Bioremediation meets biomedicine: therapeutic translation of microbial catabolism to the lysosome

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Summary

Lysosomal degradation of damaged macromolecules is imperfect: many cell types accumulate lysosomal aggregates with age. Some such deposits are known or are strongly suspected to cause age-related disorders such as atherosclerosis and neurodegeration; it is possible that they also influence the rate of aging in general. Lysosomal degradation involves extensive cooperation between the participating enzymes: each generates a substrate for others until breakdown of the target material to recyclable units (such as amino acids) is complete. Hence, the age-related accumulation of lysosomal aggregates might be markedly retarded, or even reversed, by introducing just a few bacterial or fungal enzymes—"xenohydrolases"—that can degrade molecules that our natural machinery cannot. This article examines the feasibility and biomedical potential of such lysosomal enhancement as an approach to retarding or treating age-related physiological decline and disease.

The lysosome is a severely neglected organelle. Since its discovery in the 1950s [1] it has suffered from an image crisis: its unglamorous role as the cell's garbage disposal unit has proved a disincentive to study it. Today it receives more attention but still far less than cellular components engaged in biosynthetic processes. The briefest bout of industrial action by human garbage collectors suffices, however, to remind us how crucial such processes are. From the biomedical standpoint, this is illustrated by the fact that genetic deficiency for any of numerous lysosomal enzymes involved in degradation of intracellular waste results in progressive impairment of general cellular and organismal function, being fatal at an early age. Such disorders are collectively termed lysosomal storage diseases (LSDs); they will not be reviewed in detail here because excellent surveys of them have appeared recently [2-4].

Healthy individuals also accumulate apparently undegradable material in lysosomes, albeit much more slowly. In fact, the best-studied such aggregate—variously termed "lipofuscin" or "age pigment"—was first described >100 years before its enclosing organelle [5]. LSDs can therefore be considered as a category of what Martin termed "segmental progeroid syndromes" [6]: accelerated

occurrence of a subset of the age-related molecular or cellular changes that occur in all of us at a relatively much slower rate. A striking characteristic of the LSDs is that the rate of accumulation of aggregates is many times greater than normal, even though only one of at least a few dozen enzymes is missing. This cannot be explained on the basis that one enzyme is responsible for the majority of all lysosomal catabolism because the loss of any one of several such enzymes causes greatly accelerated accumulation.

On closer inspection, the apparently "rate-limiting" role of so many enzymes is not surprising. The material that enters the lysosome is an extremely heterogeneous collection of lipids, proteins and other macromolecules in varying states of disrepair as a consequence of oxidative and other attack, as well as undamaged ones marked for degradation as part of normal cellular function. Dissecting such a morass into the small molecules that the lysosome releases for reuse in cellular biosynthetic processes is analogous to totally dismantling a car: one requires different tools at different steps, and the lack of any one tool brings the entire process to a halt, with only some of the car's parts available for reuse and the remainder having to be stored indefinitely in the junkyard.

Aggregates accumulate in essentially all long-lived cells because the diluting action of cell division is absent and the only other logically available option, periodic exocytosis, is evidently disfavoured (possibly because the material would merely be transported to the kidneys, the filtration function of which might be severely compromised, or else sequestered indefinitely in the extracellular space where it might eventually cause other problems). The gross composition of these aggregates varies between cell types. Several of them are implicated in the genesis of age-related pathologies. Hence it would be potentially of great benefit to identify interventions that could slow or reverse this accumulation. However, nothing has yet been reproducibly found to retard, let alone reverse, the accrual of lysosomal aggregates.

Here I consider the feasibility of a hitherto unexplored approach to this problem: augmentation of the lysosomal catabolic machinery with "xenohydrolases", enzymes identified in other organisms that can degrade material that our existing apparatus cannot. Such enzymes should only need to break down a small minority of the molecular structures present in these aggregates to have a substantial effect because by doing so they will create and/or expose previously inaccessible substrates for enzymes we already have. Lysosomal function seems to be impaired by such aggregates [7], but not abolished, indicating that new hydrolases are continually (albeit ineffectively) targeted to aggregate-laden lysosomes. Hence, addition of only a few suitably competent enzymes could potentially lead to the wholesale disaggregation of large granules of previously indigestible material (Fig. 1). This strategy, although undoubtedly ambitious and long term, has a probability of success and a potential biomedical impact that amply justify its exploration. It contrasts with the more obvious (and doubtless easier) option of simply up-regulating our existing enzymes. That approach would be very unlikely to confer much benefit, because the extremely slow rate of accumulation of aggregates implies that our existing enzymes cannot possibly be saturated (i.e. competent but present in inadequate amounts): rather, the constituents of these aggregates must be truly undegradable by human lysosomal enzymes.

Late-onset LSDs: the case for xenohydrolases

The disorders conventionally classified as LSDs all begin by early adulthood and result in very premature death [2-4]. For example, absence of TPP1, an enzyme that cleaves three amino acids from the N-terminus of a protein, causes late infantile neuronal ceroid lipofuscinosis due largely to a severely reduced ability to degrade subunit c of the mitochondrial ATP synthase [4]. Their combined incidence is relatively low—one in ~7700 live births [3]. Several diseases with much

later ages of onset have an etiology that clearly qualifies them to be classified as LSDs, however; moreover, some of them are among the most prevalent and serious diseases of old age.

By the age of ten, the aorta wall of all humans contains foam cells—macrophages that have entered it for the purpose of digesting modified lipoproteins that have become trapped there, and that have become so engorged with this material that they have become unable to maintain normal function [8]. Because foam cells are no longer able to take up much additional material, additional macrophages must enter the intima for this purpose. However, the foam cells are not removed. With age, therefore, the abundance of these cells progressively rises and their distribution spreads from the aorta to more and more of the arterial network [8]. In some individuals, nothing more ever happens; foam cells of typical centenarians are relatively abundant but do not seem to do them any harm [9]. In many of us, however, the presence of these cells eventually triggers a dysregulated proliferative response in nearby vascular smooth muscle cells, which envelop the foam cells and in due course form an atherosclerotic plaque. The pathological effects of such lesions, including heart attacks and strokes, are collectively the leading cause of death in the developed world [10].

Heart attacks are extremely common—but at least they kill people relatively quickly. It could be argued that diseases that inflict very long-term debilitation on a large proportion of the population are even more serious. Prominent among such ailments are the neurodegenerative diseases, principally Alzheimer's and Parkinson's diseases. All such diseases are characterised by the accumulation of aggregates in certain areas of the brain, with distributions and compositions that vary between diseases. It has proven difficult to demonstrate conclusively that these aggregates cause the pathology but the hypothesis that they do remains the leading interpretation. The subcellular localisation of these aggregates is unclear in some cases, but tangles in Alzheimer's disease (AD) are definitely cytosolic and quite probably lysosomal [11,12]. The amyloid plaques in AD are extracellular but recent advances in immunisation against their major component, the peptide Aβ (amyloid beta), have opened up the possibility of sequestering that material within the lysosomal compartment of microglia [13,14]. Unfortunately, this material is only poorly degraded after uptake [14]. Hence, both of the aggregates implicated in the pathogenesis of AD are potentially amenable to improved clearance by enhancement of lysosomal proteolytic capacity.

A non-life-threatening, but still debilitating, disease of old age is macular degeneration, the leading cause of blindness in the elderly. There is good evidence that this is also a LSD. The cells of the pigmented epithelium continually phagocytose fragments of the photoreceptor cells in front of them, thereby removing photodamaged material such as rhodopsin and retinal. The lysosomes of these cells thus accumulate certain compounds that result from this photodamage, especially A2E, a molecule formed by a sequence of reactions between retinal and phosphatidyl ethanolamine [15]. At high concentrations this compound evidently sensitises the pigmented epithelium to serious light-induced damage including lysosomal rupture, which results in cell death [16]. Even at physiological concentrations, however, subsequent degradation of photoreceptor material is impaired [16]. This would thus be prevented if the lysosomes within which A2E accumulates were able to degrade it.

Finally, it is possible (though by no means proven) that lysosomal aggregates are involved in the subtler decline exhibited by other non-dividing cell types, such as cardiomyocytes. The aggregate found in such cells in normal aging, lipofuscin, differs from those mentioned above in that no single molecule predominates in its composition. This does not, however, imply that lipofuscin would be any harder to degrade by a lysosomal enhancement strategy than the substances discussed above, because the obstacles to degradation are not whole macromolecules but particular molecular structures. The inhibitory effect of excessive lipofuscin on lysosomes' ability to autophagocytose and digest material that they otherwise degrade fully has been documented *in vitro* by Brunk's and

Boulton's groups [7,16-19]. Lipofuscin occupies about 10% of cell volume in old hearts [20], so it is possible that it is damaging *in vivo* too.

Why should useful xenohydrolases exist? If they do, how can they be found?

Circumstantial evidence that organisms exist that can break down the substances discussed above is immediately apparent: these substances do not accumulate in the environment. They are not peculiar to humans—the processes by which they are formed occur within the corresponding organs of all mammals, albeit at different rates—so any that is truly refractory to degradation by any soil microbe should be evident in the soil. Confirming this, preliminary attempts to identify soil bacteria that can degrade lipofuscin were immediately successful [21]; the energy-rich nature of such aggregates provides for the simple selection procedure of exposing bacteria to the target substance in the absence of other nutrients and selecting those that form colonies. Soil bacteria have been isolated in this way that can degrade numerous highly recalcitrant pollutants such as trinitrotoluene [22], so this strategy is well established.

More challenging is to identify the specific enzyme (or enzymes) that degrade the target substance. As discussed above, the quest is not for the entire collection of enzymes necessary to reduce the substance to low molecular weight fragments, but rather the first of those enzymes, the action of which exposes previously unavailable molecular features that could be substrates for human lysosomal enzymes. Thus, one approach is subtractive genomics—the identification of a range of closely related strains of which some can degrade the target substance and others cannot, and scanning of their genomes for genes the presence or absence of which correlates with this ability. Alternatively, and depending on the genetic tractability of the bacterium in question, a more efficient approach might be to mutagenise strains that are competent to break down the target substance and isolate subcultures that have lost this ability, following which mapping and sequencing could identify the relevant gene(s).

Bacteria are not the only organisms in which such enzymes are likely to be present; fungi also have a spectacularly wide range of catabolic abilities [23]. An important reason to investigate fungi is that the cytosol of soil bacteria is typically near to neutral pH, whereas the fungal vacuole is acidified to a pH comparable to that of the lysosome [24]. The activity and stability of lysosomal hydrolases are typically lowered at cytosolic pH [25], perhaps for reasons of cellular robustness in the event of lysosomal rupture; hence, an enzyme with promising substrate specificity but too high a pH optimum might be of little use (although *in vitro* mutagenesis under appropriately selective conditions might overcome this).

Issues of delivery and safety

Once identified, such genes must be introduced into mammalian cells in such a way as to exploit their products' catabolic activity. *In vivo* delivery faces the same difficulties as all other applications of gene therapy, and will not be addressed in detail here, because excellent reviews of progress in this area have appeared recently [26-28]. Moreover, LSDs have been the specific focus of several gene therapy initiatives over recent years, involving targeting to many cell types [29-31]. However, there are some important obstacles to enhancement of normal lysosomal function, over and above those that confront gene therapy for LSDs or other inborn genetic deficiencies.

First, the xenohydrolase must be targeted to the lysosome after synthesis. Targeting of proteins to the lysosome occurs by two major means: a vacuolar route via the Golgi [32] and a receptor-mediated direct uptake [33]. Both pathways are now sufficiently understood to allow the design of appropriate modifications of the transgene that would cause its targeting to the lysosome: the receptor-mediated pathway uses a consensus N-terminal amino acid sequence, whereas the vacuolar pathway operates by covalent attachment of mannose-6-phosphate, the site of attachment of which is again determined by local sequence. Thus, it could be quite simple to cause transgene-encoded enzymes to be appropriately targeted—particularly for enzymes identified in fungi, because the mechanisms of targeting to the vacuole are quite similar to those for mammalian lysosomes [34].

It is possible that a more severe challenge will be the response of the immune system. Transgenic enzymes would probably be targeted by the proteasome and fragments presented on the cell surface, just as occurs for most other proteins [35], so successfully transfected cells would be in danger of immune system attack as a result of their presentation of non-self peptides. Indeed, in some gene therapy trials, patients whose genetic defect is a point mutation have been preferred, to minimise the incidence of peptides that would be seen as foreign [36]. However, two approaches to tackling this problem are evident. First, the immune system can be induced to tolerate new proteins: extensive work on tolerisation strategies is ongoing, both for the treatment of patients with pathogenic deletion mutations and for xenotransplantation tolerance [37,38]. Secondly, the extremely slow accumulation of the substances that must be degraded indicates that the xenohydrolase need be expressed only at extremely low levels, possibly below the limit of detection by immune surveillance. A variation on this option would be to make expression only occasional and brief. This is particularly attractive because it could be implemented by placing the transgene under a drug-inducible promoter; this would allow expression to be combined with transient (and perhaps very robust) immunosuppression treatment, which would be discontinued after a lag period sufficient to ensure that xenohydrolase fragments were no longer being presented on the cell surface. Just one such "spring cleaning" could potentially reverse decades' worth of buildup of aggregates.

A further safety issue concerns the presence of these enzymes outside the lysosomal compartment. Any catabolic enzyme must be selective for its target material, not attacking anything functional. This is another argument for investigating fungi as sources of such enzymes, because the avoidance of cytosolic toxicity will already be built into them (possibly by pH-specificity, possibly by other means). Cytosolic toxicity is not the only potential problem: some mammalian lysosomal enzymes are detectable in the extracellular space, possibly not only "on purpose" [9] but also as a side-effect of the role that lysosomes have in repairing large-scale damage to the cell membrane [40]. pH-dependence of the enzyme's activity could well be adequately protective here too, however. Finally, it might be possible to engineer (or simply select) xenohydrolases that are synthesised as proenzymes that would be inactive until processed within the lysosome.

Conclusion

The restriction of the term "lysosomal storage disease" to early-onset conditions is unjustified: vastly the most prevalent disorders with a lysosomal storage etiology are late-onset ones. These include atherosclerosis, macular degeneration and probably diverse neurodegenerative disorders, as well as possibly some aspects of aging in general. Treatments for these diseases have been, and continue to be, energetically sought; nevertheless, they remain major causes of morbidity and mortality in all developed nations. The possibility of identifying enzymes capable of degrading the lysosomal aggregates that cause these diseases has yet to be explored. It is highly ambitious but its

enormous biomedical potential and apparent feasibility argue strongly for its expeditious investigation.

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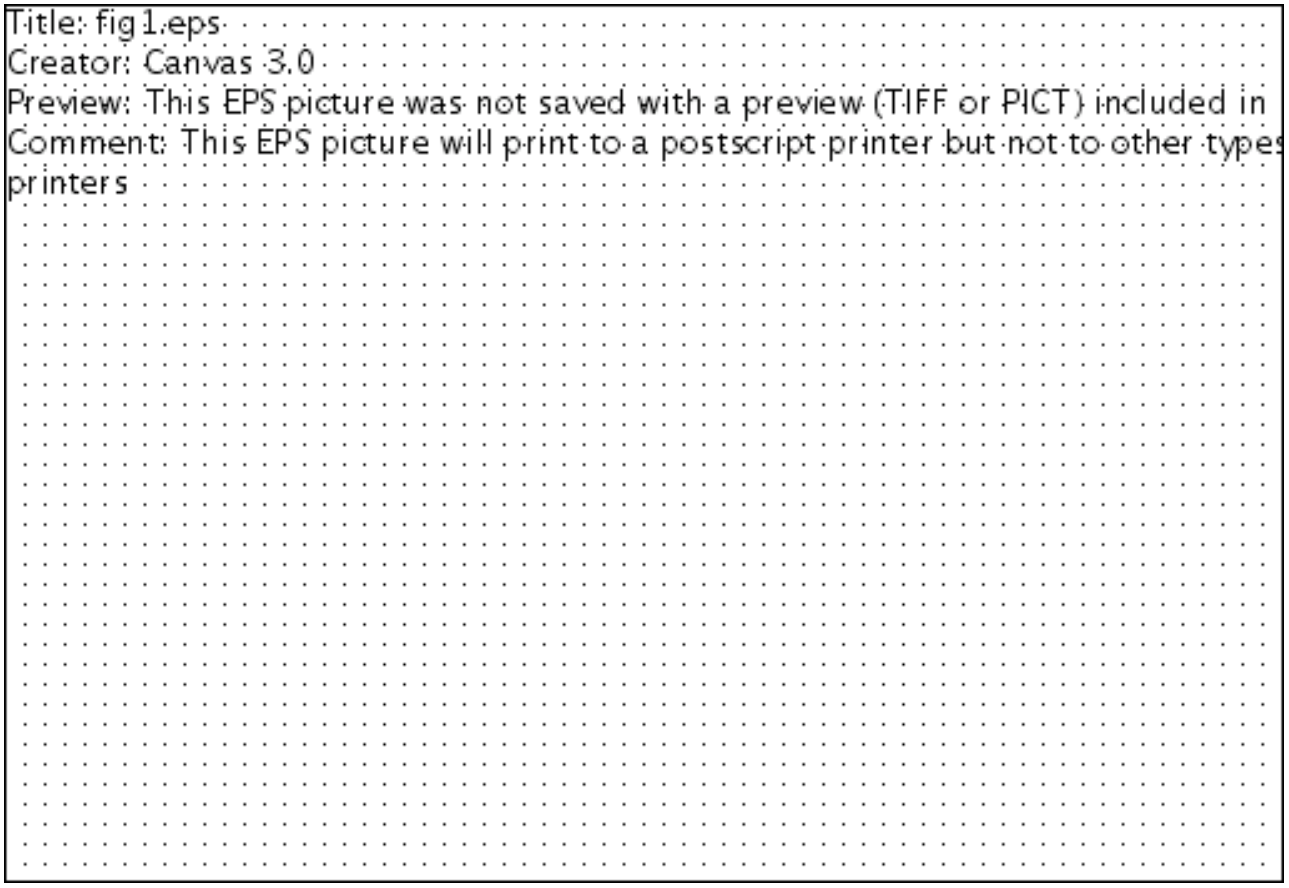

Figure 1. Cells perpetually target many types of macromolecule for degradation by various routes. The lysosome is the route of last resort; when its degradative capacity fails, the only remaining option is indefinite sequestration. Impairment of lysosomal hydrolysis in lysosomal storage diseases (LSDs) accelerates the accumulation of undegraded material and brings early death. Conversely, enhancement of lysosomal hydrolysis with xenohydrolases should retard and reverse this accumulation and might be a key component of a panel of interventions to prevent and cure major age-related diseases and extend longevity.