Alzheimer's, atherosclerosis, and aggregates: A role for bacterial degradation

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Abstract
Several of the most prevalent and severe age-related diseases, notably Alzheimer's disease and atherosclerosis, feature the accumulation of undegradable aggregates within the lysosomes of disease-affected cells. At an early point in disease progression, the breakdown of lysosomal contents by the resident catabolic enzymes stops working properly. A return of lysosomal enzymatic activity to pre-disease levels may restore aggregate elimination. In this review, a method of bioremediation-derived lysosomal enzyme enhancement is proposed, featuring the cellular introduction of microbial-isolated enzymes, or xenoenzymes. The benefits and challenges of using xenoenzymes to breakdown aggregates are discussed. As the size of our elderly population grows, the incidence of age-related diseases will increase, making necessary the exploration of radical, but potentially powerful, therapeutic strategies.

Key words
Bioremediation, atherosclerosis, Alzheimer's disease, lysosome, aggregate accumulation, lysosome storage disease, age-related storage disease, xenoenzyme.

Introduction
Mammalian cells possess numerous components that degrade damaged or otherwise unwanted proteins. These range from individual proteolytic enzymes present within the intracellular and extracellular environments, to the most versatile degradation organelle, the lysosome. The enzymatic arsenal present in lysosomes is normally able to break down virtually any substance present within cells. In some cases however, these substances become resistant to enzymatic action, or lysosomal function becomes impaired, leading to accumulation within lysosomes and, on a larger scale, accumulation within the affected tissue. This accumulation often triggers an inflammatory response, ultimately leading to diminishing cell and tissue function, and premature death.[1]

Traditional lysosomal storage diseases are characterized by a deficiency in either the production or functionality of a specific lysosome enzyme resulting in accumulation of undegraded product.[2] Most are rare, progressive and life-threatening, and the question now arises whether this term should also encompass more common "age-related storage diseases" characterized by a failure of lysosomal function and aggregate accumulation, such as atherosclerosis and Alzheimer's disease (AD).

A vast amount of evidence exists supporting the progressive impairment of cellular function and the subsequent inflammatory response, which characterize both atherosclerosis and AD. In both cases,

there is strong evidence specifically highlighting the role of aggregate accumulation in disease progression: cholesterol and its related molecules in atherosclerosis,[3] and the proteins tau and amyloid beta (A_) in AD.[4]

Age-related storage diseases: Atherosclerosis

Atherosclerosis is the underlying pathophysiology for the majority of heart attacks and strokes and to date, the primary focus of therapeutic approaches to preventing such events.[5] A characteristic finding in atherosclerotic plaques is the presence of foam cells - macrophages laden with cholesterol and cholesteryl esters. Within foam cells, much of the internalized cholesterol becomes trapped within the lysosome (Figure 1).[6] Regardless of how this happens, what is certain is that degradation of lipids becomes deranged within the atherosclerotic plaque,[7-10] and as the disease advances, lysosomal activity becomes ever more limited, promoting further lipid accumulation within the foam cell.[11] This can cause cell death and further exacerbate the problem.[12, 13] Any therapy that restores lysosomal function will inevitably help curb the transformation of the plaque into an unstable structure prone to rupture.

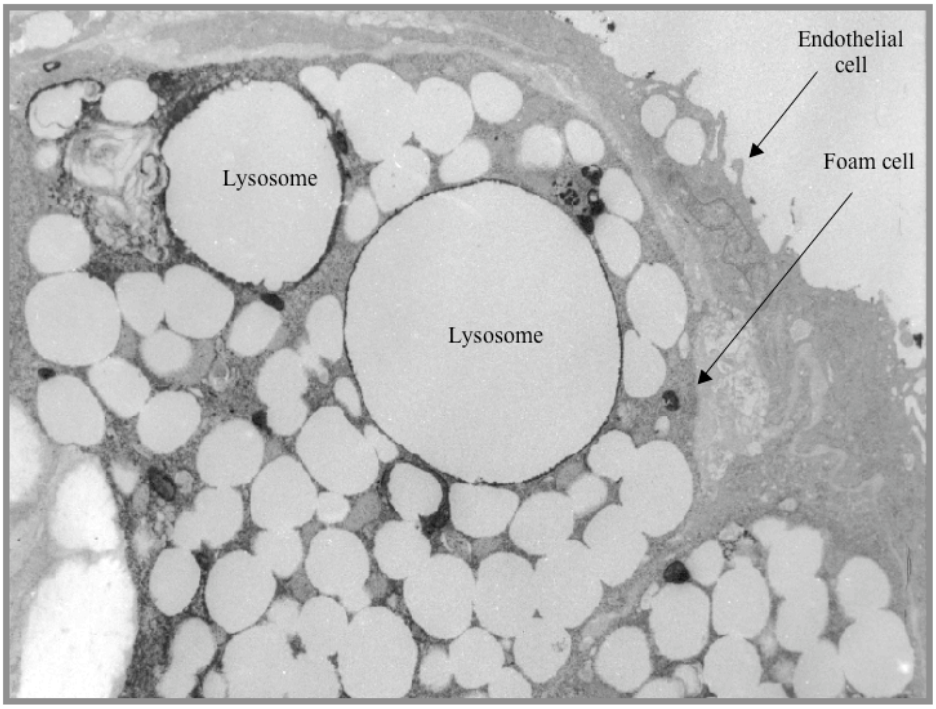

Figure 1. Electron micrograph of lipid-engorged lysosomes within a foam cell in an atherosclerotic plaque. The cytoplasm of the foam cell also contains the characteristic lipid droplets.

Age-related storage diseases: Alzheimer's disease

Alzheimer's disease remains one of the most common causes of debilitation in the industrialized world.[4] The defining characteristics of AD are the existence of extracellular _-amyloid plaques, whose main component is A_, and the intracellular neurofibrillary tangle (NFT), whose main constituent is the structural protein tau, in a hyperphosphorylated state.[4] How extracellular A_ causes cell death in AD is unclear, and a number of reports suggest intracellular A_ may be the culprit.[14-17] Transgenic mouse studies indicate synaptic, behavioral and physiologic functions decline in concert with accumulation of A_ in the brain, but before the appearance of A_ plaques,[14-16] and a post-

mortem analysis of human brain reported cognitive dysfunction best correlated with soluble A_, rather than its insoluble or extracellular forms.[17] Much of this intracellular A_ accumulates in the endosomal-lysosomal system of neurons (Figure 2).[18, 19] Endosome abnormalities have been identified as some of the earliest neuropathic features of AD, and may also be linked to cell death cascades.[20] Restoration of endosomal-lysosomal function may therefore correct a fundamental precursor ofAD.

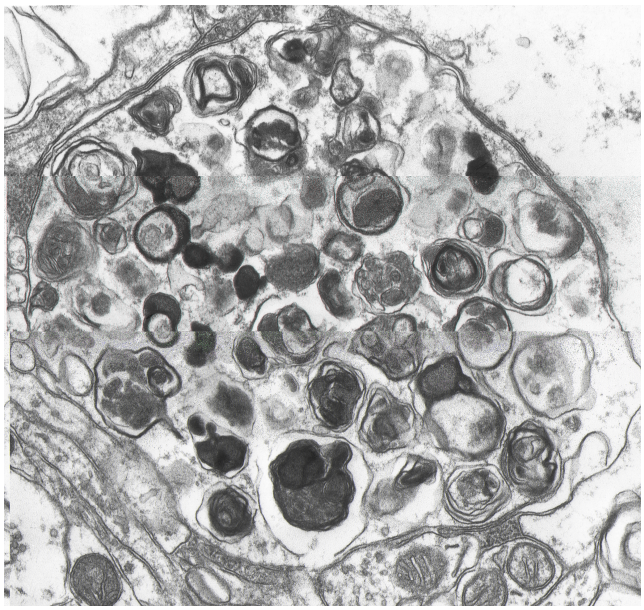

Figure 2. Electron micrograph of a dystrophic neurite, filled with incompletely degraded material in aberrant autophagosomes. Multilamellar structures may be serially autophagocytosed material. This state may result from failure of autophagosomes to fuse with lysosomes, a key step in degrading large structures such as mitochondria.

Lysosomal aggregates: A therapeutic target?

Lysosomes are powerful organelles of degradation and intralysosomal aggregate accumulation clearly hampers lysosomal activity, representing an early step towards cell dysfunction and death in these disease states.[12, 13, 19, 20] One therapeutic option in these age-related storage diseases, therefore, would be to focus on treatments that would be capable of either supplementing endogenous lysosomal activity to stimulate the degradation of lysosomal aggregates, or introducing enzymes that digest aggregates that are resistant to endogenous lysosomal activity. This is a viable therapeutic strategy based on a foundation of success, as enzymatic enhancement has been utilized for other lysosomal storage disorders, namely, Gaucher disease,[21] Fabry disease,[22] and mucopolysaccharidosis type I disease.[23] Presented below is a proposal describing a novel way to stimulate the degradation of lysosomal aggregates, by using enzymes derived from a rather unlikely, and unorthodox, source.[7, 24-26]

Bioremediation

The issue of undegradable aggregates in nature is not a new one. As our society has become more industrialized, the breadth and depth of waste products that exist in our environment has increased. Although both industry and consumers have begun to address this problem, as demonstrated by the relatively recent push for biodegradable consumer goods and community recycling programs, the

environmental accumulation continues. It has been noticed, however, that some of the discarded materials that normally would be considered to be non-biodegradable accumulate in the environment at a rate slower than would be expected.[27]

Where is this material going? It transpires this slower rate of accumulation is a result of degradation by specialized soil microbes. Soil is a haven for an immense variety of bacterial and fungal species and like other ecosystems, the competition for nutrients is fierce. Some microbes use "brawn" to flourish in an ecosystem, exploiting a higher growth rate than their competitors, while other microbes secure their niche by the use of "brain," developing the ability to digest materials that provide a sufficiently abundant energy source, but that competing organisms cannot access. This concept, first presented in 1952, is known as the "the microbial infallibility hypothesis."[28] Simply put, it postulates that any abundant, energy-rich organic material that is hard to degrade will provide the selective pressure necessary to evolve the machinery necessary to degrade it.

The soundness of this concept has been proven to such a degree that a flourishing subfield of the environmental decontamination industry has developed, termed bioremediation. Here, microbes isolated from contaminated post-industrial soil are screened for colonies that possess the ability to degrade the toxic substance. Usually bacteria, they are scaled-up in the laboratory and then returned to decontaminate the soil to a point safe for residential use.[7, 29] This procedure has been used successfully to decontaminate sites of TNT,[30] dioxins,[31] PCBs,[32] and rubber.[33]

So, how is this relevant to atherosclerosis and AD? First noted by the author in 2002,[25] a biomedical application for bioremediation technology was initially put forth with the realization that one specific type of ecosystem is enriched in human remains – graveyards. Although graveyards accumulate bones, they do not accumulate cholesterol or A_, suggesting the presence of microbes capable of metabolizing such age-related aggregates. The catabolic enzymes distilled from these microbes may thus possess intrinsic therapeutic value. In theory, through screening graveyard-isolated microbes it should be possible to uncover bioremediation agents capable of treating specific or multiple human lysosomal deficits by stimulating the endogenous degradation of lysosomal aggregates, or introducing xenoenzymes to digest the aggregates themselves. A grand plan – but how exactly would one carry out this process of "environmental decontamination *in vivo*?"

From bioremediation to biomedical application

It is important to note that the endpoint for this research is not the exposure of patients to the isolated microbes; several steps are necessary to move from environmental bioremediation to biomedical application in humans (Table 1). Albeit ambitious, the steps are conceptually straightforward, and the issues of feasibility and potential of such lysosomal enhancement by foreign biological material – or xenoenzymes – has been discussed in detail elsewhere.[7, 24-26] Briefly, microbes from the environment in question are cultured in an environment where the chosen target aggregate is the only energy source available. With starvation as the selection pressure, bacterial colonies that develop the ability to utilize the target aggregate will emerge, from which the enzymes responsible for digestion of the aggregates may be identified through various mutagenic, chemical, and genomic techniques. Once the activities of the isolated enzymes are confirmed in mammalian cell cultures, testing may be moved to animals. Finally, assay competence will be confirmed in mouse models of age-related storage diseases, and only then will the product be ready for testing in humans. Although only at the first stage of this process, results from very preliminary experiments suggest that isolation of such xenoenzymes may indeed be feasible.[24] Using artificial lipofuscin, which is a granular waste material that accumulates in the lysosomes of aged muscle, heart, liver and nerve cells, bacteria have

been isolated from a 17th Century plague site that possess the ability to grow using this material as their only energy source.[24] The journey towards the use of xenoenzymes to treat age-related storage diseases has clearly begun. However, many roadblocks still need to be overcome.

Isolate competent strains; select by starvation
Identify the enzymes (mutagenesis, chemistry, genomics)
Make lysosome-targeted transgenes, assay cell toxicity
Assay competence in vitro (more mutagenesis/selection)
Construct transgenic mice, assay toxicity in vivo
Assay competence in disease mouse models
Test in humans as for lysosomal storage diseases

Table 1. Necessary milestones for the development of a biomedical application for bioremediation-based products.

Xenoenzymes: Efficacy and delivery

Key issues that must be addressed before attempting human treatment are xenoenzyme efficacy and delivery: will these enzymes work in the human environment, and how do we get them to the sites of aggregate accumulation in sufficient amounts to exert a therapeutic effect?

With regard to efficacy, whether enzymes isolated from bacterial sources will retain their function in a mammalian environment may not be a significant concern. There already exist a number of examples of molecules that have been isolated and retain their activities, from both bacterial (e.g., β-galactosidase – a colorimetric indicator used in transgenic mouse development) and other non-mammalian sources (e.g., green fluorescent protein (GFP), from jellyfish).[34] In fact, the functional activity of the native compound has been improved in some cases through genetic mutations, such as enhanced GFP, a fluorescent protein derived from GFP but significantly brighter.[35]

When considering delivery of these xenoenzymes to the lysosomes within the cells of affected tissues, it is important to note that the amount of xenoenzymes needed to initiate lysosomal aggregate degeneration may be quite small. While the rapid and complete removal of the aggregates might require large quantities of the active enzyme, it is important to remember that the goal of this strategy is not aggregate annihilation, but to produce a therapeutic effect. All that is required is to "tip the balance" within the lysosome in favor of removal of the aggregate and then the biochemical synergy of existing lysosomal catabolic enzymes should be able to do the rest.

Regardless of the quantity required, the xenoenzymes must still be delivered to the lysosomes of affected cells. And again, the issue of delivering a foreign protein, or its host gene, into a specific cell is not unique to biochemical applications of bioremediation. To date three approaches to introducing xenoenzymes into the affected lysosome have been identified, which are discussed in greater detail elsewhere.[26] The first is to introduce the gene encoding the xenoenzyme into the cells by somatic gene therapy. Much progress has been made in viral-mediated gene delivery, including in neurons.[36-38] Targeting the xenoenzyme gene to the lysosome will require minor genetic alterations so that the correct protein signals are expressed to organize its delivery to the lysosome, and may be accomplished by chaperone-mediated autophagy – a receptor-mediated pathway that delivers various lysosomal substrates.[39, 40]

Another option would be to use *ex vivo* cell therapy, involving the introduction of the xenoenzyme gene into patient cells outside the body, which are then expanded and transplanted back into the patient. A significant advantage of this method is that cells can be screened prior to implantation to ensure that only those with the desired modification are introduced. While significant progress in this technology has been achieved in recent years, including a clinical trials involving implantation in the brain,[41] various technical issues must still be overcome before this can be considered a viable option.

A third method would be enzyme replacement therapy, involving injection of the purified xenoenzyme into the affected individual. An additional step in this procedure is to provide a signal to assist in the targeting of the xenoenzymes to appropriate affected tissue. This could be achieved through glycosylation, a process involving the addition of sugar moieties to the xenoenzyme surface to help the target tissue identify it as a "friendly" compound.[42] Thus, with appropriate glycosylation, targeted tissue uptake becomes attainable. This is already a well-established delivery technique used in the treatment of a number of lysosomal disorders.[43]

Safety: Avoiding side-effects

When bioremediation moves from basic and pre-clinical research to clinical testing, safety will be a real concern, given the origin of the active moieties. Some of these safety concerns can be anticipated, and are discussed below.

Immunogenicity

The development of inhibitory antibodies is an issue for many biological therapies, as can be seen in the fields of hemophilia and multiple sclerosis, for example.[44, 45] While immune reactions in current applications of enzyme replacement have been noted, they are initially mild and tend to decline over the years.[46, 47] This may be because enzymes introduced into the cell by endocytosis are not typically presented on major histocompatibility class I complexes, unlike those synthesized within the cell. Even when foreign proteins are expressed within a cell, however, the immune system can be trained to tolerate them, despite some initial immunogenic reactions. If gene therapy is ever to be applied therapeutically with any regularity, such tolerization is vital and is thus aggressively researched, with promising results.[48]

Toxicity outside the lysosome

The desired xenoenzymes are catabolic in nature, and for them to degrade essential cellular components in addition to their intended target aggregate would be disastrous. Luckily, this can be made highly unlikely. Even if lysosomal integrity were compromised, the xenoenzymes, selected for their efficacy within the acidic pH of the lysosomal environment,[49] would essentially be inactive once emerging into the neutral pH of the cytoplasm. Another ingenious safety trick provided by mammalian evolution is the way some catabolic enzymes are initially constructed: some mammalian lysosomal enzymes are synthesized as inactive pro-enzymes, and only become the active enzyme when a short peptide leader sequence is cleaved upon lysosome internalization.[50] Theoretically, such an approach could be incorporated into the final design of the xenoenzyme. Ultimately, the underlying strategy will be to choose xenoenzymes of the highest specificity for their intended target aggregate material.

Conclusion

As the size of our elderly population grows, the incidence of age-related diseases increases, and thus makes necessary the exploration of radical, but potentially powerful, therapeutic strategies.

Enhancement of lysosomal function may be the only promising approach to ameliorate the degradation of the accumulating aggregates associated with these serious diseases. A therapy derived from the field of bioremediation, featuring the use of xenoenzymatic activity, may be a suitable means to provide future lysosomal enzyme enhancement.

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