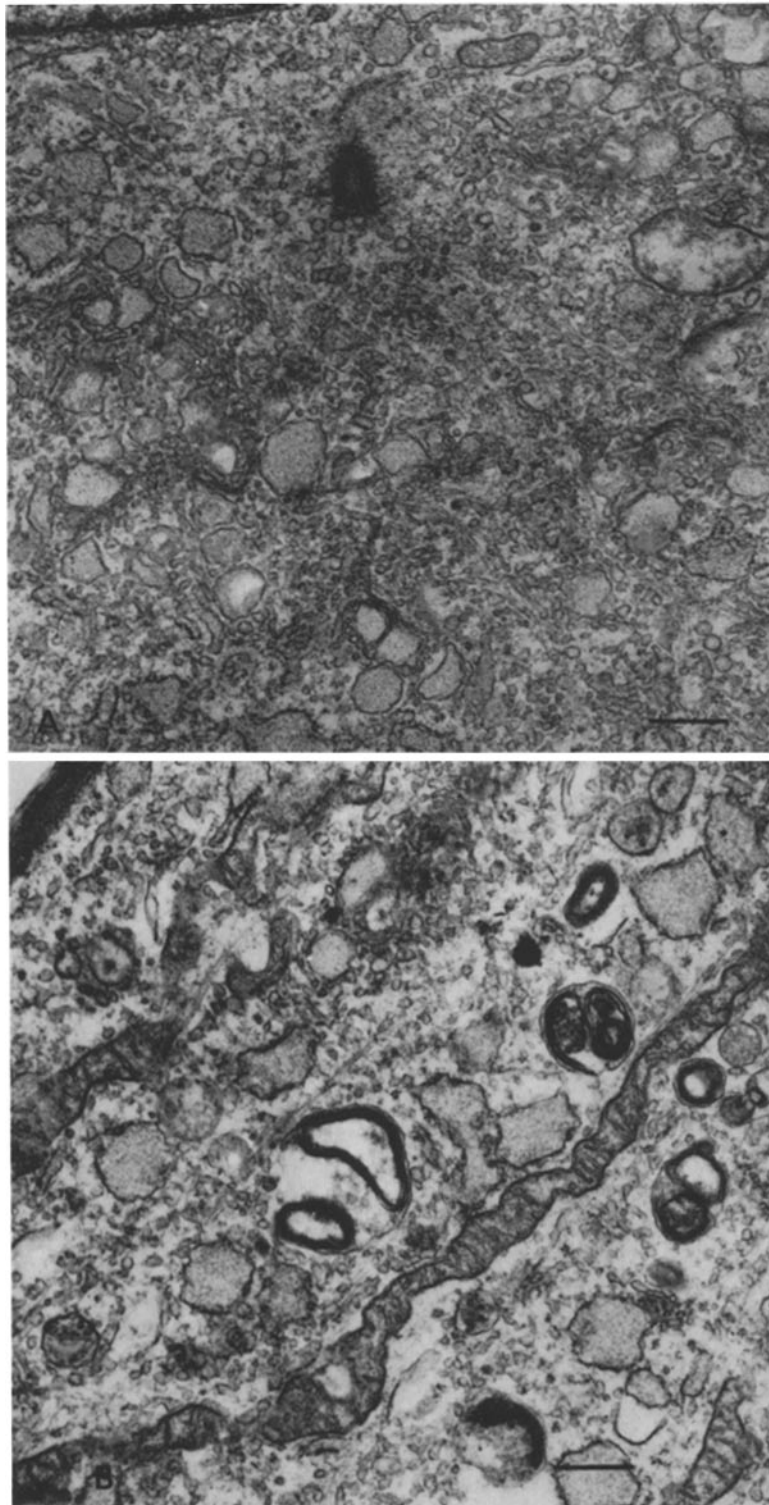
FIGURE 1   Electron micrographs of cultured human fibroblasts. (A) Cell line MCH 20: normal fibroblast with a small section of nuclear membrane visible at upper left. Dark body at upper center is a centriole. Bar, 500 nm. (B) Cell line WG 280: DMD fibroblast showing several lamellar bodies and swollen cisternae. Bar, 500 nm.

while only 43% of the normal counterparts possessed similar organelles.

Multiple cells were examined for each cell line, and almost without exception the dystrophic cells possessed more lamellar bodies. The sole exception was cell line GM497 which, although it is a normal control line, possessed a number of

myelin figures approaching that of dystrophic cells (Table I). The reason for this high value in GM497 cells is not known.

Since lamellar body formation is not infrequently associated with other lysosomal abnormalities, we further examined the lysosomal morphology of the cells at the light microscope level. For these studies the lysosomes were viewed by fluorescence
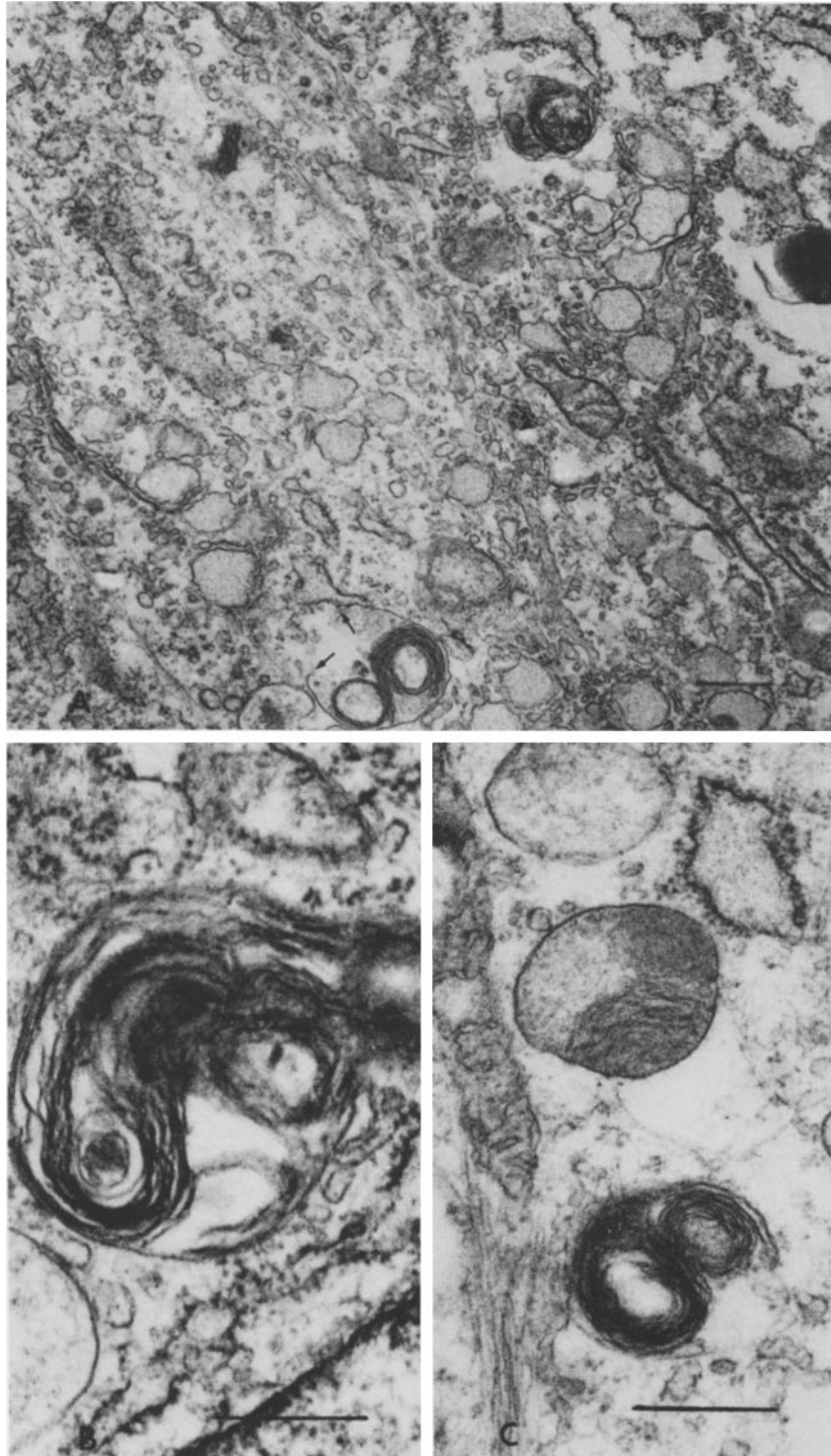
FIGURE 2 Electron micrographs of representative DMD cells. (A) Cell line WG 280: DMD fibroblast showing two characteristic lamellar bodies. Note the occurrence of the lamellar body enclosed within a disrupted membrane (arrows). Bar, 500 nm. (B) Higher magnification of a typical lamellar body viewed in the perinuclear region of cell line WG 448 DMD fibroblast. Bar, 500 nm. (C) High magnification of two lamellar bodies seen within the cytosol of cell line WG 348 DMD fibroblast. Note that in the lower lamellar body the surrounding membrane is noncontiguous. Bar, 500 nm.

microscopy after exposure of the cells to acridine orange. This membrane-permeable, fluorescent dye is concentrated in lysosomes as a result of protonation of the dye at the low internal pH of the lysosomal lumen which decreases the membrane permeability of the dye molecule (29). As the dye becomes more concentrated there is a shift in the emission spectrum

from green to orange so that the lysosomes are easily distinguished in the fluorescence microscope as bright, punctate, orange inclusions on a green cytoplasmic background. Using this technique, we saw that the subcellular localization of lysosomes of both normal and DMD cells was primarily in the perinuclear region. No difference in the average number, size,

fluorescence intensity, or distribution of normal and DMD acridine orange particles was observed in micrographs subjected to double-blind evaluation.

Particle Density Studies

Rome et al. (26) recently described a procedure for separating two species of lysosomal organelles in cultured human fibroblasts. These particles were resolved on self-generating, colloidal silica-polyvinylpyrrolidone density gradients, yielding a dense peak of lysosomal enzyme activity which, according to the authors, is free of contamination with other organelles, and a more buoyant peak which sedimented with several other organelles. It has been postulated that the dense peak is derived primarily from the residual bodies and the buoyant one from fragmented pieces of the Golgi-endoplasmic reticulum-lysosome (GERL) network (26). To determine whether the enzymatic and morphological changes we have observed in DMD cells are associated with an alteration in either of these lysosomal subclasses, we studied the density characteristics of DMD and normal fibroblast lysosomes by this procedure. We found that the components of the colloidal silica-polyvinylpyrrolidone gradient interfere with the assay procedure for DAP-I, so the density distribution of this enzyme could not be accurately determined. The distribution of two other lysosomal enzymes from postnuclear supernates of six normal and six DMD fibroblast lines separated on these self-generating density gradients is shown in Fig. 3. The results are in agreement with Rome et al. in that two distinct populations of lysosomal particles having differing density characteristics were resolved in both normal and DMD cells, in addition to the enzyme activity associated with the nonsedimented overlay fractions. The data show that the amount of enzyme activity appearing in the soluble overlay peak varies, depending on the particular lysosomal enzyme studied. Thus, the majority of β-glucuronidase activity appears in the nonsedimented peak, whereas β-glucosidase, being firmly bound to the lysosomal particles (4), appears mostly in the sedimented peaks of the gradient. (The β-glucosidase activity remaining in the overlay is probably microvesiculated rather than truly soluble, in view of the results in Fig. 4 described below showing that the 30,000 g supernate
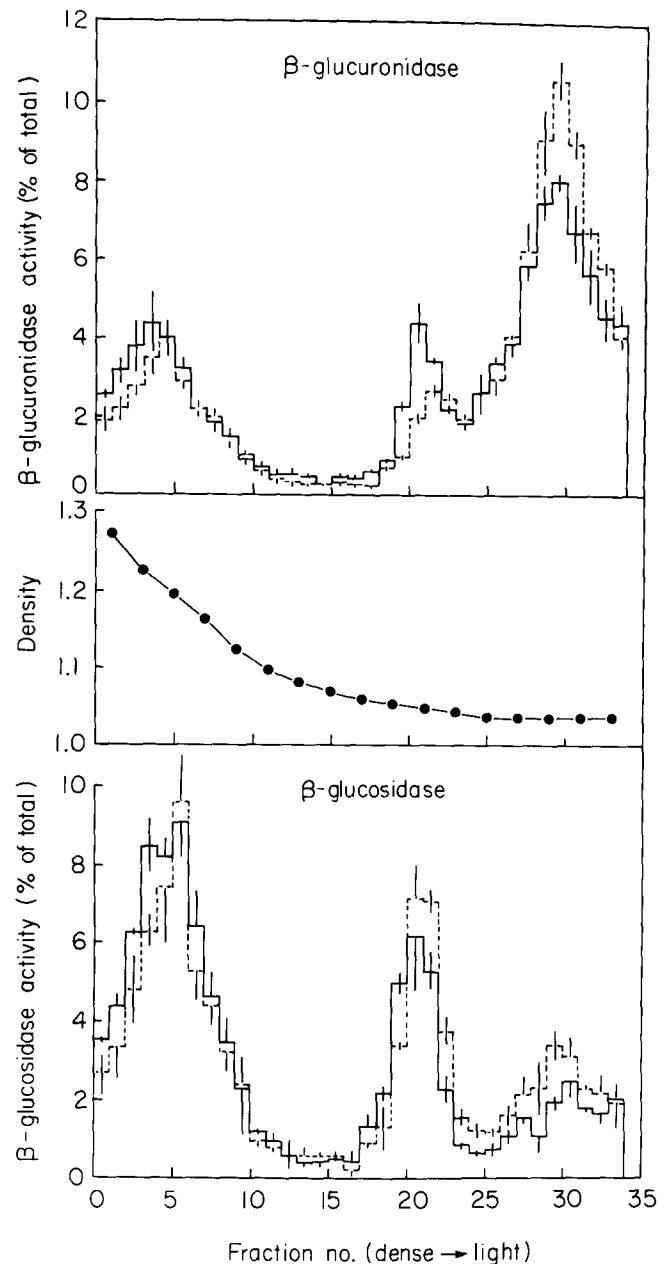

FIGURE 3 Distribution of lysosomal enzyme activity on a self-generating colloidal silica-polyvinylpyrrolidone gradient for two different enzymes. Peak at the lightest density represents soluble activity remaining with the sucrose overlay, while the middle- and high-density peaks represent the GERL and lysosome fractions, respectively. Solid lines show average values for six different normal lines measured as three sets of paired pools. Dotted lines show average values for six different DMD lines, also measured as three sets of paired pools.

TABLE I

Occurrence of Lamellar Bodies in the Perinuclear Region of Human Fibroblasts

Designation	Status	No. of fields viewed	No. of fields positive	No. of lamellar bodies	Avg. field
MCH 20	Normal	3	0	0	0.0
MCH 31	Normal	4	1	3	0.8
MCH 35	Normal	8	3	5	0.6
GM 2987	Normal	8	3	4	0.5
GM 497	Normal	12	8	38	3.2
Average	Normal				1.0
W.G. 280	Dystrophic	12	8	39	3.3
W.G. 348	Dystrophic	6	5	20	3.3
W.G. 433	Dystrophic	5	5	106	21.2
W.G. 448	Dystrophic	4	4	17	4.3
W.G. 502	Dystrophic	13	11	103	7.9
A.B. 001	Dystrophic	3	3	14	4.7
Average	Dystrophic				7.5
					$P < 0.01$

displays some structure-linked latency of β-glucosidase activity after exposure to detergent.) Comparison of the normal and DMD postnuclear supernate did not reveal qualitative changes in the sedimentation pattern of lysosomal enzymes, as depicted in Fig. 3. The morphological abnormalities described above in the DMD cells do not, therefore, appear to be associated with a significant change in the density characteristics of the two populations of lysosomes resolved on these density gradients. There is, however, a small but statistically significant difference ($P < 0.009$) in the distribution of the peaks of β-glucuronidase

30,000 g pellet   30,000 g supernatant

N-acetylgalactosaminidase activity

β-glucosidase activity

Dipeptidyl aminopeptidase I activity
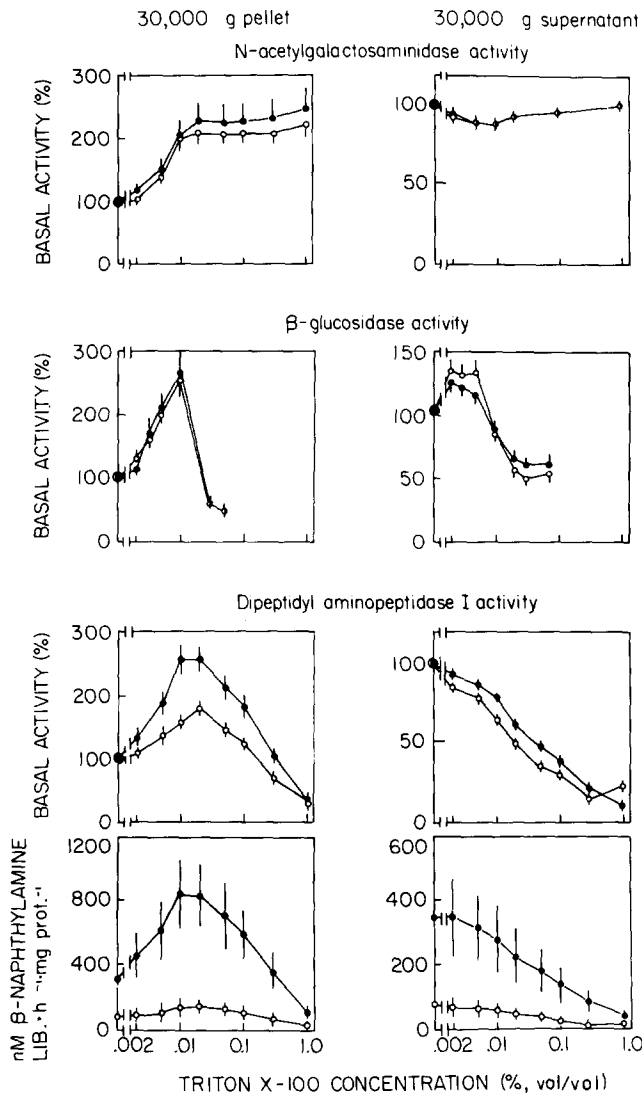
TRITON X-100 CONCENTRATION (%, vol/vol)

FIGURE 4   Detergent activation of three lysosomal enzymes expressed as percent of activity in the absence of Triton X-100. Left-hand panels show the effect of the detergent on enzyme activity in isolated lysosomes, while the right-hand panels show the effect of detergent on enzyme activity of soluble, nonparticulate enzyme from disrupted lysosomes. Specific activity of DAP-I for both fractions is also presented to show that the marked decrease in total DMD DAP-I activity previously reported (8) is also apparent under these conditions. The data are given as mean ± SEM, $n = 6$ (normal, shown in closed circles) or $n = 8$ (dystrophic, shown in open circles).

with less activity appearing in the DMD GERL fraction and correspondingly more activity in the nonsedimenting overlay peak. No other significant differences in lysosomal density profiles were observed.

Enzyme Latency and Osmotic Sedimentation Studies

The enzymes of isolated lysosomes are separated from exogenous substrate by the topologically closed membrane of the lysosomal organelle. Consequently, these enzymes do not display maximal activity in intact lysosomes unless this membrane barrier is compromised. The permeability barrier can be disturbed by a number of procedures including hypotonic (osmotic) stress, chemical disruption with detergents, or mechan-

ical disruption such as sonication. The additional enzyme activity that appears after disruption of the permeability barrier is termed the latency of the enzyme. The extent of structure-linked latency for a particular lysosomal enzyme depends upon the characteristics of the permeability of the lysosomal membrane to the substrate and cofactors for that enzyme. Latency studies can thus be applied to studies of the general permeability properties of the lysosomal membrane (24).

Structure-linked latency of three lysosomal enzymes was examined using increasing concentrations of the detergent Triton X-100 to disrupt the lysosomal membrane. The increase in activity caused by the release of latent enzyme from the lysosome is plotted in Fig. 4 as the percentage increase over basal activity (100%) observed in the absence of detergent. The left-hand panels of Fig. 4 show that all three of these enzymes were activated to the extent of ~250% above basal (detergent-free) activity, but that their sensitivity to enzyme inhibition by Triton X-100 was markedly different. N-acetyl-galactosaminidase (GalNAcase) was not inhibited at any concentration of detergent, and its latency curve therefore presents a monophasic increase in activity which reaches a plateau at a concentration of Triton X-100 corresponding to its critical micellar concentration (CMC) (CMC = 0.017%, vol/vol). β-Glucosidase, on the other hand, displayed a marked inhibition by Triton X-100 so that its latency curve is biphasic with activity increasing up to the CMC and sharply declining thereafter. DAP-I was less sensitive to inhibition by Triton X-100 and the shape of its latency curve therefore appears as intermediate between those of the above two enzymes, displaying a typical rising phase followed by a more gradual inhibitory phase as the CMC is exceeded. It should be emphasized that the rising and falling phases of these detergent latency curves presumably result from independent mechanisms. The rising "activation" phase is the result of an interaction of the detergent with the lysosomal membrane whereas the inhibitory phase results from a more direct interaction between detergent and enzyme. To examine the inhibitory effects of the detergent more closely, we studied the effect of Triton X-100 on sonicated preparations containing only solubilized lysosomal enzymes, so as to eliminate the effects of the activation phase of the curves. The enzymes were released from the lysosomes by freeze-thaw (two times) of a crude cell homogenate and the 30,000 g supernate was used as solubilized enzyme. The results of these studies are shown on the right-hand panels of Fig. 4. In the case of GalNAcase, the latency phase of the curve was no longer apparent as was expected and, as there had previously been no inhibitory effect, the detergent therefore had no effect at all on the activity of the solubilized enzyme. The results for β-glucosidase show that a significant degree of latency persists in the 30,000 g supernatant fraction, a strong indication that this "soluble" fraction actually contains minute, relatively buoyant fragments of lysosomal membrane containing associated β-glucosidase activity released during homogenization and freeze-thaw treatment. This is another indication that fibroblast β-glucosidase is firmly bound to the lysosomal membrane, especially considering that other soluble lysosomal enzymes did not exhibit any latency in the supernatant fraction. In the case of DAP-I, the latency phase of the Triton X-100 curve was completely absent so that the effect of the detergent on solubilized DAP-I is purely inhibitory.

When the detergent latency characteristics of these three enzymes in normal and DMD fibroblasts were compared, there was no difference in the activation curves for GalNAcase and

β-glucosidase activities. However, there was a clear and reproducible difference in the detergent activation curves for DAP-I activity, shown in the next to the bottom, left-hand panel of Fig. 4. To make comparison of normal and DMD groups easier, the data are expressed as the percentage of the basal activity when no detergent is present. The previously reported decrease in DAP-I specific activity in DMD cells is here shown to occur at all concentrations of Triton X-100 studied (bottom left and right panels). The DAP-I latency curves demonstrate that DMD fibroblasts exhibit not only a decreased total DAP-I activity but also a significantly lower percentage of latent DAP-I activity than normal controls. The fact that this latency difference does not exist for the other lysosomal enzymes indicates that this difference is specific for the components of the DAP-I assay as opposed to a generalized leakage of enzymes or substrates across the lysosomal membrane of the DMD cells. The average of the individual values for detergent activation of the DAP-I normal and DMD cell lines (Fig. 5) shows a difference that is statistically significant ($P < 0.01$).

To further determine the relationship between DAP-I and the lysosomal membrane, we studied the release of enzymes from the lysosomal particles by hypotonic lysis. Washed lysosomal pellets were incubated with hypotonic concentrations of sucrose followed by centrifugation to separate the enzyme activity released into the medium from the activity remaining bound to the lysosomal particles.

The results are shown in Fig. 6. The shape of the osmotic sedimentation curves depends markedly upon the particular enzyme being studied, in agreement with the observations of
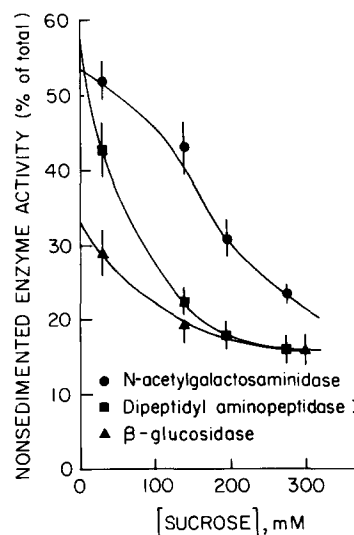
FIGURE 6   The release of lysosomal enzymes from cultured human fibroblast homogenates by hypotonic stress. Equal aliquots of washed 30,000 g pellets suspended in 300 mM sucrose were diluted with 9 vol of various sucrose solutions, producing the final sucrose concentrations shown on the abscissa. Samples of each dilution were assayed for enzymatic activity both before and after centrifugation at 30,000 g for 20 min. The results are expressed as the percentage of activity that remained in the supernate after centrifugation (mean ± SEM, n = 11). Data from dystrophic and normal fibroblasts were combined because there was no difference between the groups.

others using lysosomes from various species and organs (24). Thus, GalNAcase is representative of one class of enzymes that is released in approximate proportion to the hypotonicity of the sucrose concentration. We have operationally defined such enzymes as "soluble" lysosomal enzymes. In contrast, β-glucosidase activity is only sparingly released from the lysosomes after osmotic stress, and the results from this and other experiments discussed above have led us to conclude that this enzyme is firmly bound to the lysosomal particles. This agrees with the results obtained with β-glucosidase from other tissues (24). Finally, the osmotic sedimentation pattern of DAP-I activity was found to be intermediate between those of Gal-NAcase and β-glucosidase: slight hypotonicity causes the enzyme to sediment as though it were bound to the particles, whereas marked hypotonicity caused a shift to a soluble sedimentation pattern. Although both normal and DMD cells were examined for osmotic latency, the results from the DMD and normal lysosomes are combined in Fig. 6 because no differences were seen between the groups for any of the enzymes studied. Because the osmoprotective property of sucrose was not different in the DMD and normal lysosomes for osmotic sedimentation studies of three different types of enzymes, the fragility of the lysosomes is apparently equivalent in DMD and normal fibroblasts.

DISCUSSION

Lamellar bodies have been observed in a variety of conditions related to pathological lysosomal stress. In Tay-Sachs disease, a hereditary disorder of glycolipid metabolism with massive cerebral accumulation of GM_2 gangliosides because of the absence of the lysosomal enzyme β-D-N-acetylhexosaminidase, there is a striking abundance of lamellar bodies (27). Similarly, induction of intracellular ganglioside storage by feeding either
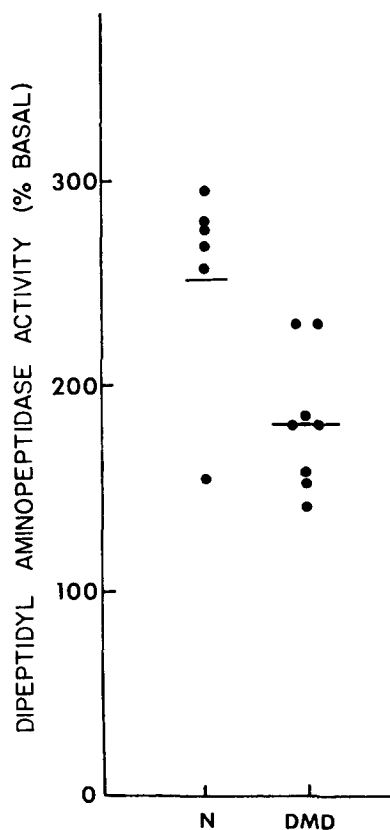
FIGURE 5   Detergent activation of lysosomal DAP-I in six normal and eight DMD fibroblast lines. Latency is expressed as percent stimulation of enzyme activity after addition of optimal concentration of Triton X-100. Difference between average latency for normal cells (255%) and that for DMD cells (180%) is significant at $P < 0.01$.

gangliosides or sulfatides to cultured spinal cord or dorsal root ganglia gives rise to lamellar body formation (30). Lamellar bodies are also seen in I-cell disease which involves deficiencies in several lysosomal enzymes because of an impaired phosphorylation of the enzymes followed by failure to re-accumulate the excreted enzymes (10). Environmental or induced stress to the lysosome is also associated with the formation of lamellar bodies. Thus, in chloroquine myopathy there is a massive accumulation of intralysosomal chloroquine which is thought to increase lysosomal pH, resulting in inhibition of acid hydrolases and the formation of abundant lamellar bodies in the muscle (18). The aminoglycoside antibiotics streptomycin and gentamycin are also sequestered by lysosomes with an associated appearance of lamellar bodies in fibroblasts and kidneys (3). Administration of other toxic compounds such as β-3-thienylalanine and triperanol, a cholesterol synthesis inhibitor, leads to focal cytoplasmic degradation with concomitant formation of lamellar bodies (32). Increasing the pH of the culture medium also results in the appearance of lamellar bodies in human fibroblasts (13). Experimental depletion of potassium results in both muscle weakness and the appearance of lamellar bodies in the affected muscle (12), a situation that is thought to mimic the inherited disorder hypokalemic periodic paralysis (7). Certain naturally occurring conditions are also associated with the occurrence of lamellar bodies. Thus, for example, the type II alveolar pneumocyte which is involved in lipid secretion normally contains lamellar and multivesicular bodies (28), and the aging human skin fibroblast in culture accumulates lamellar bodies as it becomes senescent (14).

Interestingly, many of these lysosomal-lamellar body conditions involve specific effects on muscle tissue. Tay-Sachs disease, chloroquine myopathy, potassium depletion, and hypokalemic periodic paralysis are all associated with muscle weakness. Lamellar bodies themselves are not all identical in appearance, varying in size and fine structure as a function of the cell type and the condition giving rise to their formation. Those seen in chloroquine myopathy most closely resemble the lamellar bodies we have observed in the fibroblasts of patients with DMD. Wyatt and Cox (33) have also reported finding numerous "inclusion bodies" in DMD fibroblasts, some of which look to us quite like our lamellar bodies. Their observation has, however, been challenged by Cullen and Parsons (5).

Morphometric analysis of the human skin fibroblasts reported here has revealed a statistically significant increase in the number of lamellar bodies present in the cytoplasm of DMD fibroblasts. This result reinforces our earlier observation of a lysosomal abnormality in these DMD cells evidenced by a marked reduction in DAP-I (8). Analysis of the kinetic properties of DAP-I activity in normal and DMD fibroblasts led us to conclude that the DMD enzyme is probably not structurally altered, but is rather present in a reduced number of catalytic units. This, in turn, suggests the possibility of some underlying lysosomal or cellular defect, a suggestion that is strengthened by the increased presence of lamellar bodies. However, as is clear from the preceding discussion, lamellar bodies appear to be the common crossroad of a variety of altered metabolic pathways and therefore do not, by themselves, point to a specific defect.

In the studies reported here on the further characterization of the presumptive defect underlying the lamellar body formation and the decreased DAP-I activity, we have found a significant decrease in the structure-linked latency of lysosomal

DAP-I which, we believe, is evidence of an alteration in the permeability characteristics of DMD lysosomes. It is important to note that the nature of this change does not appear to be a generalized increase in the permeability of DMD lysosomal membranes. Rather, it appears to be a selective permeability change in which structure-linked latency is altered for only one of the lysosomal enzymes studied, while latency remains normal for two other lysosomal enzymes. Because the enzyme molecules themselves are essentially membrane impermeable and remain within the lysosomes under normal conditions, the degree of structure-linked latency of lysosomal enzymes has generally been attributed to the permeability characteristics of the substrate molecules (9). Thus, a high level of substrate permeability is associated with a relative lack of latency, whereas an impermeable substrate will be associated with a high degree of latency. The permeability characteristics of essential cofactors could also affect the latency phenomenon of enzymatic reactions in the same manner that substrate permeability does. Thus, the permeability of chloride ion or of dithiothreitol could easily affect the degree of DAP-I latency because both of these are required cofactors in this enzymatic reaction. We believe, therefore, that decreased lysosomal DAP-I latency in DMD fibroblasts is an indication that the properties of the lysosomal membrane in DMD cells are altered in a relatively specific fashion. The precise nature of this alteration could be caused directly by an increased permeability of DMD lysosomal membranes or indirectly by some other factor that causes an accumulation of substrate or of one of the required cofactors. That the decreased latency is not the result of a unique property of the Triton X-100 detergent is shown by the occurrence of similar latency differences with a wide variety of other detergents (data not shown).

Because we have now described three discrete alterations in the properties of DMD lysosomes, the question arises whether there is a correlation between the degree of severity of one factor and that of another. The three DMD-associated fibroblast abnormalities are: decreased DAP-I-specific activity, decreased DAP-I structure-linked latency, and increased lamellar body formation. Thus, it is possible that the decreased structure-linked latency could be a direct consequence of decreased DAP-I concentration. This could occur, for example, if DAP-I were bound to the lysosomal membrane with a K_d similar to the normal DAP-I concentration in the lysosome so that a decrease in enzyme concentration would decrease the percent of DAP-I bound. If the activities of the bound and unbound forms were different, this could in turn give rise to an apparent change in structure-linked latency. This possibility can be analyzed by calculation of the correlation index, r^2, which is a measure of the degree of interdependence of these two variables. The correlation index for DAP-I specific activity and DAP-I structure-linked latency in normal cells is $r^2 = 0.24$ and in DMD cells $r^2 = 0.12$. Neither of these weak correlations is statistically significant ($P < 0.32$ and $P < 0.39$, respectively). Because the range of normal DAP-I activities evaluated is quite broad (8), this argues strongly against the possibility that the decreased latency in DMD cells could be caused simply by reduced levels of the enzyme. On the other hand, if the normal and DMD groups are considered as a single set, the correlation index increases to $r^2 = 0.51$ with $P < 0.01$. This significant, positive correlation is consistent with the presence of a common underlying abnormality that affects both of these parameters.

The relationship between the appearance of lamellar bodies and the decrease in structure-linked latency of lysosomal DAP-

I activity in DMD fibroblasts is unclear at this time. These two parameters are only weakly associated as shown by a correlation index of $r^2 = 0.015$, and speculation as to the nature of their relationship would be premature. However, both of these effects appear to be related to abnormalities in the fibroblast lysosomes, and further characterization of lysosomal function in cultured human skin fibroblasts from DMD patients may lead to the discovery of other phenotypic alterations in these nonmuscle cells. Such findings are potentially applicable to the development of methods of carrier detection and pre- and postnatal diagnosis of DMD as well as providing potential insight into the fundamental etiology of the disease process itself.

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