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Foreseeable pharmaceutical repair of age-related extracellular damage

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Abstract

Various molecular and cellular alterations to our tissues accumulate throughout life as intrinsic side-effects of metabolism. These alterations are initially harmless, but some, which we may term "damage", are pathogenic when sufficiently abundant. The slowness of their accumulation explains why decline of tissue and organismal function generally does not appear until the age of 40 or older. Aging is thus best viewed as a two-part process in which metabolism causes accumulating damage and sufficiently abundant damage causes pathology. Hence, a promising approach to avoiding age-related pathology is periodically to repair the various types of damage and so maintain them at a sub-pathogenic level. Some examples of such types of damage are intracellular and others extracellular. Several types of intracellular damage are highly challenging – sophisticated cellular and genetic therapies will be needed to combat them, which are surely at least 20 years away and maybe much more. Extracellular damage, by contrast, generally appears more amenable to pharmaceutical repair which may be feasible in a shorter timeframe. In this article, the major types of age-related extracellular damage and promising avenues for their repair are reviewed.

Introduction

The predominant view within biogerontology remains that technologies that can substantially extend the healthy human lifespan are still a long way off. The basis for this pessimism is that since aging is not an evolved process but rather a product of evolutionary neglect [1], it is inherently chaotic and as complex as metabolism itself. Although a newcomer to biology may feel that tremendous progress was attained in the past century with regard to understanding metabolism, biologists appreciate that this progress is purely relative, and that in truth much remains to be done to unravel metabolism's complexity. This has led most biogerontologists to the view that the only strategy to postpone aging in the foreseeable future is to elicit latent life-extension responses that we already possess, such as the response to caloric restriction (CR) [2,3]. (Just how beneficial CR itself, let alone its pharmacological emulation, will be in humans remains highly debatable [4].)

However, this is not a clear-cut proof that major postponement of aging is a distant prospect – indeed, the way to achieve that postponement may be in sight, albeit by no means imminent. One basis for this view is a scheme popularly known as SENS, for "Strategies for Engineered Negligible Senescence" [5-7], which seeks to sidestep the complexities both of metabolism and of age-related pathology by attacking the phenomena that link the former to the latter: accumulating molecular and cellular changes, hereafter

referred to by the simple term "damage", that are intrinsic side-effects of metabolism, accumulating in an ongoing manner from before birth but becoming pathogenic only after middle age. This approach derives its plausibility from two key observations: first, those intermediate phenomena are not nearly as complex as the causes and effects that they connect, and second, the innumerable and mostly undiscovered specifics of those connections – which aspects of metabolism are most to blame for which types of damage, and which types of damage are the most to blame for which types of eventual pathology – are irrelevant to the potential efficacy of periodically repairing all such damage, which is what the SENS scheme aims to do.

The types of damage that the SENS interventions seek to repair fall into seven classes, as shown in Table 1. This classification is based on the types of therapy likely to repair the damage, rather than the molecular nature of the damage itself. For example, mitochondrial mutations may most plausibly be obviated (not actually repaired, in this case, but their pathogenicity eliminated) by inserting suitably modified copies of the 13 protein-coding genes of the mitochondrial DNA into the nucleus that would maintain mitochondrial function if the presence of any mitochondral mutations [8,9]: this is clearly useless for nuclear mutations, even though the chemistry of the two types of damage is identical. It is nevertheless legitimate to consider "mitochondrial mutations" as a single category, because the repair is essentially the same in all tissues even though the details will surely vary between tissues.

Type of age-related damage	Suggested	Proposed repair (or obviation)
Cell loss, cell atrophy	1955	Stem cells, growth factors, exercise
Senescent/toxic cells	1965	Ablation of unwanted cells
Nuclear mutations/epimutations	1959/1982	"Whole-body Interdiction of Lengthening of Telomeres"
Mitochondrial mutations	1972	Allotopic expression of 13 proteins
Extracellular cross-links	1981	AGE-breaking molecules
Extracellular aggregates	1907	Immune-mediated phagocytosis
Lysosomal aggregates	1959	Transgenic microbial hydrolases

Table 1. The seven major categories of age-related "damage" (accumulating and eventually pathogenic side-effects of metabolism), dates in which their contribution to age-related decline was first suggested, and some ways to repair or obviate them.

This article will focus on the three SENS strands that are in a meaningful sense extracellular. These are of particular interest from the perspective of foreseeable therapeutic interventions, because they are (as will be discussed) further advanced than the other SENS components.

- One is extracellular by definition: indigestible aggregates that accumulate in the extracellular space and for which there is evidence of toxicity. The best-known such aggregates are the senile plaques seen in Alzheimer's disease (AD).
- The second is extracellular by virtue of our biology: covalent cross-links between functional proteins, typically (though not always) resulting from reaction with sugars. These reactions occur within cells too, but it seems likely that intracellular cross-linking can be neglected, because intracellular proteins do not survive for a significant fraction of the human lifespan except in the case of aggregates, which are not functional. Extracellular structural proteins, by contrast – those comprising the artery wall or the lens, for example – are not turned over, and cross-linking of those proteins can thus accumulate during life with eventually pathological consequences.
- The third is extracellular only in a narrow sense – it is intercellular. Cells that become actively toxic (via the secretion of toxic substances – cells that are toxic solely because of their uncontrolled division, namely cancer cells, are not addressed here) are an accumulating feature of aging. The requirement is to kill them rather than repair them, so for present purposes they are usefully thought of as

extracellular toxins to other cells. This also relates to a fourth SENS strand, mitochondrial mutations, because a leading hypothesis for the mechanism whereby these mutations contribute to aging is that they oxidise circulating material and thereby introduce toxins into mitochondrially healthy cells elsewhere.

In line with the philosophy just outlined, this article will focus on interventions that *reverse* the progression of these categories of damage and will only incidentally mention therapies that merely *retard* that progression. This is not least because the latter are more numerous than true "rejuvenation" interventions and have been the topic of excellent recent reviews [10-12].

Extracellular aggregates

As just mentioned, the best-studied extracellular aggregate associated with aging and age-related pathology is the senile plaque in AD. Various other aggregates also accumulate with aging (see Table 2) and will be surveyed below.

Aggregate (major constituent)	Organ/tissue affected	Suggested consequences
Abeta (Aβ)	Brain	Alzheimer's disease
Amylin	Pancreas	Type 2 diabetes
Transthyretin	Heart, others	Cardiomyopathy

Table 2. Some types of extracellular aggregates that accumulate with age and their suggested pathogenic consequences.

Senile plaques in Alzheimer's disease

The senile plaque is one of the two defining histological features of AD, the other being the neurofibrillary tangles found inside pyramidal neurons [13]. Unlike tangles, plaques are extracellular. Intense debate rages concerning whether these structures 1) cause the cognitive decline of Alzheimer's patients, 2) are harmless epiphenomena or 3) are positively beneficial in retarding the disease [14]. However, if they are beneficial, it must be the *aggregation* of their main component protein (amyloid beta, Aβ) that is beneficial and not the actual *aggregates*, as the latter are necessarily inert by virtue of their low surface-to-volume ratio. Thus, it is reasonable to pursue ways in which senile plaques could be removed.

A strategy for eliminating such aggregates is to dissolve them. However, this would increase the concentration of the monomeric or oligomeric proteins of which the aggregate was composed. If aggregation is indeed a defence against excessive concentrations of a toxic protein that (for whatever reason) cannot be cleared, reversing aggregation will be deleterious. Nonetheless, since aggregation may *not* be beneficial, it is legitimate to explore such approaches. Compounds currently being explored to dissolve senile plaques include beta-breakers [15], DAPH [16] and dopamine [17,18]. Alternatively, one might eliminate the aggregates without dissolving them. The only system yet developed for achieving this is to immunise against the aggregated material (in this case Aβ in AD), presumably causing microglia to internalise it and degrade it intracellularly. Success with this approach in mice was reported in 1999 [19] and led rapidly to clinical trials [20]. Unfortunately, 6% of the subjects developed encephalopathic side-effects [21], causing the phase IIa trial to be terminated prematurely. This side-effect was probably mediated by a T cell response [22], suggesting various refinements: most simply, rather than injecting Aβ and stimulating an immune reaction, one could induce "passive immunity" by injecting anti-Aβ antibodies, which would induce a B cell-mediated reaction [23]. A more sophisticated option is to create a chimeric protein consisting of a portion of Aβ known to be a B cell epitope but not a T cell one, linked to a T cell epitope that is a genuinely foreign protein [24]. This chimeric protein will then induce a full immune response involving both T and B cell activities, but the self-reactivity will be only by B cells.

It must be stressed that there is much more to do to repair a brain suffering from AD than to remove the senile plaques. The neurofibrillary tangles must also be removed; this may to some extent occur spontaneously on removal of the plaques, but a considerable residue remains [25]. Moreover, neurons that have died during progression of the disease must be replaced, either by stimulating endogenous neurogenesis [26] or by introducing stem or precursor cells that differentiate appropriately [27]. These challenges are beyond the scope of this paper; the intracellular aggregate issue is addressed elsewhere [28,29].

Other extracellular deposits

Type 2 diabetes is associated, in 90% of cases, with the extracellular accumulation of material mainly consisting of a 37-amino-acid peptide called islet amyloid protein or amylin [30]. Possible mechanisms for the production and deposition of amylin fibrils include the depletion of insulin, since insulin heterodimerises with amylin and prevents fibril formation [31]. This would create a positive feedback loop, because amylin fibrils seem to promote death of insulin-producing pancreatic beta cells [32]. As with Aβ, controversy surrounds whether soluble oligomers or actual fibrils, or both, are the toxic amylin species. There are as yet no reports of attempts to eliminate islet amyloid by vaccination. However, amylin fibrils have been found within macrophages, implying that islet amyloid is naturally phagocytosed and thus that stimulation of this process is plausible [33]. Unfortunately, this also implies that islet amyloid resists degradation within the macrophage lysosome; hence, enhanced lysosomal function [28,29] may be required to clear islet amyloid efficiently.

Immunoglobulin light chain also forms amyloidogenic fibrils. In this case the result is a disorder of plasma cells causing widespread deposition of amyloid [34]. Unlike AD or diabetes this is a disease with rapid progression and a mean survival time of only a year or two, because the source of the amyloidogenic peptide is a neoplastic and rapidly expanding plasma cell clone. Thus, the clone is the most direct therapeutic target but may be as resistant to elimination as other cancers, so alternative interventions, especially against the amyloid, merit exploration. Recently there has been progress in passive immunisation against immunoglobulin amyloid [35].

A protein particularly well-studied in respect of amyloid deposition is transthyretin. Many sequence variants are known that greatly accelerate the accumulation of transthyretin amyloid, leading to symptoms at an early age [36]. However, wild-type transthyretin is also somewhat amyloidogenic and causes senile systemic amyloidosis (SSA), in which amyloid accumulates in various organs in old age, particularly the heart [37]. SSA is seen in 25% of those over 80 but is normally asymptomatic; however, it can cause congestive heart failure, and it is a common cause of death in extremely elderly people [38]. Again, an immunological approach to clearance of this amyloid seems promising.

Extracellular cross-links

As noted above, the only intracellular proteins long-lived enough to accumulate cross-links are ones that have been transported to the lysosome for destruction but have resisted degradation. These proteins may certainly be harmful once abundant, but that problem can be addressed by complete destruction of the proteins in question, as discussed elsewhere [28,29]. The extracellular space, on the other hand, contains long-lived proteinaceous structures: examples are the artery wall and the lens of the eye. These structures often require elasticity to function (Table 3): for example, visual accommodation depends on the elasticity of the lens, and the elasticity of the artery wall is crucial to its ability to withstand the constantly oscillating blood pressure without tearing. This elasticity exists because of the regularity of the inter-polypeptide linkages that are introduced when the material is first synthesised. The non-enzymatic addition of ectopic, randomly-distributed covalent linkages between neighbouring proteins reduces this elasticity [39].

Material	Organ/tissue affected	Suggested consequences
Artery wall	Artery	Hypertension
Lens	Eye	Long sight
Renal basement membrane	Kidney	Nephritis

Table 3. Some extracellular structures in which extracellular cross-links accumulate with age and their suggested pathogenic consequences.

Glycoxidation-induced cross-links

Some protein-protein cross-links form from simple oxidation reactions. The best-known example is the dityrosine link [40]. In abundance, however, these structures are far outweighed by those deriving from a complex sequence of reactions termed glycoxidation [41]. This process begins with the reaction of a circulating sugar molecule, typically glucose or fructose, with the side-chain of an amino acid (typically lysine) to form a Schiff base. In the best-characterised variant of glycoxidation, this is followed first by an intramolecular rearrangement of the Schiff base to form an Amadori product and then by an oxidation event to form an advanced glycoxidation end-product, or AGE. (There are variant pathways in which cross-links are formed without an Amadori product intermediate [42].) The creation of the initial Schiff base is readily reversible, so that only a minority ever proceed to form Amadori products, and the Amadori rearrangement is also somewhat reversible. The AGE, by contrast, is highly stable. The cross-link problem arises because some of the possible oxidation steps involve a neighbouring amino acid and create a cross-link. The best-studied such structure is pentosidine, which is extremely stable and is also fluorescent [43].

Acid-labile glycoxidation-induced cross-links

However, pentosidine accounts for at most 1% of the total glycoxidation-induced cross-linking of structural proteins in vivo [44]. This has, naturally, led to a search for more abundant cross-link species. Unfortunately, that search has been hindered by technical realities: to identify a molecular structure within a dense proteinaceous material such as cartilage or lens, the standard first step is acid hydrolysis to disintegrate the material [45], and all but the most stable cross-links (such as pentosidine) are thereby destroyed before they can be identified, let alone quantified.

The impasse thus created has recently led researchers to methods that are less brutal to the tissue being analysed. These involve digestion with proteases, which cleave only the peptide backbone of the constituent proteins and do not require severely acid pH to function [46]. The products of this digestion can be analysed by mass spectrometry to identify those whose molecular weight does not correspond to one or a few unmodified amino acids. The digestion process requires up to six proteases, each with different reaction conditions.

Nonetheless, this approach has been successful. The first and (as yet) most significant breakthrough was Lederer's discovery of glucosepane, a bizarre structure featuring a seven-membered ring [47]. Glucosepane is roughly 100 times more abundant than pentosidine in various long-lived structural tissues. Quite recently, a second acid-labile structure named K2P was isolated from lens, in which it is even more abundant than glucosepane [48].

The alert reader may by now be growing restive at the lack of mention of interventions to repair extracellular cross-linking. The reason is that the cross-links mentioned thus far are not amenable to removal by any method yet found. There is hope, however, as will now be outlined.

AGE-breakers

Over a decade ago, a paper appeared in a rather obscure journal suggesting a reaction scheme for the formation of a particular type of AGE cross-link, the alpha-dicarbonyl linkage [49]. The possibility that

this result was more significant than its place of publication might imply was raised by the fact that its senior author was Anthony Cerami, the pioneer of the idea that glycoxidation might be a substantial contributor to mammalian aging. Sure enough, three years later a report building on this work appeared in *Nature* and attracted considerable attention [50]. The *Nature* paper reported that a new drug, *N*-phenacylthiazolium bromide (PTB), could do something never previously reported: it could actually break an AGE cross-link, rather than merely inhibit its formation.

The paper reported the effects of PTB on the ability of tail tendons isolated from diabetic rats to be digested by cyanogen bromide. This is a measure of the degree of cross-linking of the tendon and, when compared with young tendons, is a good estimator of the degree of non-enzymatic cross-linking in this essentially non-turning-over material. PTB caused old tendons to be nearly as easily digested as young ones. But critically, this was not the effect of lifelong administration of PTB to the rats, but rather a short immersion in PTB after isolation. In other words, PTB was genuinely breaking a large proportion of the non-enzymatically derived cross-links, rather than just inhibiting their formation.

Subsequent reports from the same group, which had by this time moved into the biotech sector as Alteon, confirmed and extended this initial study. PTB was rapidly improved into PMTC, phenacyldimethylthiazolium chloride, which is both more active and more stable. PMTC was for several years referred to by Alteon and others as ALT-711 and has recently been renamed alagebrium chloride. PMTC was found to rejuvenate glycoxidation-sensitive tissues in dogs [51] and monkeys [52], and quickly moved to clinical trials [53]. It has yet to advance to phase III trials, partly (and quite understandably) because the phase II trials performed thus far have investigated systolic hypertension and have suffered from a large placebo effect which precluded the statistical significance of PMTC's effect. A strong case can be made that loss of visual accommodation, which occurs with age largely as a result of cross-linking in the lens, is a phenomenon more likely to be incontrovertibly reversed by PMTC in humans.

The substrate for PMTC

Alteon and PMTC have had an additional difficulty, however: there is almost uniform skepticism within the mainstream academic glycoxidation community concerning what (if anything) PMTC actually does. While not doubting the published data, senior researchers are concerned not only at the failure of phase II trials to reach statistical significance but also at the chemistry involved. The initial report of this chemistry has not been followed up by evidence on important matters such as whether PMTC acts catalytically (discussed further below). Furthermore, alpha-dicarbonyl links have actually never been shown to occur *in vivo* (though they are plausible products of certain glycoxidation reaction sequences). Moreover, alpha-dicarbonyl linkages are predicted to be highly unstable, further challenging the theory that they are abundant enough (and accumulate enough with age) for their cleavage to be the true explanation for the physiological effects of PMTC [54]. Broadly speaking there are three schools of thought regarding PMTC among senior academics: 1) it does essentially nothing, 2) it inhibits but does not reverse the accumulation of cross-links, and 3) it breaks something or other but the published reaction scheme is incorrect.

The relationship between academics and biotech varies widely, of course, but this would seem to be a prime example of a stand-off that has benefited nobody. (Alteon have sought long-term partnerships with large pharmaceutical firms for at least six years without success.) All that can be done at this point is to offer a somewhat subjective – informed, but nonetheless necessarily personal – interpretation, as follows.

The theory that PMTC does essentially nothing [54] cannot be sustained. While the phase II clinical trials have failed to meet their pre-assigned endpoints, pre-clinical results [50-52] do not share this shortcoming.

The theory that PMTC only inhibits cross-link formation [55] is also inconsistent with pre-clinical data. Moreover, it appears also to conflict with clinical results, in that arterial stiffness returns after withdrawal of PMTC over a matter of weeks, orders of magnitude more rapidly than it initially emerged prior to

treatment [53]. The only explanation of this observation that seems available is that PMTC is truly breaking cross-links but that the resulting molecular structures are highly prone to reformation of the cross-links, such that newly-restored elasticity persists only as a result of repeated cycles of cross-link cleavage and re-formation.

[Note: in principle, the same kinetics could actually occur as a result of a purely cross-link-inhibiting action of PMTC, if there is a major class of cross-link that is *naturally* short-lived, spontaneously disintegrating and re-forming with a rather short half-life. In this case, the increase in stiffness with age could still result from a progressive rise in the equilibrium level of such links, and if PMTC simply binds (reversibly, but with slower kinetics) to the broken links and thus stops them from re-forming, the effect on reduction and reappearance of stiffness would be as observed. However, given the absence of any proposal for specific chemistry along these lines, this interpretation is highly speculative.]

The theory that PMTC is working as published a decade ago also seems doubtful, not least because the scheme described there consists of a release of intact PMTC following cleavage and thus predicts catalytic, superstoichiometric activity. This prediction has been tested independently [55] and was not supported. If PMTC is not catalytic, the explanation may be that it cleaves a cross-link and then remains attached to one side of the erstwhile link, with a slow but significant rate of dissociation that leaves the link in a state competent to re-form. This would be consistent with cross-link re-formation over weeks *in vivo* and also with stoichiometric consumption of PMTC in short-term assays in vitro.

Finally, the theory that PMTC is performing a radically different cross-link-breaking role than that published by Cerami and his colleagues also seems fragile, in view of the lack of alternative suggestions for its mechanism. In particular, none of the abundant cross-link species so far identified *in vivo* seems to be a plausible substrate for PMTC.

This appears to leave one plausible alternative to explain the data currently available: that PMTC is indeed cleaving alpha-dicarbonyl linkages (albeit perhaps not by the published mechanism), that these linkages really are highly abundant *in vivo*, and that they are stable enough *in vivo* to accumulate with age but not stable enough to survive enzymatic endoproteolysis (let alone acid digestion). Unfortunately, however, this hypothesis is not readily testable, since, to do so, one would require an analysis method gentle enough not to destroy these linkages before they are identified.

This situation might be changed, however, by the discovery of improved variants of PMTC that leave cleaved cross-links in a state where they will not re-form. The continuous administration of a drug – even one as free of side-effects as PMTC has so far appeared to be – is always inferior to the periodic administration of one whose effects last long after the drug has been withdrawn. A derivative of PMTC (or, alternatively, a separate drug co-administered with PMTC) that not only cleaved alpha-dicarbonyl linkages but also left them non-reactive would thus be a major therapeutic breakthrough. In scientific terms it would also be a major advance, because the cleaved structures would probably be distinctive both in structure and in molecular weight, allowing their facile identification. This, in turn, might identify (or at least give strong hints to) the precise type of cross-link that was originally present, the pathway by which it arose and the pathway by which PMTC cleaved it. At the least, it would reveal the abundance of the crosslinks that PMTC cleaves.

Other AGE-breakers and AGE-breaking strategies

An Indian company, Torrent Pharmaceuticals, have reported the development of a drug that cleaves biologically relevant cross-links and have partnered with Novartis to bring it to market. However, at the time of writing nothing has been published on this compound, so further comment is impossible.

A further topic of note in relation to protein-protein cross-links is the prospect of developing drugs that cleave structures more stable than alpha-dicarbonyl linkages. Glucosepane and K2P, being acid-labile and abundant, are more tempting targets than highly stable species such as pentosidine. A point in favour of the plausibility of finding such drugs is that, like alpha-dicarbonyl linkages, glucosepane and K2P are

dissimilar to any functional chemical structures seen in mammals, so even drugs with rather broad substrate specificity might be adequately benign. At this point, however, interest in seeking such drugs appears limited [56].

Enzymatic approaches to cross-link-cleavage are also conceivable and may be attractive if some highly abundant cross-links prove refractory to cleavage by adequately specific small-molecule drugs. Enzymes exist naturally that eliminate Amadori products by converting them back to Schiff bases – these have been termed "amadoriases" and are phylogenetically widespread, emphasising their importance [57] – but no enzymes are known that cleave any class of AGE cross-link. This is perhaps due to the unavailability of ATP in the extracellular medium, which precludes the coupling of a possibly quite severely endothermic reaction (the breaking of a stable cross-link) to the canonical exothermic one, ATP hydrolysis. A possible avenue to solve this is suggested by the remarkable protein MGMT, also known as ATase, which performs a DNA repair function that is non-catalytic. ATase transfers an alkyl group from guanine in DNA onto one of its own cysteine residues, and this is irreversible, so the protein is then degraded [58]. This "one-shot" strategy overcomes the thermodynamic barrier imposed by the stability of alkylguanine by creating a reaction product (alkylcysteine) that is even more stable and simply forgoing the highly endothermic step of restoring it to cysteine. In principle, a similar approach could be used to cleave "low-energy" protein-protein cross-links.

Toxic cells

The third and last SENS category that can be described as extracellular is the accumulation of cells that secrete substances toxic to other cells (Table 4). Such cells arise by various mechanisms, some of them as yet poorly understood. Hence, in this article, as with the whole of SENS, the emphasis in seeking foreseeable therapies is not on preventing the formation of such cells but on eliminating them.

Cell type	Organ/tissue affected	Suggested consequences
Visceral adipocytes	Abdominal cavity	Type 2 diabetes
Clonally expanded T lymphocytes	Circulation, spleen	Immunosenescence
Senescent cells	Many tissues	Cancer
Mitochondrially mutant cells	Mainly post-mitotic cell types	Oxidative stress

Table 4. Dysfunctional and possibly toxic cells that accumulate with age and their suggested pathogenic consequences.

Replicative senescence and similar phenotypes in vivo

The phenomenon of replicative senescence discovered by Hayflick [59] was initially observed in dermal fibroblasts, the cell type in which it is still most frequently studied. Novices sometimes presume that this is evidence for the *in vivo* relevance of replicative senescence, since the skin is a continually renewing tissue, but this is of course an error, as only the epidermis is continually renewing: the dermis, by contrast, comprises cells that are generally quiescent and divide only on stimulation, such as to seal a wound. The dermis of an elderly individual does not, therefore, consist of cells close to their limit of replicative potential (their "Hayflick limit"): indeed, they are on average imperceptibly closer to that limit than those in young dermis [60]. Furthermore, continually renewing tissues such as the epidermis or the blood are maintained by stem cells that are thought to divide considerably more often in a human lifetime than the 50-90 divisions of which fibroblasts are capable. They achieve this by expressing low levels of telomerase, an enzyme that adds copies of the 6bp sequence TTAGGG to the ends of chromosomes [61]: this compensates for the "end-replication problem" [62,63] that progressively shortens chromosomes with each cell division and has been shown to be the sole cause of replicative senescence in various human cell types (including dermal fibroblasts) [64,65]. Hence, many biogerontologists now doubt that replicative senescence plays any role whatsoever in human aging.

However, cells resembling those that have reached replicative senescence *in vitro* are in fact found in human tissues. A beta-galactosidase with a low pH optimum, which is diagnostic of the senescent state *in vitro* and has thus been termed senescence-associated (SA) beta-galactosidase, is seen at elevated levels in occasional cells in various human tissues [66] and is abundant in one tissue, the articular cartilage [67].

It currently seems likely that these cells predominantly arise not from excessive cell division and concomitant telomere shortening but from DNA damage, specifically double-strand breaks. The senescent phenotype can be induced *in vitro* by irradiation [68] and is thought to be triggered by chromosome breakage. Moreover, cells expressing high SA-beta-galactosidase are seen in mouse tissues too [69]. This is significant because mice are extremely unlikely to have any cells that have become senescent because of excessive cell division, for two reasons: their telomeres are about five times as long as human telomeres, and they express telomerase ubiquitously.

Whatever their origin, however, since such cells do exist *in vivo* and accumulate with age, we must consider whether they may be harmful, and if so, what to do about them. In tissues where they are rare, they may nonetheless be harmful, because they secrete proteins that may promote tumorigenesis. The senescent cell cannot itself become cancerous, since by definition its division is permanently arrested, but division of neighbouring cells may be stimulated by these secreted molecules, and if those cells are already pre-cancerous this may trigger an additional mutational event on the road towards the full neoplastic phenotype [70].

Eliminating senescent cells

There are formally two ways to reduce the number of senescent cells in a tissue: kill them or "de-senesce" them. In practice these two options may overlap, because the senescent phenotype is often characterised by resistance to apoptosis: since senescence is a response to damage, and since apoptosis is a common response to the very same type of damage (DNA damage), a cell released from the senescent state may promptly undergo apoptosis. Killing rare cells is unlikely to compromise their host tissue even if compensatory cell division does not occur. However, where senescent cells are abundant, as in cartilage, there may be a need to stimulate proliferation of remaining cells or of precursors in order to restore appropriate cell number for tissue integrity.

Killing a senescent cell can be achieved by the same types of technique as for any other cell identifiable by its gene expression. There are two major options. The first is immunotherapy: to trigger the immune system to react to some diagnostic protein that is particularly highly expressed in the cell type of interest and eliminate those cells by virtue of the fragments of that protein that are presented on the cell surface by class I MHC molecules. This might initially seem likely to have severe side-effects, as the cells in question are necessarily expressing only "self" proteins and an immune response might thus attack many healthy cells too. In fact, however, there is a threshold level of systemic expression below which this does not occur, as demonstrated by the fact that, for example, telomerase can be used as a nearly universal tumour antigen despite being expressed at trace levels by stem cells [71]. Nonetheless, no work attempting to eliminate senescent cells by this method is known to the author at present.

The second generic approach to killing cells that express high levels of a given protein is to introduce (by somatic gene therapy, for example) a "suicide gene" encoding a highly toxic protein such as thymidine kinase, but under a promoter that will be activated only in the presence of the diagnostic protein. This approach can be developed and refined first *in vitro* and then by germ-line transformation in mice, and work is ongoing to implement it [72].

De-senescing senescent cells

Reversing the senescent phenotype has proceeded somewhat further. The senescent phenotype appears to require the expression of p53 or p16, since inactivation of p53 allows cells to resume replication when levels of p16 are also low [73]. (Interestingly, even when senescence is induced by telomere shortening, it cannot be reversed by introducing telomerase.) This intervention has not yet been demonstrated *in vivo*, however.

Mitochondrially mutant cells

For reasons that remain incompletely understood (though hypotheses abound [74]), loss-of-function mutations in the mitochondrial DNA (mtDNA) sometimes exhibit a selective advantage in the continuous mitochondrial turnover that occurs in non- or slowly-dividing cells [75,76]. This results in cells becoming entirely deprived of oxidative phosphorylation capacity by virtue of being taken over by a clonal expansion of copies of a mutant mtDNA sequence at the expense of wild-type mtDNA. In some tissues, such cells probably die quite rapidly and are replaced by cell division. In others, however, they appear to survive indefinitely. This survival must require some remarkable metabolic acrobatics on the part of such cells, possibly via a scheme [77] (since termed the "reductive hotspot hypothesis", RHH [78-80]) whereby they may survive by up-regulating plasma membrane electron transport. This is a process that might generate superoxide on the outer surface of the cell, oxidising circulating material such as low-density lipoproteins, which could in turn be systemically toxic. Accordingly, if this model is correct, mitochondrially mutant cells are within the category under discussion in this section, toxic cells.

Eliminating mitochondrially mutant cells

The system on which such cells are proposed to rely for their internal redox homeostasis, the plasma membrane redox system (PMRS), is ubiquitous [81] but not considered likely to be vital for mitochondrially healthy cells, at least not in the short term. Thus, a drug that inhibits the PMRS should be fatal to mitochondria mutant cells within a timeframe that has minimal side-effects on mitochondrially normal cells (but see below). Immunotherapy may also be an option, since some PMRS components are present only at low levels in most cells [82].

A major potential drawback of the above strategy presents itself, however. Because skeletal muscle fibres are so long and thin, and also because mitochondria within muscle fibres are relatively immobile, the clonal expansion of mutant mtDNA proceeds only slowly within an affected fibre, gradually expanding along it from an initial OXPHOS-negative zone but only ever reaching a few millimetres in length – a small fraction of the total length of a typical muscle fibre [83]. This may mean that PMRS inhibitors might have severe side-effects on muscle mass: death of an OXPHOS-negative segment could sever the fibre, separating half of it from the neuromuscular junction and thereby causing death of that half even though it has normal mitochondria. Indeed, the spontaneous atrophy of OXPHOS-negative muscle fibre segments has been reported [84] (though, as noted above, it appears to be a stochastic event that many fibres avoid indefinitely) and has been proposed as a substantial contributor to sarcopenia [85].

Restoring OXPHOS to mitochondrially mutant cells

Therefore, there is a case for exploring ways to remedy the metabolic defect in mitochondrially mutant cells rather than killing them. Various options have been proposed.

One is to reverse the selective advantage enjoyed by mutant mtDNA. In mitochondriopathies (diseases typically characterised by a high abundance of mutant mtDNA molecules all of which carry the same lesion), this may be achievable by sequence-specific inhibition of mtDNA replication, for instance using peptide nucleic acids [86]. In normal aging, however, even though each affected cell is taken over by copies of a single mutation, different cells have different mutations, so this seems inapplicable. A more practical approach may be to exploit the fact that mutant mitochondria have a reduced proton gradient (though not zero, because the net consumption of ATP within the mitochondria and consequent reversal of direction of the phosphate and adenine nucleotide carriers generates a residual proton gradient). A modest proton gradient is necessary for import of the several hundred mitochondrial proteins that are encoded by nuclear genes. Hence, a mild inhibitor of protein import might decisively impede biogenesis of mutant mitochondria while affecting normal mitochondria only minimally. In cells lacking wild-type mitochondria this would probably lead to cell death, but in muscle fibres it might – if the dose can be well controlled – allow the affected segment to be progressively repopulated by proliferating wild-type mitochondria from either end.

The remaining approaches will require considerable advances in somatic gene therapy before they can be therapeutically relevant, so they are only briefly described here. Repopulation of mutant mitochondria with functional mtDNA [87] faces the challenge that any successfully rejuvenated mitochondria will surely be lost as fast as they are made, by the same mechanism that clonally expanded the mutant species in the first place. An ingenious way around this is to engineer the new mtDNA so that it possesses an additional gene encoding a restriction enzyme that cuts normal mtDNA, and also lacks that enzyme's restriction site [88]. Such mtDNA would, in theory, be able to destroy and replace all native mtDNA (whether mutant or wild-type).

A second option is to restore the electron transport aspect of OXPHOS without the ATP-generating component. The idea here is that cells bearing mitochondrial mutants are toxic not because they are short of ATP, but only by virtue of their need to export electrons at a high rate in order to maintain internal redox homeostasis. If intramitochondrial NADH could be recycled using intracellular electron acceptors, excessive electron export would not occur. Some yeasts possess a single-polypeptide enzyme that performs the same electron-transport function as Complex I (i.e. NADH-ubiquinone oxidoreductase oxidising intramitochondrial NADH) but which does not pump protons. This enzyme (which is naturally nuclear-coded) has been expressed in mammalian cells and found to rescue their viability in medium that does not support growth of control cells lacking complex I: the residual proton-pumping of complexes III and IV suffices [89]. Similarly, plants' alternative oxidase bypasses complexes III and IV by acting as a non-proton-pumping ubiquinol oxidase [90]. Cells expressing both these enzymes could thus maintain internal redox stability in the presence of any mtDNA mutation. Their combined activity in the normal cell population would uncouple their mitochondria and be lethal, but the rarity of OXPHOS-negative cells *in vivo* means that if selective and modest expression only in those cells can be achieved this is a plausible therapeutic strategy to render such cells harmless.

Finally there is the option, known as allotopic expression, of inserting the 13 protein-coding genes of the mtDNA into the nuclear genome, with alterations so that their products would be directed to the mitochondria, imported using the same machinery that imports naturally nuclear-coded mitochondrial proteins, and assembled into the OXPHOS enzyme complexes as normal, thus rescuing the mtDNA mutation [8]. Unfortunately, these proteins are very hydrophobic and consequently resistant to import. However, recent advances, both in *bona fide* allotopic expression in CHO cells [91] and in the identification of these genes in species in which they have been transferred to the nucleus during evolution [92-94], justify confidence that allotopic expression is a feasible goal in the foreseeable future *in vitro* and thereafter *in vivo*.

Visceral adipocytes

Though less conspicuous than subcutaneous fat, excess adipose tissue within the abdominal cavity (generally termed visceral fat) appears to be more prejudicial to health. It releases hormones that interfere with insulin signalling and promote insulin resistance and diabetes [95]. As such, it appears to be a major mediator of the health risks associated with obesity.

Eliminating visceral fat

Surgical removal of visceral fat from diabetic rats rapidly and comprehensively eliminates their pathology [96]. Whether the same intervention would reverse diabetic complications that had arisen over a much longer period during normal aging is unclear, of course, but this result is at least highly suggestive.

Surgical removal is not an attractive option for treating humans, however. While liposuction is a viable therapy for subcutaneous fat, the visceral fat coats the major abdominal organs and may thus be hard to remove mechanically. However, the immunotherapy and suicide-gene approaches described above for senescent cells seem potentially feasible here.

Rendering visceral fat benign

Recently Unger's group demonstrated the possibility of restoring visceral adipocytes to a benign state: they found that high doses of leptin caused visceral fat to activate mitochondrial energy metabolism, rapidly burning fat stores and ceasing to secrete hormones thought to be responsible for insulin resistance [97].

Anergic T and B cells

As noted earlier, stem cells of continuously renewing tissues express trace levels of telomerase. Interestingly, transit amplifying cells (those undergoing differentiation after stem cell division) generally exhibit rather higher telomerase activity [98]. This is perhaps to be expected, since they are dividing more rapidly than the stem cells themselves, but it is more paradoxical when one recalls that after perhaps a dozen divisions such cells become post-mitotic and short-lived (with the exception of T and B cells, discussed below). This may indicate that haematopoietic and epithelial cells (including stem cells) express telomerase only during mitosis.

Thus, maybe other cell types undergoing rapid division might also activate telomerase even when it was previously suppressed. A conspicuous example is the lymphocyte: specifically T and B cells, which expand rapidly on stimulation by their cognate antigen. These cell types indeed express telomerase while expanding, and not when unstimulated [99].

This raises a paradox, however. Humans' tight telomerase regulation is thought to be an anti-tumour mechanism: since in humans (unlike mice) tumours must divide >>100 times before reaching a clinically relevant size, they must activate telomerase in order to be lethal. (In fact, 5-10% of tumours activate an alternative and poorly-characterised method of telomere extension termed ALT [100], but that is beyond the scope of this review.) This would clearly fail if simple stimulation of cell division promptly turned telomerase on! Accordingly, telomerase activation must be strictly tissue-specific and regulated by other signals in addition to those that stimulate cell division. Moreover, there must be robust mechanisms to re-suppress telomerase as soon as rapid cell division ends. Such signals are presently unknown.

The T and B lymphocytes are examples of such cells. One class, the cytotoxic T cell, is of interest here, because certain antigens – especially various herpesviruses, and most especially the cytomegalovirus (CMV), which infects most humans – are not fully eliminated from the body by the immune system and continue to stimulate proliferation of their cognate T cells throughout life. This results in enormous clonal expansion of anti-CMV T cells [101]. The presence of these clones appears to suppress the whole immune system, possibly by monopolising the "immunological space" (a hypothetical limit on the total circulating T cell population) [102].

Eliminating large anergic T cell clones

Ostensibly, this problem can only be addressed by somehow eliminating the CMV infection. However, these clones are not only very large: they are also inactive [103]. They do not divide readily in response to antigen, partly because of diminished reactivation of telomerase and consequent shortening of telomeres, and are referred to as anergic. Hence, a viable option may be to target such cells for apoptosis; far from allowing CMV to resurface, this would allow new anti-CMV cells (and, indeed, cells reactive to other new antigens) to proliferate without hindrance.

Rejuvenating anergic T cells

Restoring normal function to anergic T cells is also plausible. Their resistance to proliferative stimuli may not be the appropriate target, as restoration of proliferation (by, for example, stimulating telomerase activity) may promote tumorigenicity. However, the anergic phenotype may itself be a target. Anergic cytotoxic ($CD8^+$) T cells are distinguished by loss of the cell surface marker CD28 [104]. Constitutive re-expression of CD28 may restore function of these cells. Whether it would also restore (finite) proliferative capacity has yet to be determined.

Conclusion

Aging consists of a vast array of interacting processes. Postponing aging by "cleaning up" metabolism so that these processes occur more slowly is thus a daunting challenge. The SENS approach to postponing aging is based on the observation that this complexity, and our lack of knowledge thereof, can largely be side-stepped by attacking not the deleterious processes themselves but their immediate consequences, the lifelong accumulation of molecular and cellular changes that are pathogenic once they exceed a certain threshold. While treating the downstream pathologies themselves is a losing battle, the initiating damage is altogether simpler in nature and hence in amenability to repair. Among the various types of such damage, those involving the extracellular space (whether directly, in the form of aggregates or cross-links, or indirectly, as intercellular toxicity via contamination of the extracellular milieu) appear at present to be the more amenable to intervention by drugs that are either already in development or trials or are feasible targets for development in a reasonably short timeframe. The postponement of aging will without doubt be by far the biggest biomedical market in history; accordingly, now is the time for those with the appropriate resources to position themselves to profit from it by the development of relevant drugs.

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