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Extracellular Glycation Crosslinks: Prospects for Removal

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ABSTRACT

Extracellular aging-accumulating molecular damage by glycation, oxidation, and crosslinking of long-lived extracellular proteins, mainly collagen and elastin-is a major cause of several important human aging pathologies. Crosslinking increases mechanical stiffness of blood vessels and urinary bladder. Crosslinking impairs the functioning of the kidney, heart, retina, and other tissues and organs. Glycation adducts trigger inflammatory signaling, provoking tissue damage and cancers. Crosslinking tightens up the extracellular matrix (ECM), hardening it against natural turnover processes. Known crosslink breakers (e.g., alagebrium, of the thiazolium halide family) are only partly effective because they break only a subset of AGE crosslink structures (sugar-derived α-diketone bridges). So far, no agent has been found that breaks the prevalent glucosepane and K2P crosslink structures. Enzymes that would be able to recognize and disassemble glycation products may be too big to migrate into the ECM and repair collagen or elastin *in vivo*. Two approaches to therapy development are presented here. ECM *turnover enhancement* would enhance natural processes to digest old ECM and replace it with new. It will be important to tune the collagen degradation to a rate slow enough to prevent dire side-effects, such as hemorrhage from leaky blood vessels as collagen molecules are removed and replaced. *Glycation breaker discovery* would use high-throughput screening and rational drug design to find molecules that are able to break glucosepane crosslinks and K2P crosslinks of extracellular proteins. Candidates would be further screened for selectivity and toxicity in order to avoid damage to other molecules.

INTRODUCTION

EVERYONE IS GETTING OLDER. Human aging is a grave problem. Each minute humans live, the stuff between the cells, the *extracellular matrix* (ECM), undergoes gradual changes that increase progressively over time. This extracellular aging is an important mechanism in the interrelated processes of human senescence. Over time, chemical and mechanical changes accumulate in persistent materials surrounding the cells that profoundly affect the cells themselves, as well as their growth, development, and even death. Changes in mechanical properties affect many aspects of physiology: blood flow, lens focusing and cataracts, glaucoma, bladder distensibility, joint function, and erectile function. Chemical changes can trigger destructive inflammation and even cancer.[1] The proteins, collagen and elastin, in the extracellular matrix (ECM), stay in place for a very long time. Molecular modifications can remain unrepaired and accumulate with age.

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It is now apparent that some types of accumulating chemical modifications are damaging to human physiologic functioning. Accumulating *extra*cellular molecular modifications play major roles in the etiologies of age-associated physical declines and illnesses. As the molecular mechanisms become clearer, opportunities and directions for goal-oriented research are coming into view. This paper presents an overview of the problems and prospective solutions.

EXTRACELLULAR MATRIX AND ITS STIFFENING WITH AGE

Between cells, tissues are held together by an extracellular matrix (ECM) of protein strands, glycoproteins, and proteoglycans, bathed in blood or lymph. The structural protein strands can stay around for many years, and they are exposed to harsh chemicals that increasingly modify them over time, adversely affecting their physical properties. The most abundant protein in ECM is *collagen*. It is found in several variants throughout the body, principally as a strong, straight structural fiber. Another important structural extracellular protein fiber is *elastin*, whose wrinkled meshwork provides elastic properties that are very important to tissues.

Reactive sugars in the blood and lymph chemically attack proteins. Most of this sugar is glucose, which has an aldehyde group at one end that spontaneously bonds to an amino group of a protein, usually at a lysine residue. This covalent bonding is called *glycation* to distinguish it from beneficial, enzymatically controlled glycosylation of proteins (Fig. 1).[2–4]

The sugar hanging onto the protein is called an *adduct*. Over time, glycation adducts on a protein chain may covalently bond to a second protein chain, forming a permanent *crosslink* between the protein chains.

Although collagen and elastin are strengthened by beneficial crosslinks[3,4] that do not involve sugar, the additional glycation adducts and crosslinks interfere directly with the mechanical properties of structural collagen and elastin fibers, generating pathogenic consequences. Glycation crosslinks bind together adjacent protein strands, reducing flexibility and elasticity of the tissue. Atherosclerotic plaque formation involves glycation crosslinks between endothelial proteins and circulating proteins and fragments in the blood.[2]

Extracellular matrix turnover

In most tissues, ECM is digested and replaced by cells such as fibroblasts. The turnover half-life of collagen is characteristically different in each different human tissue.[5] To the extent that ECM is digested, removed, and replaced, any chemical modifications or damage, such as glycation, also would be removed. However, crosslinks and adducts *mechanically* restrict the ability of fibroblasts, chondrocytes, and enzymes to turn over ECM. In contrast, sugar is a *small* molecule, able to fit in narrow spaces between collagen strands. When it crosslinks them, they are drawn even more tightly together, hardening and stiffening the collagen. Enzymes are much larger molecules than sugars. Some digestive enzymes can chew into the collagen from the outside, but any crosslink-breaking enzyme is physically restricted from access to its target.

As the number of crosslinks increases over time, the collagen fibrils are pulled more tightly together, making the ECM less accessible to cells and enzymes that are attempting to digest and turn over the ECM. Consequently, the turnover rate will slow down, which will extend turnover time further, allowing more time for more crosslinks to form. This vicious cycle causes an exponential increase in crosslinking with age, exactly what is found in human skin, cartilage, and lens.[5]

The population of fibroblasts in each person also changes in character as that person gets older.[6] Fibroblasts may decline in their ability to turn over collagen. Thus, even as accumulating crosslinks are making collagen tougher with age, the fibroblast population may be losing their ability to replace it.

In terms of abundance, almost all glycation crosslinks so far found (or inferred to exist) in humans are one of the three following molecular structures:

1. Glucosepane[5]
2. α-Diketone linkers[2]

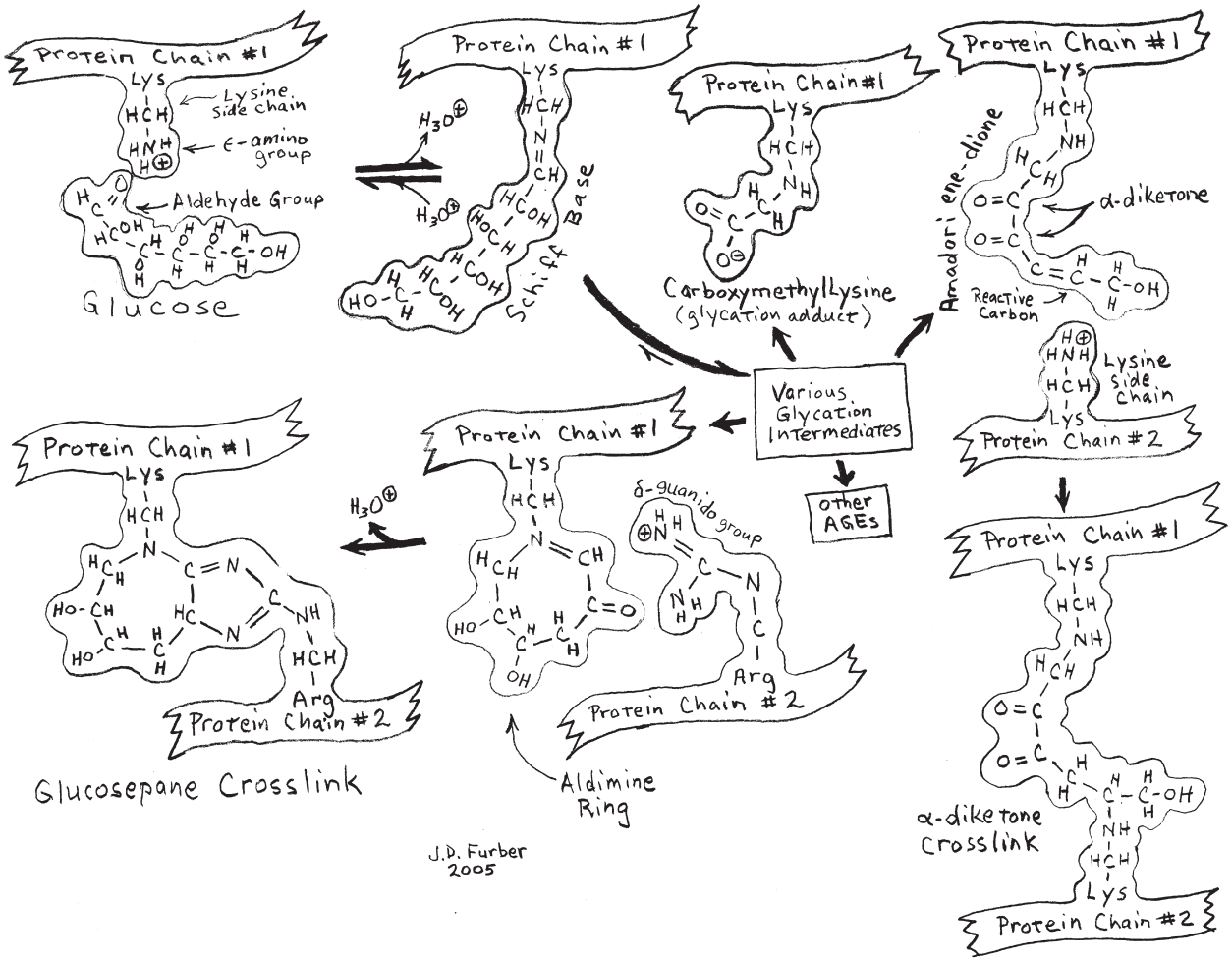

FIG. 1. Glycation and crosslinking: reactive aldehyde group on glucose attacks reactive ϵ-amino group on lysine residue in protein chain #1, forming an unstable *Schiff base,* which may either come apart or proceed to form various intermediates and end products. One intermediate is the *Amadori product*, which may rearrange to form the *Amadori-ene-dione,* which can attack another lysine ε-amino group in protein chain #2, forming a stable, covalent crosslink that binds the two protein strands together. This is the structure of the *α-diketone crosslink*. Another intermediate forms as the sugar adduct cyclizes to an intramolecular aldimine, which then is attacked by an arginine δ-guanido side chain in protein chain #2, forming the stable *glucosepane* crosslink. *Carboxymethyllysine* (CML) forms by several pathways. Other advanced glycation end products (AGEs) are less prevalent and less important.

3. Lysine-dihydropyridinium-lysine (L2P or K2P), which accumulates in the lens of the eye[7]

So far, breakers have only been found for the α-diketone linkers. Small molecules have been made based on the catalytic activity of vitamin B_1, thiamine. These molecules incorporate the nucleophilic catalytic active site on a thiazolium ring, and attach a second nucleophilic center spaced a short distance away.

These two nucleophiles attack in tandem the adjoining carbonyl carbons of the α-diketone linkers, resulting in breakage of the crosslink.

The Alteon drug *alagebrium*, formerly designated *ALT-711*, or *3-phenyl-oxoethyl-4,5-dimethyl thiazolium chloride*, is the only crosslink breaker currently in clinical trials.[2]

Plan A: small-molecule breaker development. With only one out of three of the major crosslinks broken, that is, α-diketone crosslink breakers, it would be valuable to develop small-molecule breakers for glucosepane and K2P crosslinks. A problem like this could be approached with high-throughput screening (HTS).

Two assays are created with easily detected model crosslinks, one for glucosepane and one

for K2P. Thousands of chemical entities are tested against these assays. Any chemicals that show effectiveness would be studied to see if modifications would work better. The next step is to look for side-effects or toxicity, starting *in vitro* and in cell culture, next in small and then large animals, and finally in humans.

However, what are the options if new small-molecule breakers are not quickly found?

Research in three other areas may provide one or more alternative therapies for the crosslink problem.

Plan B: natural turnover stimulation. Fibroblasts and mesenchymal lineage cells have the innate ability to turn over collagen and elastin. The natural turnover time is many years in most human tissues. It is reasonable to expect that ways can be developed to stimulate them to increase the turnover rate. This might involve fibroblast stem-cell therapy. An autologous fibroblast from the patient might be given various growth factors in culture, and processed to modify its gene expression. A large number of copies of the stimulated fibroblast would be grown up in culture and injected into the patient.

Alternatively, it might be possible to inject fibroblast-targeted growth factors into the patient, to stimulate the activity of the patient's own fibroblasts while still in the body. If done correctly, the revitalized fibroblasts would move through collagen, digesting the old collagen as they go, and leaving freshly synthesized collagen in their wake, all properly arranged, without generating disorganized scar tissue, and without making holes in arteries or other vital structures. If elegantly designed, a fibroblast enhancement therapy also might ameliorate other pathologies of aging fibroblasts, including their dedifferentiation and secretion of inflammatory and cancer-promoting molecules.

Plan C: combination breaker therapy. Develop or discover large-molecule, enzymatic breakers for glucosepane and K2P. Then perhaps alagebrium can loosen up the ECM enough to allow these new larger molecules access to *their* targets. Working together, small diketone breakers and large glucosepane and K2P breakers might *jointly* restore the collagen to its youthful state.

Plan D: nanobots. Design and build billions of little machines to do what fibroblast and mesenchymal lineage cells do, only better. If realized, these tiny engineered devices would be able to chew through collagen, leaving fresh collagen in their wake, all properly arranged.

In the meantime, exercise, fasting, and low blood sugar levels are good for your collagen.

Kjaer and colleagues have shown that vigorous exercise induces rapid collagen turnover in human tendons.[8] Other tissues remain to be studied. Fasting and low blood sugar levels slow the rate of glycation.

Glycation inhibitors

A number of compounds are being studied as glycation inhibitors. Some are prescribed for diabetes. The easiest to obtain and least likely to cause adverse reactions are fruits and vegetables, which contain vitamins B_1, C, and E, curcumin, *N*-acetyl-cysteine, EGCG (black tea, green tea, white tea), PABA, quercetin, resveratrol, rutin, or thyme.

Parallel development to maximize early success

1. High-throughput screening for small-molecule breakers of glucosepane and K2P
2. Stimulation and acceleration of natural ECM turnover processes, such as fibroblast activity
3. Rational design or screening for enzymatic breakers of glucosepane and K2P (to be used in combination with small-molecule diketone breakers)
4. Development of nanobots to turn over old collagen and replace it with new collagen

CONCLUSION

Like exercise and fasting, glycation inhibitors and agents which lower blood-sugar levels will help to slow the rate of glycation, easing the urgency of collagen turnover. Nevertheless, crosslinks accumulate over time, causing pathologies, unless crosslink breakers are developed or ECM turnover is enhanced to match

the rate of AGE formation. Extracellular rejuvenation, in combination with other therapies to reverse other aspects of human aging, will work together to rejuvenate the whole person. A major research and development effort is required to create these therapies and make them available to people and pets sooner rather than later.

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