The Role of Lysosomal Enzymes in Killing of Mammalian Cells by the Lysosomotropic Detergent N-Dodecylimidazole

Patricia D. Wilson,* Raymond A. Firestone,* and John Lenard*

* Department of Physiology and Biophysics, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854-5635; and † Department of Membrane and Arthritis Research, Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065

Abstract. The sensitivity of cultured human and hamster fibroblast cells to killing by the lysosomotropic detergent N-dodecylimidazole (C₁₂-Im) was investigated as a function of cellular levels of general lysosomal hydrolase activity, and specifically of cysteine cathepsin activity. Fibroblasts from patients with mucolipidosis II (I-cell disease) lack mannose-6-phosphate-containing proteins, and therefore possess only 10-15% of the normal level of most lysosomal hydrolases. I-cell fibroblasts are about one-half as sensitive to killing by C₁₂-Im as are normal human fibroblasts. Overall lysosomal enzyme levels of CHO cells were experimentally manipulated in several ways without affecting cell viability: (a) Growth in the presence of 10 mM ammonium chloride resulted in a gradual decrease in lysosomal enzyme content to 10-20% of control values within 3 d. Subsequent removal of ammonium chloride from the growth medium resulted in an increase in lysosomal enzymes, to ~125% of control values within 24 h. (b) Treatment with 80 mM sucrose caused extensive vacuolization within 2 h; lysosomal enzyme levels remained at control levels for at least 6 h, but increased 15-fold after 24 h of treatment. (c) Treatment with concanavalin A (50 µg/ml) also caused rapid (within 2 h) vacuolation with a sevenfold rise in lysosomal enzyme levels occurring only after 24 h. The sensitivity of these experimentally manipulated cells to killing by C₁₂-Im always paralleled the measured intracellular lysosomal enzyme levels: lower levels were associated with decreased sensitivity while higher levels were associated with increased sensitivity, regardless of the degree of vacuolization of the cells. The cytotoxicity of the cysteine proteases (chiefly cathepsin L in our cells) was tested by inactivating them with the irreversible inhibitor E-64 (100 µg/ml). Cell viability, protein levels, and other lysosomal enzymes were unaffected, but cysteine cathepsin activity was reduced to <20% of control values. E-64-treated cells were almost completely resistant to C₁₂-Im treatment, although lysosomal disruption appeared normal by fluorescent visualization of Lucifer Yellow CH-loaded cells. It is concluded that cysteine cathepsins are the major or sole cytotoxic agents released from lysosomes by C₁₂-Im. These observations also confirm the previous conclusions that C₁₂-Im kills cells as a consequence of lysosomal disruption.

De Duve et al. applied the term "lysosomotropic" to various weakly basic amines that share the property of becoming concentrated, often several hundred-fold, within the lysosomes of cells to which they are added (7). Lysosomotropic amines possess this property because they diffuse readily across membranes in their uncharged form but become trapped in their protonated (nondiffusible) form in membrane-bound compartments of low pH. Following this concept, Firestone et al. synthesized several compounds designed to combine lysosomotropism with detergent activity (8–10). One effective lysosomotropic detergent is N-dodecylimidazole (C₁₂-Im), which combines imidazole, a base of intermediate pK, with a long hydrocarbon chain. It was reasoned that accumulation of the protonated form of this compound above a certain concentration (related to its critical micelle concentration) should cause it to acquire detergent-like properties, resulting in lysis of the lysosomal membrane and subsequent cell death. Consistent with this expectation, lysosomotropic detergents were found to be cytotoxic to several types of cultured cells at low concentrations. When added at the same concentrations to erythrocytes, which lack lysosomes, the lysosomotropic detergents had no hemolytic effect (8–10, 19).

Miller et al. have previously studied the mechanism whereby C₁₂-Im and related compounds kill baby hamster kidney (BHK)/21F cells, a line of cultured hamster fibroblasts (19). The results showed a strong association between lysosomal disruption and cytotoxicity. The compounds were
shown to accumulate in lysosomes and to rapidly increase the permeability of the lysosomal membranes toward ions and small molecules. Some protection against cell killing was afforded by methylamine, nigericin, and monensin, agents that raise lysosomal pH without damaging lysosomal integrity. Finally, effects on the lysosomes preceded by at least 1 h any other indication of cytotoxicity, such as inhibition of protein or nucleic acid synthesis, cell rounding, or cell leakage (19).

Although these results were consistent with a lysosomal role in cell killing they were not definitive. Specifically, lysosomotropic detergents should act similarly on all acidic organelles; endosomes (lacking lysosomal hydrolases) and the trans elements of the Golgi complex both have recently been found to be acidic (3, 18, 27, 28), so the possibility that disruption of these organelles could be cytotoxic must be considered. Further, if lysosomal disruption is the cytotoxic event, the question of just how such disruption promotes cell death had not been addressed. Agents that disrupt lysosomes are widely believed to kill cells by allowing leakage of lysosomal hydrolases into the cytoplasm (2, 7) but direct evidence for this idea seems never to have been reported.

We now provide evidence of a central role for lysosomal enzymes in general, and cysteine cathepsins in particular, in fibroblast cell killing by C\textsubscript{12}-Im. We show that sensitivity closely follows the cellular lysosomal enzyme levels when these are manipulated in several different ways, or when they are altered by genetic disease. These results support previous suggestions that lysosomal disruption is a necessary event in the development of cytotoxicity by C\textsubscript{12}-Im (8–10, 14, 19).

Materials and Methods

Cells

Normal human fibroblasts (GM5757) and human fibroblasts isolated from an individual with I-cell disease (mucolipidosis II, GM3066), obtained from the Human Genetic Mutant Cell Repository (Camden, NJ), were grown and passaged exactly as recommended by the Cell Repository. Chinese hamster ovary (CHO) fibroblasts were grown in Hams F12 medium containing 5% FBS (Gibco, Grand Island, NY). CHO cells were maintained in glass stock bottles in a humidified incubator in 5% CO\textsubscript{2}/95% air at 37°C and passed once or twice weekly using trypsin/EDTA (Gibco). For individual experiments, cells were subcultured in 12-well plastic tissue culture clusters (Costar, Data Packaging Corp., Cambridge, MA) using amounts of cells sufficient for the monolayer to grow to 12/25, 50, or 100% confluency in 1–3 d.

Reagents

Concanavalin A (Con A) was obtained from Pharmacia (Uppsala, Sweden). Enzyme substrates and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). 1-trans-epoxy succinyl-leucyl amido (4-guanido) butane (E-64), carbobenzyloxycarbonylarginyl-7-(4-methyl) coumarylamide (Z-arg-arg-MeCou), and carbobenzyloxycarbonylarginyl-methylcoumarylamide (Z-arg-MeCou) were purchased from Peptide Institute (Osaka, Japan). C\textsubscript{12}-Im was prepared as previously described (9).

Cell Treatments

CHO cells were subcultured at different confluencies for additional periods in medium containing 10 mM NH\textsubscript{4}Cl, 10 mM NH\textsubscript{4}Cl plus 10 mM mannos-6-phosphate (M6P), 80 mM sucrose, 50 μg/ml Con A, and 10, 50, or 100 μg/ml E-64. In some cases the cells were cultured for up to four additional days after removal of the NH\textsubscript{4}Cl or E-64. Lucifer Yellow CH (1 mg/ml) was added to the medium for microscopic observation of fluid phase endocytosis, processing of this fluid phase marker to the lysosomes, and lysosomal disruption by C\textsubscript{12}-Im (19).

All experiments were carried out in triplicate. In each 12-well cluster duplicate wells were washed twice with Hams F12 medium containing 15 mM Hepes buffer and extracted with 1% SDS for cellular protein determination, or with 0.5% Triton-X-100 for cellular β-hexosaminidase, LDH, and cathepsin activities. Cells in the remaining wells were incubated for 2 h at 37°C in 500 μl of Hams/Hepes buffer, pH 7.6 containing 0, 10, 20, 40, 60, 80, 100, or 120 μg/ml of C\textsubscript{12}-Im. 100-μl samples of medium were then removed from each well to determine release of the cytoplasmic enzyme LDH, an indication of cell death.

Enzyme Analyses

Beta-hexosaminidase was assayed using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside as substrate (II). Cathepsins were assayed fluorometrically by the methods of Barrett and Kirschke (5, 18). Z-phenyl-arg-MeCou was used as substrate to measure cathepsins B + L. Hydrolysis of Z-arg-arg-MeCou, a specific substrate for cathepsin B (17), was just above the level of detection (~3% of that of Z-phe-arg-MeCou) so that accurate measurements of this enzyme activity could not be obtained. LDH activity was assayed according to the method of Wroblewski and LaDue (31), and protein by the method of Lowry (66).

Results

I-Cell Disease Human Fibroblasts

Fibroblasts isolated from patients with I-cell disease and grown in continuous culture are deficient in one of the enzymes needed to attach M6P to lysosomal proteins (12, 25). Lysosomal enzymes are therefore not efficiently targeted to lysosomes in I-cell fibroblasts, but instead are secreted into the medium (20). In consequence, I-cell fibroblasts are deficient in virtually all known lysosomal hydrolases, generally possessing 10–15% of the levels present in normal fibroblasts (13, 20). I-cell fibroblasts were compared with normal human fibroblasts for sensitivity to killing by C\textsubscript{12}-Im. Cell killing of both normal and I-cell fibroblasts showed sigmoidal dose dependence (not shown) as found earlier in BHK cells (19). Fig. 1 shows that I-cell fibroblasts were less sensitive than normal fibroblasts at all levels of cell confluency tested. Sensitivity decreased with increasing confluency for both normal and I-cell fibroblasts, as was previously reported for BHK cells (19). The specific activity of β-hexosaminidase (a M6P-containing enzyme) increased somewhat with increasing confluency in both cell lines, but in I-cell fibroblasts was consistently 10–20% of that found in the normal fibroblast line.

![Figure 1](image-url)
Effects of Ammonium Chloride on CHO Fibroblasts

Lysosomotropic amines such as ammonium chloride have been shown to greatly enhance secretion of lysosomal enzymes by fibroblasts. The secreted enzymes are of the "high-uptake" form bearing the M6P recognition marker, suggesting that the amine disrupts the process(es) by which the enzymes are normally targeted to the lysosomes (6).

Fig. 2 shows that intracellular β-hexosaminidase levels decreased gradually to 5-15% of control values when grown over several days in the presence of 10 mM ammonium chloride. Parallel reductions in the levels of all other lysosomal hydrolases are assumed since ammonium chloride causes secretion of enzymes on the basis of their phosphomannosyl recognition markers (6). The addition of 10 mM M6P to inhibit reuptake of the secreted enzymes did not significantly alter the rate or extent of the reduction in intracellular activity (Fig. 2). The rate of reduction shown in Fig. 2 was independent of starting cell density. Subconfluent cells treated with medium containing 10 mM ammonium chloride showed normal growth together with loss of β-hexosaminidase activity similar to that shown in Fig. 2. Both the total cell protein and the specific activity of LDH, a cytoplasmic enzyme, remained constant and were indistinguishable from control cells (Fig. 2).

The dose dependence of cell killing of CHO cells by C$_{12}$-Im depleted to different levels of their lysosomal hydrolases is shown in Fig. 3. A close correlation was found between β-hexosaminidase levels and sensitivity to killing by C$_{12}$-Im.

The effects of ammonium chloride were reversible. Upon removal of ammonium chloride from the growth medium, β-hexosaminidase levels remained unchanged for 8 h after which they rose to supranormal values (125% of control) within 24 h (Fig. 4). Sensitivity to killing by C$_{12}$-Im was restored in parallel: the cells became hypersensitive when β-hexosaminidase levels increased to supranormal values (Fig. 5).

Ohkuma and Poole showed that the rise in lysosomal pH induced by addition of ammonium chloride was rapidly reversed upon removal after ~20 min of exposure (21). It may be questioned, however, whether similar rapid reversibility occurred after the extended (3-7 d) treatment with ammonium chloride used here. Observations of Lucifer Yellow CH uptake (19) suggested that recovery of normal endocytic function and processing was essentially immediate even after prolonged exposure to ammonium chloride. Ammonium chloride was removed after 3 d and Lucifer Yellow CH (1 mg/ml) immediately added to the medium. Punctate dots were seen inside the cell within 2-3 min. These were indistinguishable in rate of formation and extent from those seen

Figure 2. Effects of ammonium chloride on CHO fibroblasts. Cells were cultured for 1-7 d in normal growth medium containing 10 mM NH$_4$Cl and/or 10 mM M6P, where indicated. After various lengths of exposure, the cells were washed with Hams/Hepes and cellular extracts prepared for determination of total cell protein, LDH, and β-hexosaminidase. Protein is expressed as percent of value on day 0. (△) NH$_4$Cl, β-hexosaminidase; (△) NH$_4$Cl + M6P, β-hexosaminidase; (●) NH$_4$Cl, protein; (○) NH$_4$Cl, LDH.

Figure 3. Cell killing by C$_{12}$-Im in CHO cells depleted of β-hexosaminidase by various times of NH$_4$Cl treatment. Cells were incubated for 2 h at 37°C in Hams/Hepes buffer, pH 7.6 containing various concentrations of C$_{12}$-Im and medium removed for LDH measurement. The levels of intracellular β-hexosaminidase, relative to controls, were: (●), 12%; (△), 26%; (○), 61%; and (△), 100%.

Figure 4. Reversibility of NH$_4$Cl effect on cellular β-hexosaminidase activity. CHO cells, deficient in β-hexosaminidase after 3.5 d of NH$_4$Cl (10 mM) treatment were cultured in fresh growth medium lacking NH$_4$Cl and cellular extracts prepared at various times after removal, for measurement of: total intracellular protein, (●); LDH, (○); and β-hexosaminidase, (△). Results are expressed relative to values in control cells that had not been treated with NH$_4$Cl.

Figure 5. Effects of removal of NH$_4$Cl on sensitivity of CHO cells to killing by C$_{12}$-Im. Cells were incubated for 2 h at 37°C in various concentrations of C$_{12}$-Im and media removed for LDH measurement. Intracellular levels of β-hexosaminidase were 100% before NH$_4$Cl treatment (●); 26% after NH$_4$Cl for 3.5 d (○); 135% 24 h after removal of NH$_4$Cl (●).
in control cells, and are thought to represent endocytic vesicles; they were not seen if Lucifer Yellow CH was added in the presence of ammonium chloride. Within 30 min fluorescence was seen in larger patches in the perinuclear region, suggesting that processing of the dye to the lysosomes had occurred; this was similar to that seen in control cells. These observations suggest that normal endocytic function and processing to lysosomes were reestablished essentially instantaneously upon removal of ammonium chloride.

**Effects of Sucrose and Con A**

Both sucrose and nontoxic lectins such as Con A cause rapid and extensive vacuolization of cells. (22, 24). The addition of either sucrose (80 mM) or Con A (50 μg/ml) to CHO cells caused extensive vacuolization, readily visible by microscopy, within 1–2 h. Total cellular protein was unchanged by either treatment for up to 48 h (Figs. 6 and 8). Intracellular β-hexosaminidase levels remained unchanged for at least 6 h, but dramatic increases were evident by 24 h (15-fold for sucrose, sevenfold for Con A; Figs. 6 and 8). Cytoplasmic LDH also increased although by a smaller amount (Figs. 6 and 8). To assess the effect on sensitivity of vacuolization with and without increased lysosomal enzyme, cell killing by C2-Im was determined 4 and 24 h after addition of reagent. Sensitivity remained essentially at control values 4 h after addition of sucrose or Con A, although vacuolization was already extensive (Figs. 7 and 9). Increased sensitivity was observed at 24 h, however, after lysosomal enzyme levels had increased (Figs. 7 and 9). In both these experiments, therefore, sensitivity increased with increased lysosomal enzyme levels but was not affected by extensive vacuolization, which substantially increased the intracellular acidic volume (24).

**Effects of E-64**

E-64 has been characterized as a nontoxic active site directed irreversible inhibitor specific for cellular cysteine protein-
Figure 12. Fluorescence micrographs of CHO cells labeled for 4 h with Lucifer Yellow CH (1 mg/ml) in the absence (a–c) or presence (d–f) of E-64 (100 μg/ml). The presence of E-64 did not alter the punctate distribution of Lucifer Yellow CH into a lysosome-like pattern (a and d). Subsequent addition of Ct2-IIm caused moderate (25 μg/ml; b and e) or extensive (75 μg/ml; c and f) lysosomal leakage, as indicated by diffuse cytoplasmic fluorescence, regardless of prior treatment with E-64.
The cytotoxicity of C$_2$-Im is shown here to closely parallel the cellular content of lysosomal enzymes under a variety of conditions. This provides strong supporting evidence that the disruption of lysosomes, rather than of any other acidic organelles, causes cell death. Furthermore, extensive proliferation of acidic vacuoles, which occurs rapidly upon treatment of cells with sucrose or Con A (22, 24), does not increase sensitivity to C$_2$-Im unless it is accompanied by increased levels of lysosomal enzymes (>4 and 24 h points; Figs. 7 and 9). This conclusion is consistent with the discovery, by Okada and Rechsteiner, of a procedure by which pinocytic vesicles may be osmotically lysed inside the cell without effect on cell viability (23).

It has frequently been noted that lysosomal disruption by any of a variety of agents is cytotoxic, and it was assumed that this arose by inappropriate hydrolytic action in the cytoplasm (2, 7). An implication is that lowering lysosomal enzyme levels should therefore decrease the cytotoxic effects of lysosomal disruption. This is tested here for the first time, and the results support a cytotoxic role for lysosomal hydrodases.

The methods used to alter enzyme levels, as shown in Figs. 1–9, were expected to affect a broad range of enzyme activities similarly. I-cell fibroblasts are known to be deficient in the levels of all the M6P-containing enzymes, a population that is responsible for virtually all the known lysosomal enzyme activities in fibroblasts (13, 20). Similarly, treatment of cells with ammonium chloride results in secretion of the whole spectrum of M6P-containing enzymes (26). Increased levels of several lysosomal enzymes after growth of hamster fibroblasts in sucrose-containing medium have been previously reported (29) although the increases were much smaller than those found here (Fig. 6). In view of the extensive proliferation of the lysosome-like compartment caused by both sucrose and Con A (21, 23), an "across-the-board" increase in enzyme activity levels seems likely to be induced by these agents.

Inhibition of cysteine cathepsin activity by E-64, on the other hand, has been reported to be quite specific (4), and none of the other enzyme activities measured by us were significantly altered by E-64 treatment (Fig. 10). Lysosomal disruption by C$_2$-Im appeared normal in the presence of C$_2$-Im (Fig. 12). The almost complete resistance conferred by E-64 to killing by C$_2$-Im (Fig. 11) therefore permits the conclusion that the cysteine cathepsins are the major or sole cytotoxic agents released from lysosomes by C$_2$-Im. This finding incidentally confirms the central role of lysosomal disruption in cell killing by C$_2$-Im.

The intracellular level of cysteine cathepsins is probably not the only determinant of sensitivity to killing by C$_2$-Im. It may not account for the decreased sensitivity of confluent or slowly growing cells, which appears to be a quite general feature observed in every mammalian cell line we have examined, and also in yeast cells (14). In normal human fibroblasts, I-cell fibroblasts, and CHO cells we have observed that the β-hexosaminidase levels increased with increasing cell density. Similar increases in different lysosomal enzymes as cell density increases and growth slows have been reported by others using various cell lines (30); it is also true in yeast (1). An increase in sensitivity of confluent or stationary phase cells to killing by C$_2$-Im would be expected on this basis, yet the opposite, a markedly decreased sensitivity, is consistently observed (Fig. 1: also, references 14, 19). One possible explanation for this growth dependence is that the cytotoxic cathepsin is specifically decreased at high cell density. Other possibilities include: a lower pH in the lysosomes of sparse, rapidly growing cells; growth-dependent differences in the proteins or lipids of lysosomal membranes that alter their susceptibility to disruption by detergents; growth-dependent changes in concentration of a cytoplasmic inhibitor of the cysteine cathepsin(s); or growth-dependent differences in the cell's ability to transport or accumulate the lysosomotropic detergent.

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