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Corresponding Author	Family Name	**Furber**
	Particle	
	Given Name	**John D.**
	Suffix	
	Division	
	Organization	Legendary Pharmaceuticals
	Address	PO Box 14200, Gainesville, FL, 32604, USA
	Email	johnfurber@LegendaryPharma.com

Abstract

Accumulating extracellular molecular modifications play major roles in the etiologies of age-associated physical declines and illnesses. The most important changes are caused by glycation, lipoxidation, cross-linking, and cleavage of the long-lasting extracellular structural proteins (LESPs): collagen, elastin, fibronectin, and laminin. A series of reactions results in several stable structures referred to as “advanced glycation endproducts” (AGEs) and “advanced lipoxidation endproducts” (ALEs). LESP modifications contribute to debilitation in several ways: stiffening and weakening tissues, inciting inflammatory damage, and creating an unhealthy environment for the body’s cells. Some amelioration and postponement of LESP aging can be achieved through dietary composition choices, fasting or calorie restriction, and ingesting foods, herbs, or substances that inhibit glycation or lipoxidation. Exercise and crosslink-breaking substances can repair some damage, thus producing partial rejuvenation. Proposals have been made to look for additional crosslink breakers and deglycators to destroy the full range of AGEs. This author anticipates that repair and rejuvenation of a wide range of extracellular aging and damage may be achieved by stimulating fibroblast-lineage cells to more rapidly turn over and regenerate the extracellular matrix.

Chapter 19
Repairing Extracellular Aging and Glycation

John D. Furber

Contents

J.D. Furber (✉)
Legendary Pharmaceuticals, PO Box 14200, Gainesville, FL 32604, USA
e-mail: johnfurber@LegendaryPharma.com

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19.1 Introduction: Pathologies Caused by Aging Extracellular Proteins

Stiffness, arthritis, and cataracts have long been associated with aging humans and other mammals. In recent decades, important biochemical bases of these, and other, progressive age-associated pathologies have been identified. They are caused, at least in part, by accumulating chemical modifications to long-lasting structural proteins in the extracellular matrix (ECM) (Kohn 1978; Cerami et al. 1987; Vasan et al. 2001; Verzijl et al. 2003; DeGroot et al. 2004). Over time, chemical and mechanical changes accumulate in long-lasting extracellular structural proteins (LESPs), profoundly affecting the growth, development, and death of cells, as well as the mechanical operation of bodily systems. The LESPs stay in place for a very long time. Molecular modifications can remain unrepaired, and accumulate with age. It is now apparent that several types of accumulating chemical modifications are especially damaging to human physiological functioning. Extracellular aging is a major player in the interrelated processes of human aging (Cerami et al. 1987; Robert et al. 2008).

Chemical reactions, importantly glycation, lipoxidation, oxidation, nitration, amino acid isomerization, each change the LESPs in the ECM, as do protein strand breaks, wound healing, scar (cicatrix) formation, photoaging of the skin, and the actions of macrophages, infections, and inflammation. Important consequences include:

- Changed mechanical properties of tissues
- Changed environmental niches for cells, which affect their health and development
- Vicious cycles of progressively increasing damage.

Three processes are especially significant causes of pathogenic LESP modifications: glycation, lipoxidation, and strand breaks (Cerami et al. 1987; Januszewski et al. 2003; Robert et al. 2008). Glycation, formerly called “nonenzymatic glycosylation”, is the spontaneous covalent bonding of a sugar to a macromolecule, such as a protein (Eble et al. 1983; Bucala and Cerami 1992). Lipoxidation occurs when oxidation of lipids produces reactive lipid fragments that covalently bond to proteins (Miyata et al. 1999). The chemical group attached to the protein is referred to as an “adduct”. The phrases, “advanced glycation endproducts” (AGEs) and “advanced lipoxidation endproducts” (ALEs) have been used to describe the wide array of chemical species that eventually result from glycation and lipoxidation reactions (Cefalu et al. 1995; Januszewski et al. 2003). Glycation and AGEs have been studied for many years in connection with diabetic complications and physiological senescence. More recently, the Baynes lab pointed out that lipoxidation pathways also create some of the same damaging endproducts (Januszewski et al. 2003; Miyata et al. 1999).

AGEs and ALEs have been established as strong contributors to many progressive diseases of aging: vascular diseases (such as atherosclerosis, systolic

hypertension, pulmonary hypertension, and poor capillary circulation) (Cerami et al. 1987; Bucala and Cerami 1992; Vaitkevicius et al. 2001;Vlassara and Palace 2003), erectile dysfunction (Usta et al. 2004, 2006); kidney disease (Vasan et al. 2001; Vlassara and Palace 2003), stiffness of joints and skin, osteoarthritis (deGroot et al. 2004; Verzijl et al. 2003), cataracts, retinopathy (Vasan et al. 2001), peripheral neuropathy (Bucala and Cerami 1992), Alzheimer's Dementia (Ulrich and Cerami 2001; Perry and Smith 2001), impaired wound healing, urinary incontinence, complications of diabetes, cardiomyopathies (such as diastolic dysfunction, left ventricular hypertrophy, and congestive heart failure) (Bucala and Cerami 1992), and solid cancers and metastasis (Taguchi et al. 2000).

In nondiabetic people, LESP aging occurs very slowly. This is consistent with our understanding that these age-associated diseases occur late in life because passage of time is required for sufficient damage to accumulate on LESPs. It is noteworthy that these same diseases emerge at an earlier age in diabetic individuals, whose average blood sugar and lipid concentrations are higher than normal, thus driving the deleterious reactions faster (Cerami et al. 1987; Bucala and Cerami 1992; Januszewski et al. 2003).

19.2 Normal Functions of the ECM

Our bodies are constructed of cells and extracellular materials. "The **extracellular matrix** consists of macromolecules secreted by cells into their immediate environment. These macromolecules form a region of noncellular material in the interstices between the cells" (Gilbert 2000). Some authors also refer to soluble extracellular materials as the "aqueous phase of the matrix" (Fawcett 1986). The structural molecules of the ECM include proteins, glycoproteins, and proteoglycans. The ECM holds cells together and co-creates the microenvironments in which they live (Spencer et al. 2007). It includes noncellular portions of bones, cartilage, tendons, and ligaments, as well as epithelial basement membranes, the renal glomerular basement membrane, and the fibrous meshworks that give strength to blood vessels, skin, tissues, and organs.

The most abundant protein in extracellular matrix is collagen. It is found in several variants throughout the body, principally as strong, straight structural fibers, providing strength to bones, cartilage, and tissues. Type IV collagen is a flat sheet that forms basement membranes.

Another important extracellular protein is elastin, whose wrinkled meshwork provides elastic properties to tissues. Elastic fibers are assembled extracellularly from elastin and several glycoproteins (Shifren and Mecham 2006). Elastic fibers form a shock-absorber to the hemodynamic pulses of the cardiovascular system. The resilience of lung tissue, arteries, and skin are due to elastic fibers (Wagenseil and Mecham 2007).

Laminin, vitronectin, and fibronectin are extracellular proteins that are important in cell adhesion, differentiation, and migration over the ECM. Integrin receptors

on cells attach to a conserved sequence of amino acids: arginine-glycine-aspartate (RGD sequence), which is part of these proteins (Gilbert 2000). The composition of the ECM influences gene expression and differentiation state in resident cells. Signals are sent to cell nuclei through receptor pathways and via cytoskeletal contacts (Spencer et al. 2007).

19.3 Maintenance and Turnover of the ECM

Natural cellular processes slowly replace the aging collagen (Bucala and Cerami 1992). The natural turnover and remodeling of ECM proteins occurs at differing rates in various tissues during aging. The average turnover time of collagen is characteristically different in each different human tissue (Sell et al. 2005). Turnover can remove aged ECM and replace it with new, undamaged ECM. However, in many human tissues, the rate of turnover is slower than the rate of AGE accumulation. Furthermore, elastic fiber repair or replacement is imperfect; there is clearly an accumulation of damaged elastin with age (Robert et al. 2008; Wagenseil and Mecham 2007; Shifren and Mecham 2006).

Turnover requires removal of old molecules and replacement by new molecules *in the proper arrangement*. To the extent that old ECM is digested, removed, and replaced, some of the chemical modifications or damage, such as glycation or isomerization, would be removed and digested or excreted to the urine (Bucala and Cerami 1992; Ahmed and Thornalley 2003; Vlassara and Palace 2003). The complex details of ECM degradation are reviewed elsewhere (Robert et al. 2008; Murphy and Reynolds 2002; Everts et al. 1996). Cells of the fibroblast lineage (FLCs), in the connective tissue, degrade and replace LESPs. FLCs include fibroblasts, chondrocytes, osteoblasts, adipocytes, smooth muscle cells, macrophages, and mesenchymal stem cells (MSCs) (Alberts et al. 2002). FLCs can secrete digestive enzymes that cleave the collagen strands so that the resulting fragments may be phagocytosed and digested further within lysosomes (Everts et al. 1996; Murphy and Reynolds 2002). Additionally, vascular endothelial cells and renal mesangial cells may participate in AGE elimination by endocytosis (Vlassara and Palace 2003). After phagocytosis and intracellular digestion, some low-molecular-weight glycated molecules may be released to the circulatory system and cleared through the kidney (Vlassara and Palace 2003).

New collagen molecules are synthesized inside the fibroblasts, as three peptide chains which twist together, like a rope, into a triple helix, stabilized by hydrogen bonds and disulfide bonds (Lodish et al. 2000; Alberts et al. 2002; Piez 2002). These rodlike procollagen molecules are secreted, by exocytosis from Golgi vesicles, into the extracellular space, where their ends are trimmed off. Fibroblasts pull and arrange them into place as intermolecular electrostatic and hydrophobic interactions guide the assembly of collagen fibrils, which can aggregate into larger collagen fibers. After assembly, collagen molecules and fibrils are stabilized and strengthened by dilysine crosslinks (Lodish et al. 2000). These beneficial crosslinks are

formed under regulated enzymatic control, and result in the mature collagen fibers. Similarly, elastin molecules are held together by beneficial di-, tri-, and tetralysine crosslinks, which are enzymatically formed after elastin strands are extruded into the ECM (Shifren and Mecham 2006; Mathews and van Holde 1990). Later, over the years, very slow processes of non-enzymatic glycation form additional crosslinks and adducts, which are pathogenic, and which accumulate over the lifetime of the collagen and elastin fibers.

Data indicate that the rate of formation of new AGEs and crosslinks per gram of collagen is the same among all of the human tissues studied. Therefore, differences in accumulation of glycated residues are apparently due to differences in collagen turnover rates of the different tissues (Verzijl et al. 2000; Sell et al. 2005). Consequently, it has been possible to use glycation accumulation to estimate turnover times for collagen in various other tissues. The results correlate well with turnover times calculated by measuring racemization of aspartate residues in collagen (Verzijl et al. 2000). Sell and colleagues reviewed collagen turnover rates in discussing their own measurements of glycation crosslinks (Sell et al. 2005). Kidney glomerular basement membrane (GBM) appears to turn over fairly quickly compared with skin, which has collagen molecules more than 15 years old. Collagen in articular cartilage reportedly has a turnover half-life of between 60 and 500 years (Verzijl et al. 2000). Sivan, et al, report a turnover half-life of cartilage in human intervertebral disks of 95 years in young adults, but turnover slows to 215 years in older adults (Sivan et al. 2008).

As the number of glycation crosslinks increases over time, the collagen fibrils are held more tightly together, making the ECM stiffer and perhaps less accessible to fibroblasts, macrophages, and enzymes that might attempt to digest and turn it over (DeGroot et al. 2001c). Furthermore, some AGEs, such as the abundant adduct, N-ε-carboxymethyllysine (CML), trigger apoptotic signals in the fibroblasts (Alikhani et al. 2005). The fibroblast population declines in number over the years, and many fibroblasts become "senescent." Senescent fibroblasts do not turn over ECM properly. Not only do they synthesize less ECM proteins but, they secrete excessive amounts of inflammatory cytokines and matrix metalloproteinases (MMPs), which digest ECM proteins without replacing them properly (Campisi 2005; Benanti et al. 2002). Similarly, articular chondrocytes decline in number and slow their production of proteoglycans, contributing to osteoarthritis and deterioration of articular cartilage (Taniguchi et al. 2009; DeGroot et al. 1999).

These events reduce ECM turnover rate, which extends turnover time, thus allowing more time for more AGEs and crosslinks to form (Vater et al. 1979; DeGroot et al. 2001a, b). These factors appear to create a vicious cycle of slowing the turnover rate (DeGroot et al. 2001c). Observations show an exponential increase in crosslinking with age in human skin (Sell et al. 1993, 2005 1993), cartilage (Verzijl et al. 2000), and lens (Cheng et al. 2004). In contrast, crosslinking increases very gradually with age in kidney GBM because the LESP turnover rate there is rapid enough to avoid a vicious cycle (Sell et al. 1993, 2005).

The rate of collagen turnover in human tendons and skeletal muscles is increased by physical exercise, as described in Section 19.5.2 (Kjær et al. 2006). Orthodontists

have long noted that fibrous joints and bone undergo increased remodeling in response to mechanical stress (Murphy and Reynolds 2002).

Inflammation induces a less desirable form of ECM remodeling. FLCs secrete additional digestive enzymes, including MMPs, to rapidly open up the ECM (Everts et al. 1996). Their purpose is to allow immune cells to move through the tissue, to search for pathogens. This rapid, inflammatory digestion of ECM is not restored as perfectly as during normal turnover and remodeling.

Scar formation is a form of ECM remodeling occurring during mammalian wound healing. It has evolved to be rapid, to mend tissues and stop fluid loss, but the resulting collagen cicatrix patch is not a perfect match to the surrounding tissue.

19.4 Age-Related Deterioration of the ECM: Anatomy, Chemistry, Structures, and Mechanisms of ECM Pathologies

A variety of processes change the LESPs during aging. Sugar, lipids, and oxygen react with ECM proteins to produce adducts and crosslinks, which we refer to as AGEs/ALEs. These reactions are variously referred to as glycation, glycoxidation, glyco-oxidation, nonezymatic glycosylation, and lipoxidation. Receptor molecules on cell surfaces react to AGEs/ALEs, triggering harmful inflammatory responses. During aging, some cells inappropriately attack the ECM by secreting extracellular proteases. LESP turnover also slows because the FLCs senesce and decline in number. Meanwhile, excess fibronectin molecules accumulate, at least in mouse skin (Labat-Robert 2004). Basement membranes thicken (Kohn 1978). Slow chemical reactions convert several protein residues to other amino acids, which may affect local shape and charge of the protein. Various serum proteins aggregate to form extracellular (EC) protein deposits referred to as amyloid. In some regions of the aging brain, protein fragments of the amyloid precursor protein (APP) aggregate extracellularly to form EC deposits, called "β-amyloid plaques", which are often associated with Alzheimer's disease.

19.4.1 Glycation Pathways

Glycation is the spontaneous covalent attachment of a sugar to a macromolecule, such as protein, phospholipid, or DNA. Occurring without the need for enzymatic facilitation, glycation is quite distinct from the beneficial, enzymatically controlled, glycosylation of proteins, glycoproteins, and proteoglycans. Interstitial fluid allows reactive sugars from the blood to diffuse to protein strands of the ECM, where a complex network of spontaneous reactions takes place, as reviewed in many references (Monnier et al. 2003; Ulrich and Cerami 2001; Rahbar and Figarola 2003; Metz et al. 2003; Furber 2006).

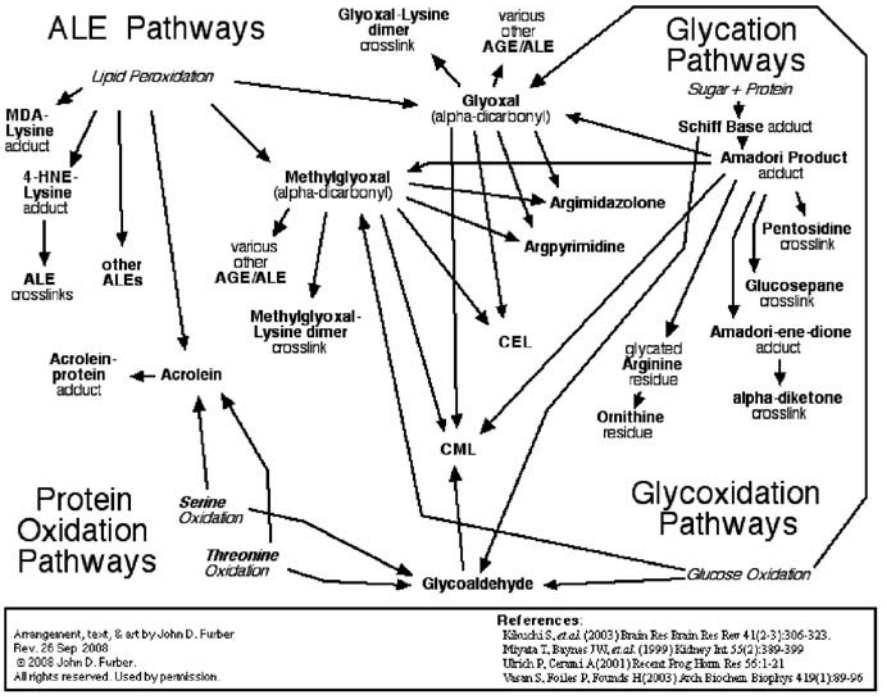

Fig. 19.1 Chemistry of ECM protein aging

The initial reaction is frequently a covalent bonding between glucose and a side chain of lysine in the protein strand (Eble et al. 1983). (See Fig. 19.1) The open-chain form of glucose has a reactive aldehyde group which attacks the reactive ε-amino group of the lysine side chain. These two groups join to form a Schiff base (Cerami et al. 1987), causing loss of lysine's positive charge.

glucose + (lysine in protein) ==> Schiff Base ==> Amadori product ==>
==> various intermediates and endproducts

The initial Schiff base is unstable and reversible, so often the glucose detaches, leaving the protein unchanged. But sometimes, the Schiff base rearranges its bonds, resulting in various structures called Amadori products. The Amadori products are also unstable, and so many revert back to the Schiff base. The rest undergo further reactions and rearrangements over time to form various stable end products, called AGEs (Cerami et al. 1987). Some of the intermediate products are quite reactive. The conversion of Amadori products to final, stable AGEs sometimes proceeds by bonding with other reactive species. The Amadori adduct on a glycated protein will sometimes bond to a reactive side group of a nearby protein chain. In this case, the former sugar becomes a permanent covalent crosslink between adjacent protein

chains or between domains of a folded protein. Several pathways are illustrated in Fig. 19.1.

Glucose is not the most reactive sugar (Ulrich and Cerami 2001), but it is by far the most abundant sugar in the blood (Cerami et al. 1987). Collagen is the most abundant ECM protein. A variety of different AGEs and AGE-crosslinks are formed in tissues via a complex brew of interacting reactions. Oxidation is involved in some of these reactions. Sometimes, glycated arginine decomposes to become ornithine (Sell and Monnier 2004).

Transition metal ions, such as copper and iron, increase the rate of glycation, probably by producing hydrogen peroxide and free radicals (Sajithlal et al. 1999; Xiao et al. 2007). Many glycation intermediates and end products, such as CML and N-ε-carboxyethyllysine (CEL) (Fig. 19.1) bind transition metals, generate free radicals, oxidize proteins and lipids, and accelerate additional glycoxidation reactions (Saxena et al. 1999; Requena and Stadtman 1999).

A variety of crosslink structures have been produced in vitro from glycated proteins and amino acids. Many of them have been found in vivo, as well . Chemically identifying crosslink structures has been difficult because some analytical procedures can destroy most AGEs before they can be characterized (Bucala and Cerami 1992; Biemel et al. 2002). At our present state of knowledge, almost all of the pathogenic extracellular glycation crosslink structures that accumulate in humans during aging appear to be one of two kinds: α-diketone crosslinks (Ulrich and Cerami 2001; Ulrich and Zhang 1997), or glucosepane (Biemel et al. 2002; Sell et al. 2005). The proposed reaction pathways forming these crosslinks are illustrated in Fig. 19.1.

The α-diketone crosslink is believed to form after a sugar adduct transforms into an Amadori ene-dione, which can attack the side chain of a lysine, cysteine, or histidine residue on a nearby protein chain. The crosslink contains two adjacent carbonyl carbons, forming an α-dicarbonyl structure called an α-diketone crosslink (Ulrich and Cerami 2001).

Glucosepane is an AGE crosslink formed between a glycated lysine residue in one protein chain and an arginine residue in a nearby chain. The side chain of arginine has a reactive δ-guanidino group, which can react with oxoaldehydes and other electrophiles. Glucosepane forms after a sugar adduct transforms into the dicarbonyl glycation adduct, dideoxyosone, which cyclizes and is attacked by the reactive guanidino group of a nearby arginine side chain. These covalently bond, forming the crosslink, glucosepane (Biemel et al. 2001).

In the condensation reactions of glycation and crosslinking, the positive charges on the lysine and the arginine are lost.

19.4.2 Lipoxidation Pathways

Oxidation and fragmentation of lipids can result in several reactive small molecules that can covalently bond to protein residue side chains. The Baynes lab has pointed out that lipoxidation reactions have some common intermediate species

with the glycation pathways, and can also result in some of the same endproducts (Januszewski et al. 2003). Important reactive intermediates common to glycation and lipoxidation are glyoxal and methylglyoxal, as illustrated in Fig. 19.1. CML and CEL adducts are common to both the AGE and ALE pathway. In contrast, other ALE protein adducts are produced by lipoxidation, but not by glycation, such as 4-hydroxynonenal-lysine (HNE-Lys) and malondialdehyde-lysine (MDA-Lys) (Miyata et al. 1999).

19.4.3 Amino Acid Isomerization, Deamidation, and Oxidation

Asparagine (L-Asn), an uncharged residue, can deamidate, via a series of reactions, to become negatively charged aspartate (L-Asp or D-Asp) or isoaspartate (L-IsoAsp or D-IsoAsp) (Clarke 2003; Shimizu et al. 2005). The change in charge or shape might have some effect on the properties of LESPs, but this has not been reported. By similar pathways, L-Asp can isomerize to D-Asp or to L-IsoAsp or D-IsoAsp (Clarke 2003; Shimizu et al. 2005). This can affect integrin binding, discussed in Section 19.4.6. Other pathological consequences of these changes have been proposed (Ritz-Timme and Collins 2002). Shimizu has observed that amyloid-β peptides in Alzheimer brains contain high levels of IsoAsp in place of Asp, and suggests that this might result in abnormal folding and deposition of β-amyloid in plaques and vascular amyloids (Shimizu et al. 2005).

Racemization of aspartate residues has been used to estimate LESP turnover rate in various tissues and at various ages, as was noted in Section 19.3 (Verzijl et al. 2000). Over time, increasing amounts of D-Asp can be detected in collagen and elastin protein chains (Ritz-Timme and Collins 2002; Sell and Monnier 2004). Although humans have an endogenous intracellular enzyme, PCMT1 or PIMT, which can reverse some of these conversions in intracellular proteins (DeVry et al. 1996; Clarke 2003), it is largely unable to access and repair ECM isomerization. Small amounts of PIMT are released into the ECM at sites of injury, but it cannot travel far into the matrix and does not reach most isomerized residues (Weber and McFadden 1997).

Proteins can be oxidized to create AGE/ALE adducts without the presence of sugar or lipids. During inflammation, macrophages produce EC hypochlorous acid in their immediate vicinity, which can oxidize nearby serine and threonine residues, resulting in acrolein, glycoaldehyde, and CML, as shown in Fig. 19.1 (Anderson et al. 1999; Miyata et al. 1999).

19.4.4 ECM Protein Strand Breakage

Over time, attacks by EC proteases, as well as simple mechanical stresses, create breaks in the protein chains of the ECM, including collagen, elastin, and fibronectin (Li et al. 1999; Wang and Lakatta 2002; Wang et al. 2003; Labat-Robert 2004;

Robert et al. 2008). In some situations, EC proteases such as MMPs are secreted by "senescent" dermal fibroblasts and other FLCs (Parrinello et al. 2005). Furthermore, senescent dermal fibroblasts downregulate TIMP-1, thus restricting normal regulation of MMP activity (Labat-Robert 2004). In other situations, proteases are secreted as part of inflammatory responses to signals from cell surface receptors, when they are activated by AGEs or by fragments of elastin or fibronectin (see Section 19.4.7 and Fig. 19.2). Skin fibroblast secretion of proteases also increases in response to sunburn (Labat-Robert 2004). Protein strand breaks can cause weakening of collagen, fragmentation of elastin and fibronectin, and loss of tissue elasticity.

It is worth remembering that ECM strand lysis and digestion are not always harmful; they are sometimes part of a controlled process of ECM turnover, remodeling, or regeneration, as described in Section 19.3. However, aging and inflammatory processes can result in excessive degradation of ECM that does not get regenerated and leads to tissues becoming thinner, weaker, or stiffer.

As it ages, elastin is degraded via a multi-step process described by Robert, weakening tissue and reducing elasticity (Robert et al. 2008). Its elastic properties arise because its hydrophobic residues gather together in puckers, when not under tensile stress, shrinking the structure. Like a spring, as stress increases, the puckers pull apart, allowing the strands to extend. When tensile force is less, they can pull together again. Over time, calcium ions and lipids bind to these

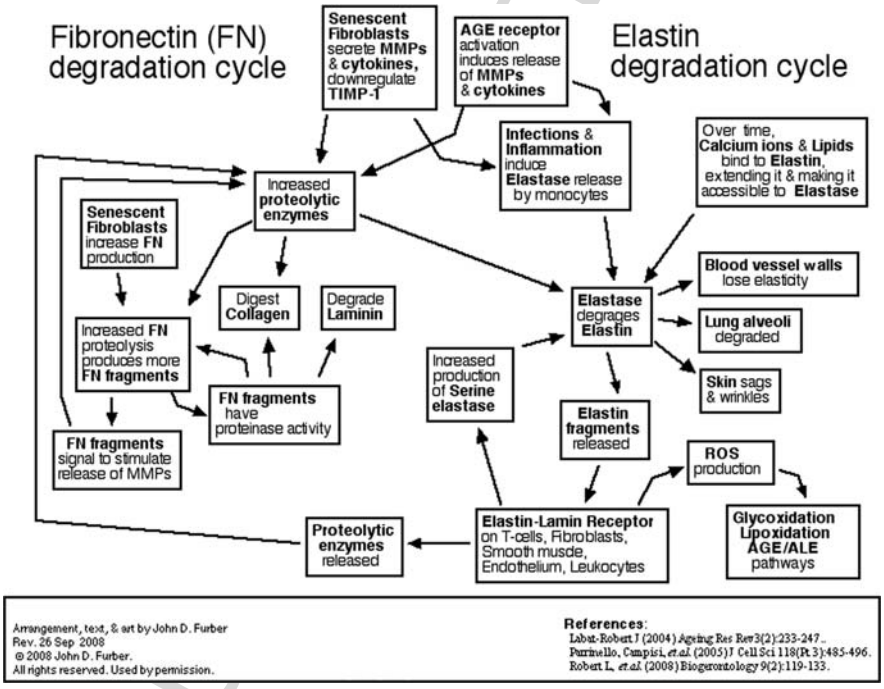

Fig. 19.2 Elastin and fibronectin degradation cycles of ECM protein strand lysis during aging

hydrophobic residues, reducing their mutual hydrophobic attractions for each other. This reduces the elasticity because it is easier for the strands stay in their extended state. Furthermore, the calcium and lipid-bound, extended elastin strands expose vulnerable sites for cutting by extracellular proteases. The lysed chains are no longer elastic, and they release protein fragments that activate inflammatory responses when they bind to the elastin-laminin receptor on cells (Robert et al. 2008), as described in Section 19.4.7 and Fig. 19.2. Like an old rubber band, the tissue loses elasticity and strength. Apparently, the elastic fibers are not readily replaced; perhaps they are never correctly replaced in arterial walls or lung alveoli (Robert et al. 2008; Wagenseil and Mecham 2007; Shifren and Mecham 2006; Finch 2007).

MMPs also lyse fibronectin strands, creating fibronectin fragments. Some fibronectin fragments are themselves proteolytic, having the ability to lyse collagen, laminin, and fibronectin. This produces a vicious cycle of LESP degradation shown in Fig. 19.2. Furthermore, some fibronectin fragments expose cryptic binding sites not available on intact fibronectin. Binding to cell surface receptors triggers a variety of deleterious cell responses (Labat-Robert 2004) described in Section 19.4.7.

19.4.5 Mechanical Consequences of Protein Alterations

Glycation adducts and crosslinks interfere directly with the mechanical properties of LESPs. Changes in charge, and the spaces occupied by adducts, can affect the conformation and behavior of proteins. Glycation adducts occupy space, and so may alter folding, shape, and function of proteins. Electrostatic charge distribution also affects folding and function. At physiological pH, the ε-amino side chain of lysine is positively charged. The guanidino side chain of arginine is also positively charged. Glycation or crosslinking converts these positively charged sites to neutral sites. Crosslinks bind together adjacent protein strands, reducing flexibility and elasticity of the tissue.

Elasticity is very important to cardiovascular function. Systolic blood pressure increases when the shock-absorbing elasticity of the artery walls is reduced (Vasan et al. 2001). High systolic blood pressure increases the risk for hemorrhagic stroke in the brain. It also increases back-pressure to the heart. The heart responds by increasing muscle mass, thickening its wall. A thicker, stiffer heart is less efficient at refilling after each contraction, resulting in diastolic heart failure (DHF). Reduced elasticity in the capillary walls restricts circulation to peripheral tissues. Mechanical elasticity of arteries is also important to maintaining healthy endothelial function, because nitric oxide (NO) signaling is reduced when stretching is limited (Zieman et al. 2007).

Glycation crosslinking of the corpus cavernosum contributes to erectile dysfunction (Usta et al. 2004, 2006). Crosslinking of the urinary bladder decreases its extensibility and capacity, resulting in the need for more frequent urination.

Glycation crosslinking is also believed to attach soluble plasma proteins to LESPs and to proteins on the surfaces of endothelial cells. This could contribute

to inflammatory immune responses, to the development of atherosclerosis, and to the thickening of basement membranes, which can impair kidney function (Ulrich and Cerami 2001; Vasan et al. 2001).

As discussed in Section 19.3, glycation crosslinks and adducts could be mechanically restricting the ability of FLCs to turn over ECM, resulting in a vicious cycle.

19.4.6 Altered Cell-Matrix Integrin Binding

Cells bind to the ECM through cell surface integrin molecules. These integrins are also essential to cell migration over and through the ECM. The integrins recognize and bind to specific peptide motifs in the EC structural proteins or glycoproteins, importantly DGEA in collagen and RGD in fibronectin, vitronectin, and laminin (Lanthier and Desrosiers 2004; Gilbert 2000). When arginine (R) or aspartate (D) in a binding motif undergoes a chemical change that alters its shape or charge, the binding strength of cells to that EC protein is reduced because their integrin receptors no longer have that RGD or DGEA sequence to bind to (Lanthier and Desrosiers 2004; Sell and Monnier 2004). As noted earlier, arginine can lose its positive charge in several ways by attachment of glycation adducts or formation of crosslinks. It can also decompose to ornithine. Aspartate can isomerize. Loss of attachment to the ECM can affect a cell's gene expression profile and differentiation state, and may increase the propensity of cells to become cancerous (Sell and Monnier 2004; Spencer et al. 2007). In some cases, cells die as a result. "The chondrocytes that produce the cartilage of our vertebrae and limbs can survive and differentiate only if they are surrounded by an extracellular matrix and are joined to that matrix through their integrins (Hirsch et al. 1997). If chondrocytes from the developing chick sternum are incubated with antibodies that block the binding of integrins to the extracellular matrix, they shrivel up and die" (Gilbert 2000).

19.4.7 Cell-Matrix Interactions: Receptors, Signaling, and Inflammation

Several distinct cell-surface receptors are activated by AGEs (Kass 2003). Other receptors are activated by fragments of lysed fibronectin or elastin.

Historically, some of the AGE receptors have had different names. Vlassara and Palace review several AGE receptors, which are found on the surfaces of various cell types (Vlassara and Palace 2003). One specific AGE receptor complex is composed of three subunits: R1, R2, and R3. Ohgami describes several other AGE receptors: RAGE, galectin-3, 80 K-H, OST-48, CD-36, SR-A-I and SR-A-II. SR-A are multiligand macrophage scavenger receptors (MSR) of the class A family. CD-36 is a multiligand scavenger receptor of the class B family (SR-B). CD-36 is expressed on macrophages and smooth muscle cells (Ohgami et al. 2001).

One specific receptor was named RAGE (Receptor of AGEs) by Stern's group (Stern et al. 2002). Stern's review of RAGE notes the complexity of the RAGE signaling system (Stern et al. 2002). RAGE is a member of the immunoglobulin superfamily of cell surface receptors. It is found on a variety of cell types, including macrophages and endothelial cells. It binds and is activated by various ligands, including amyloid fibrils, amphoterin, S100/calgranulins, CML and probably other AGEs. Upon binding a ligand, RAGE induces multiple signaling pathways within the cell (Stern et al. 2002). RAGE signaling activates inflammatory pathways, and inflammation is known to contribute to several processes important in aging (Vlassara and Palace 2003; Finch 2007). RAGE signaling also induces transdifferentiation of kidney epithelial cells to become myofibroblasts, thus impairing kidney function (Jerums et al. 2003). RAGE and CD-36 activation by AGEs/ALEs appear to contribute to the development of foam cells during atherogenesis (Vlassara and Palace 2003; Ohgami et al. 2001). RAGE activation stimulates oxidant stress and upregulates cell surface adhesion molecules and cytokines, stimulating vascular inflammation, remodeling, and atherogenesis (Zieman et al. 2007). Confusingly, some authors refer to *all* AGE receptors as "RAGE".

Not only do AGE receptors initiate signaling in response to AGE binding, but also the presence of AGE causes increased expression of the RAGE and R3 receptors (Candido et al. 2003).

The macrophage scavenger receptor (MSR, probably SR-A and CD-36), and other closely related receptors, appear to trigger an attack on AGE-modified proteins by macrophages (Araki et al. 1995). Glycation adducts on the surface of articular cartilage are major factors in the development of osteoarthritis, probably through inflammatory mechanisms (deGroot et al. 2004; Verzijl et al. 2003). Glycated peripheral nerve myelin is attacked by macrophages, contributing to peripheral neuropathy (Cerami et al. 1987). Glycation can crosslink immunoglobulins to kidney glomerular basement membrane; this then initiates complement-mediated damage (Bucala and Cerami 1992).

Although the consequences of AGE receptor activation by AGEs/ALEs are generally deleterious, these inflammation pathways are probably an inappropriate immune activity that could, on occasion, be protective against infections. AGE receptor signaling may also help to activate removal of AGE-damaged proteins by phagocytosis (Bucala and Cerami 1992).

Some glycation intermediates and end products generate free radicals, causing additional damage by oxidation and inflammation. CML generates free radicals, and is considered to be the major signaling ligand implicated in causing inflammatory diseases and cancers (Taguchi 2003; Kislinger et al. 1999; Monnier et al. 2003). The complex associations between inflammation and age-related pathologies have been reviewed in Finch's recent book, *The Biology of Human Longevity* (Finch 2007). Included is coverage of in-vivo glyco-oxidation, dietary ingestion of AGEs from cooked and processed foods, and more details on the role of AGE receptors in inflammation.

AGEs also contribute to endothelial dysfunction by degrading endothelial nitric oxide synthase (eNOS), which results in decreased NO concentrations (Bucala et al.

1991; Bucala and Cerami 1992; Dong et al. 2008). NO signaling causes vasodilation, so low NO contributes to high blood pressure. (Huang et al. 1995; Zieman et al. 2007) Decreased NO also contributes to erectile dysfunction (Haimes 2005).

As described in Section 19.4.4, elastin and fibronectin become fragmented during aging. Protein fragments from degraded elastin act as agonists binding to the elastin-laminin receptor. This upregulates the release of elastase endopeptidases, and the production of reactive oxygen species (ROS), which can cause a vicious cycle of further damage to elastin fibers, shown in Fig. 19.2 (Robert et al. 2008; Labat-Robert 2004).

Protein fragments from degraded fibronectin (Section 19.4.4 and Fig. 19.2) bind to receptors on cell surfaces, generating signals that result in inflammation, tissue degradation, and tumor progression (Labat-Robert 2004). Kume and colleages found that AGEs in cell culture inhibited the proliferation of human MSCs, induced apoptosis, and inhibited differentiation into adipose tissue, cartilage, and bone (Kume et al. 2005).

19.4.8 Extracellular Amyloidosis

"Amyloidosis is a clinical disorder caused by extracellular deposition of insoluble abnormal fibrils, derived from aggregation of misfolded, normally soluble, protein. About 23 different unrelated proteins are known to form amyloid fibrils in vivo" (Pepys 2006). Pepys further notes that these extracellular deposits interfere with the proper functioning of the surrounding tissues, resulting in pathologies that can become fatal. Although amyloidosis is rarely cited as a cause of human death, one type, transthyretin (TTR-amyloid) is frequently found at autopsy in the hearts, kidneys, and lungs of people aged over 80 (Pepys 2006). The first population-based autopsy study found TTR-amyloidosis in 25% of humans aged 85 or more from southern Finland (Tanskanen et al. 2008). Pepys and Lachmann propose that amyloidosis may contribute to several diseases of the elderly. Furthermore, they suggest that if not addressed, TTR-amyloidosis might become a more serious problem at transcentenarian ages if human lifespan is increased by successful treatment of other age-associated diseases (Pepys 2006; Lachmann and Hawkins 2006).

Amyloid deposits resist attack by phagocytosis and most enzymes. Apparently the SAP protein, normally found in blood, binds to amyloid deposits and protects them. An experimental therapy, directed at SAP, is currently in human trials. The drug crosslinks soluble SAP, thus preventing it from binding to amyloid deposits. If the therapy is successful, the body's natural scavengers would then clear up the amyloid deposits (Pepys 2006; Lachmann and Hawkins 2006).

Nattokinase is a bacterial serine protease enzyme found in the fermented Japanese soybean food called, "*natto*". Preliminary experiments have shown that this enzyme can degrade several kinds of amyloid molecules in vitro (Hsu et al. 2009). It is interesting that it remains active in the bloodstream after oral assimilation, and that it is part of a traditional human food. Further research is

needed to determine whether it can clear up TTR-amyloid or other deposits in older people. Even if not, its structure might inform future rational drug design efforts (Section 19.5.8).

19.4.9 β-Amyloid Plaques in the Brain

Extracellular deposits (β-amyloid plaques) of amyloid-β protein (A-β) accumulate in some brains as they age. A significant constituent is a 42 amino acid fragment of APP, "amyloid-$\beta_{1\text{-}42}$" or "A-β_{42}". Although often associated with Alzheimer's disease, there is considerable debate regarding whether these β-amyloid plaques are very harmful (Castellani et al. 2007). However, it is generally agreed that *in solution*, A-β_{42} produces reactive oxygen species (ROS), which can damage nearby neurons. *Soluble* A-β_{42} also activates RAGE, which contributes to neurotoxicity (Sturchler et al. 2008). Importantly, the plaques are in dissociable equilibrium with the soluble A-β_{42}, and thus can serve as a reservoir of the toxic species (Adlard et al. 2008).

19.5 Present and Possible Future Therapeutic Approaches for Better Maintenance and Repair of the ECM

This section examines prospects for therapies to slow AGE formation or to repair EC damage. The importance of glycation in diabetes and aging has led to searches for therapies that inhibit the glycation reactions or safely remove the products of glycation. Glycoxidation moieties, AGEs, and crosslinks might be chemically removed from ECM by drugs or bioengineered enzymes. Enhancement of natural ECM turnover and replacement could regenerate damaged tissues.

19.5.1 Diet, Fasting, and Calorie Restriction

As a non-enzymatic chemical reaction, we would expect glycation rate to increase with greater blood sugar concentration (Eble et al. 1983). Where glycation rate exceeds turnover rate, we expect to see accumulation of AGEs. In fact, the glycation rate does change with glucose concentration as expected. AGE/ALE accumulation rate is higher in diabetics, who have higher average blood sugar and lipid levels. Glycation rate decreases with calorie restriction, which lowers average blood sugar level. Rats fed calorie-restricted diets have less glycation crosslinking than rats that consume more calories (Lingelbach et al. 2000; Cefalu et al. 1995). Furthermore, Snell dwarf mice produce no growth hormone, and consequently have lower average blood sugar levels than control animals. Collagen glycation rates increase more slowly with age in Snell dwarves, they have much lower rates of cancer in old age, and they live longer (Flurkey et al. 2001; Alderman et al. 2009).

Thinking about therapeutic regimens, although it would be impossible to reduce the blood sugar and lipid concentrations to zero, average levels could be lowered by exercise, periodic fasting, or by constant or intermittent calorie restriction. Consequently, any of these alone, or in combination, should slow the rate of glycation.

High-temperature cooking produces AGEs/ALEs which, if ingested, would contribute to the body's AGE burden. The greatest quantity are created by frying or broiling foods containing fats or meats. Few are found in boiled or raw vegetarian foods (Goldberg et al. 2004). High levels of heat-stable glycation adduct residues, CML and CEL, were found in pasteurized and sterilized milk (Ahmed et al. 2005).

Inflammatory markers in the blood of diabetic humans and animals increased substantially after a few weeks on a high-AGE diet (Vlassara et al. 2002). This indicates that AGEs/ALEs do enter the systemic circulation from food digestion and increase inflammation. Similarly, although CR often improves the health and extends the lifespan of laboratory mice, when nondiabetic mice are maintained on a CR diet that is cooked to increase dietary AGEs, they have higher serum AGEs, oxidative stress, inflammatory markers, organ damage, and shorter lifespans than matched CR controls that received the same total calories, but not cooked food (Cai et al. 2008). A cautious person with an interest in optimizing health and lifespan might choose diets that minimize ingested AGEs and ALEs.

19.5.2 Exercise

Exercise increases the rate of turnover of collagen in human tendons and skeletal muscles, resulting in improved strength and flexibility (Kjær et al. 2006). As Kjær and colleagues observe, tendons contain fibroblasts. Weight-bearing exercise induces the surrounding tissue to release growth factors (IGF-1, IGF-1 binding proteins, TGF-β, and IL-6), which induce fibroblasts to remodel the collagen of the ECM. Collagen degradation is increased during the first day after exercise. However, new collagen synthesis is upregulated in tendon and in skeletal muscle for the first three days following intense exercise. Thus, they caution, to prevent overuse injury, it is important to space out exercise sessions. "If training sessions are too close to one another, an athlete may not gain maximum benefit from the stimulated collagen synthesis, but is instead likely to be in a net state of collagen catabolism." (Kjær et al. 2006).

There appears to be a synergistic benefit to combining exercise and crosslink breaker therapy (described in Section 19.5.4).

19.5.3 Inhibitors of Glycation, Lipoxidation, and AGE Formation

Many of the studies of potential glycation inhibitors do not look at LESP glycation in normally aging humans. They look instead at levels of soluble AGEs and reactive

glycation intermediates in the blood of diabetic humans and rats. These blood levels do not accumulate over time, so they are not useful as biomarkers of aging. To the extent that a glycation inhibitor could reduce these blood levels in nondiabetic humans, then it *might* slow the rate of accumulation of glycation adducts and crosslinks in the extracellular matrix. That, however, is speculative at this time.

There are several intervention points in the cascade of events leading to production of AGEs/ALEs (including AGE crosslinks). Table 19.1 lists several dozen compounds that inhibit AGE production. Some are lipid membrane soluble, while others are hydrophilic. Many of these inhibitory compounds exhibit multiple modes of action, such as: trapping reactive carbonyls, interacting with dicarbonyls, quenching ROS, preventing autoxidation, chelating metals such as copper, inhibiting nitric oxide synthase (NOS), combatting inflammation, binding to glucose, inhibiting crosslinking of proteins, inhibiting early Amadori reactions, or inhibiting post-Amadori reactions. A few of these inhibitors are also able to break AGE crosslinks after they have formed. Crosslink breakers are examined in more detail in the next section.

Table 19.1 Inhibitors of glycation

Inhibitor	Notes	Ref
ALT-946 = N-(2-acetamidoethyl) hydrazinecarboximidamide HCl		V1,T,J,R3
ALT-462 = triazine derivative		V1
ALT-486 = benzoic acid derivative		V1
aminoguanidine = pimagedine	DCI, EAi, TRC, NOSI, MC, SAi	V1,T,J,R3
ascorbate = vitamin C	AO	R3
aspirin	AOp, AO, AI	R3
benfotiamine	LS	R3
benzoic acid	AOp, AO	R3
carnosine = β-alanylhistidine	AO, MC, TRC	R3
carotenoids	AO	R3
cinnamon, aqueous extract	AO, TRC	P
curcumin	AO, AI, Ci	R3
cysteine	TG	F, S
desferoxamine		R3
diaminophenazine = 2,3 DAP	DCI, MC	R3
Diclofenac = Voltran	AI	R3
EGCG = epigallocatechin gallate	Ci	W
fasting	BGL	F
garlic		A
glutathione	TG	F, S
histidine	MC, TRC	
Ibuprofen	AI	R3
Indomethacin	AI	R3
Inositol	AO, Gb	R3
LR-9 = 4-(2-naphtylcarboxamido) phenoxyisobutyric acid	MC, TRC	R3
LR-series # 20, 102	MC,PAi, CB	R3
LR-23	CB	R3

Table 19.1 (continued)

Inhibitor	Notes	Ref
LR-90	MC, PAi, TRC	R3
luteolin	AO,EAi, PAi, Ci	W
metformin = Glucophage = dimethylbiguanide	DCI,EAi,PAi,CB	R0,R3
MEAG = morpholino-ethyl aminoguanidine		V1
OPB-9195 = (±)-2-isopropylidenhydrazono-4-oxo-thiazolidin-5-ylacetalinide	DCI, MC	V1, R3
PABA	AOp, AO	R3
D-penicillamine		R3
pentoxyfylline		R0,R3
Pioglitazone	DCI, MC	R0,R3
Probucol	AO	R3
Pyridoxamine	PAi,LEi,DCI, MC	Me,V1,R3
quercetin	AO, EAi, Ci	W
resveratrol = 3,4,5-trihydroxystilbene		R3
rutin	EAi, Ci	W
salicylic acid	AO, AOp	R3
Tenilsetam = (+)-3-(-2-thienyl)-2-piperazine	Ci	R3
thiamine pyrophosphate = Vitamin B1	PAi	V1, R3
thyme		Mo
Tocopherol = vitamin E	AO	R3

Abbreviations: *AO* Antioxidant, *AI* Antiinflammatory, *BGL* Lowers blood glucose, *CB* Cross link breaker, *Ci* Inhibits cross link formation, *DAOi* Diamine oxidase inhibitor, *DCI* Interacts with dicarbonyls, *EAi* Early Amadori stage inhibitor, *Gb* Binds to glucose, *LS* Lipid soluble, *LEi* Lipoxidation endproduct inhibitor, *MC* Metal chelator, *AOp* Prevents autoxidation, *PAi* Post Amadori inhibition, *SAi* Inhibits semicarbazide-sensitive amine oxidase, *TG* Transglycation, *TRC* Traps reactive carbonyls

References: A = Ahmad et al. 2007; F = Furber 2006; J = Jerums et al. 2003; Me = Metz et al. 2003; Mo = Morimitsu et al. 1995; P = Peng et al. 2008; R0 = Rahbar et al. 2000; R3 = Rahbar and Figarola 2003; S = Szwergold 2005; T = Thornalley 2003; V = Vasan et al. 2001; W = Wu and Yen 2005

Some well-known antioxidant or anti-inflammatory substances appear to inhibit AGE formation: aspirin (Bucala and Cerami 1992), ibuprofen, inositol, probucol, vitamins C and E, carotenoids, salicylic acid, PABA, and benzoic acid. Rahbar and Figarola conclude that because not all antioxidants inhibit AGE formation, those that do are employing another mechanism of action. They note that in clinical trials of diabetic patients, treatments with antioxidants that don't inhibit AGE formation do not improve their condition (Rahbar and Figarola 2003). Aspirin acetylates specific primary amino groups, thereby blocking their glycation (Bucala and Cerami 1992).

Aminoguanidine (AG or pimagedine) has been well studied in clinical trials of diabetic patients. It is a nucleophilic compound that traps reactive carbonyl groups (Ulrich and Cerami 2001). In addition to inhibiting AGE formation, it also inhibits NOS (Jerums et al. 2003). However, there have been safety concerns and apparently low clinical efficacy (Thornalley 2003). Human side effects included pernicious

anemia and anti-nuclear antibodies. In rat studies, pancreas and kidney tumors developed (Rahbar and Figarola 2003).

Pyridoxamine (PM) is the 4-aminomethyl form of vitamin B6. PM inhibits formation of AGEs and ALEs, apparently by reacting with dicarbonyl intermediates. In diabetic rats, oral PM stayed in the blood longer, and had greater therapeutic benefit than similar doses of AG (Metz 2003). The Baynes lab has showed that PM breaks dicarbonyl compounds in vitro (Yang et al. 2003). Although they were unable to show in vivo breaking of AGEs, this might be worthy of further study by other labs.

Some radical trapping compounds alter branchpoints in the AGE formation reaction network, inhibiting the formation of some AGEs, while increasing the formation of others. For example, 6-dimethylaminopyridoxamine (dmaPM) and Trolox each inhibit the formation of glucosepane crosslinks in vitro, but increase the production of other glycation products (Culbertson et al. 2003). This is especially interesting because, as discussed in Section 19.5.4.4, no breaker for glucosepane crosslinks has yet been identified.

Metformin (*N*,*N*-dimethylimidodicarbonimidic diamide mono-hydrochloride) (glucophage) (pKa = 12.4) is a drug prescribed to improve glucose tolerance in type-2 diabetes. It has also been shown to inhibit glycation in vitro (Rahbar et al. 2000), to bind dicarbonyl glycation intermediates, inactivating them (Beisswenger and Ruggiero-Lopez 2003), and to break glycation crosslinks in vitro (Rahbar and Figarola 2003).

Benfotiamine is a lipid soluble analog of thiamine (vitamin B1). In diabetic rats, it effectively reversed neuropathy and reduced accumulation of glycation intermediates (Stracke et al. 2001). Its effect on normally aging humans has not been reported. However, its mode of action seems to control pathways that are induced by diabetic hyperglycemia (Hammes et al. 2003). It would therefore not be helpful in nondiabetic situations, such as normal aging.

Carnosine (β-alanyl-L-histidine) is a dipeptide that is heavily marketed as a nutritional supplement. Its putative ability to inhibit protein glycation or crosslinking in humans is still under investigation. Hipkiss, who has been studying carnosine for years, notes that "carnosine *may* be an effective anti-glycating agent, *at least in model systems*" (emphasis added) (Hipkiss 2005).

Glutathione and cysteine may have anti-glycating ability. The glucose-lysine Schiff base can spontaneously donate its sugar moiety to nucleophiles such as cysteine and glutathione, restoring the protein to its original, unglycated condition. This has been observed in vitro without any enzymes present. The sugar binds to the sulfur atom of the cysteine. Szwergold et al. propose that this reaction also occurs spontaneously within cells, and that the glycated glutathione or cysteine is then pumped out of the cell. In support of their model is the observation that glycated cysteine is found in human urine, and that levels are higher in diabetic urine (Szwergold et al. 2005). They did not comment on the possibility of transglycation taking sugar from extracellular collagen. Cysteine and even glutathione may be small enough to go wherever glucose goes among the collagen molecules. Thus, there may be possible benefits to therapeutic use of oral N-acetylcyteine (NAC) or parenteral glutathione to increase concentrations of these nucleophiles in the extracellular fluid

that bathes collagen. NAC is commonly available as a nutritional supplement. Some clinics offer intravenous glutathione injections. Note however, that this reaction deglycates only the earliest step in the glycation pathway. After the glycation has proceeded to form Amadori products, AGEs, or crosslinks, transglycation does not occur. Nonetheless, even partial inhibition of glycation may be beneficial.

In general, AGE inhibitors are tested in vitro and in vivo. In diabetic models, they slow down the rates of physiological deterioration to some extent. However, for long-lasting benefits and rejuvenation, we must look for therapies that actually reverse or repair accumulated LESP damage, which has already occurred, including crosslinks, glycation, fragmentation, and lipoxidation.

19.5.4 Deglycators and Crosslink Breakers

Within mammalian cells, endogenous mechanisms exist for reversing glycation (Section 19.5.4.1). Outside cells, in the ECM, glycation is destroyed wherever the ECM is turned over. Several approaches are being explored to design therapies to break crosslinks or remove glycation adducts on ECM proteins. Some are based on small molecule drug designs. Others are based on adapting strategies from intracellular enzymes or fungal enzymes. A significant consideration is that much of the collagen matrix is densely packed so that glycation crosslinks may not be accessible to large enzyme molecules. If a large enzyme cannot travel to its target crosslink, it cannot break it. Perhaps this problem might be circumvented if small molecule crosslink breakers could loosen up the ECM enough for larger enzymes to get in and finish the job.

19.5.4.1 Intracellular Enzymatic Deglycation

Enzymes have been found in some cells that are able to remove Amadori adducts from intracellular proteins. In mammals, fructosamine 3-kinases (FN3Ks) have been found to act as Amadoriases. They phosphorylate Amadori products, which then spontaneously deglycate, leaving the original proteins good as new (Szwergold et al. 2001). However, Amadoriases do not work on AGEs or crosslinks, because their chemical structure is changed from the early Amadori structure. Furthermore, Amadoriases are inside the cell and they require ATP. This presents problems because crosslinked collagen is outside the cell, and a source of extracellular ATP is not available. So FN3Ks are not useful for repairing ECM (Monnier et al. 2003). However, they might serve as a starting point for future development of useful drugs or designer enzymes.

19.5.4.2 Fungal Amadoriase Enzymes

Enzymes that are able to deglycate small Amadori products, such as glycated amino acids, have been isolated from fungi. However, the enzymes discovered so far do not deglycate proteins. This is apparently due to both steric hindrance and electostatic

interactions (Monnier et al. 2003). Their mechanism is to oxidize the fructosylamino Amadori product, releasing the original unglycated amine (such as lysine), along with hydrogen peroxide and oxidized sugar (such as glucosone). Thus, they are also called *"fructosyl amine oxidases"*. An advantage of this reaction is that it does not require ATP, so it could take place outside of cells. A disadvantage is that both hydrogen peroxide and glucosone are reactive, and could cause further oxidative damage. Although these enzymes do not deglycate collagen, they have been sequenced, and the structure has been determined (Collard et al. 2008). They might suggest strategies for development of new agents.

19.5.4.3 Thiazolium Salts and Other Small Molecules

Several small molecules have been reported to have the ability to chemically cleave some of the glycation crosslinks or adducts in LESPs. Torrent Pharmaceuticals was granted several patents covering crosslink-breaking by pyridinium structures, and later published promising results with diabetic rats treated with compound "TRC4149" (Pathak et al. 2008). Rahbar, at City of Hope, was granted patents for the crosslink-breaking ability of several other structures, including metformin (Rahbar and Figarola 2003). However, his recent publications have focused on their glycation-inhibition rather than crosslink-breaking (Rahbar 2007; Figarola et al. 2008). The crosslink-breaker furthest along in human clinical trials is a thiazolium salt discovered by Cerami and colleagues.

In the early-1990s, Ulrich and Cerami were examining thiazolium compounds for their ability to interact with α-dicarbonyl structures in advanced Amadori products (Ulrich and Zhang 1997; Ulrich and Cerami 2001). These thiazolium compounds contain a nucleophilic catalytic carbon (position #2) analogous to thiamine (vitamin B-1) and a second nucleophilic carbon, attached to the nitrogen, nearby. These two carbons could interact with the two carbonyls of α-dicarbonyl structures (Vasan et al. 1996). They were surprised to discover that these compounds not only inhibited the progression of Amadori products to crosslinks, but they were also able to break model crosslinks in vitro (Ulrich and Cerami 2001). Many similar thiazolium compounds were tested and found to have crosslink-breaking activity. Patent rights were assigned to Alteon Pharmaceuticals (later renamed Synvista Therapeutics). Animal testing showed promising results in reversing collagen crosslinking, and improved functioning of kidneys, penile erections, heart, arteries, and other organ systems in aged or diabetic animals (Asif et al. 2000; Vaitkevicius et al. 2001; Usta et al. 2004, 2006). Similar beneficial results have been reported by Cheng and colleagues at the Beijing Institute of Pharmacology and Toxicology, who have been testing a structurally similar thiazolium compound, "C36" (Cheng et al. 2007).

Alteon chose alagebrium, *3-(2-phenyl-2-oxoethyl)-4,5-dimethylthiazolium chloride*, to use in their clinical trials. Early papers refer to this compound and its close relatives as "*ALT-711*". Some of the early testing was done with bromide analogs (PTB), with or without the methyl groups. PTB was abandoned by Alteon in favor of the dimethyl chloride, alagebrium, because PTB is less active and unstable (Ulrich

and Cerami 2001). PTB degrades rapidly in aqueous solution. Furthermore, bromides may have undesirable side effects (Thornalley and Minhas 1999; Vasan et al. 2001, 2003).

Alagebrium is now the crosslink breaker furthest in clinical development for human oral therapeutic use. Alagebrium appears to be effective at partially reversing some human pathologies, probably by breaking α-diketone crosslinks in collagen and elastin (Vasan et al. 1996). Possibly, it also reacts with other α-dicarbonyl glycation intermediates or endproducts, such as methylglyoxal (MGO) (Yang et al. 2003; Haimes 2007).

In 2003, the Baynes lab published a report suggesting that thiazolium bromides "do not break Maillard crosslinks in skin and tail collagen from diabetic rats" (Yang et al. 2003). This is a controversial claim, contradicting a large number of studies, which show evidence that thiazolium salts do break crosslinks in tail tendon collagen from diabetic rats (Vasan et al. 1996, 2001, 2003; Ulrich and Cerami 1997; Wolffenbuttel et al. 1998; Cheng et al. 2007). The situation is confounded because different techniques were used by different labs, so we cannot say, with certainty, why their results differ. Note, however, that the Baynes report did not use the stable alagebrium chloride, but rather, the less active, unstable bromide salts (Yang et al. 2003).

Interestingly, the Baynes group did acknowledge that the thiazolium halides produce beneficial clinical physiological results in vivo. However, they proposed different mechanisms of action. They suggested that alagebrium might be inhibiting the production of new crosslinks, as well as inhibiting glycoxidation reactions. Then, over a period of time, they reasoned, natural turnover of collagen would result in a reduction in the number of crosslinks, creating the appearance of crosslinks being broken (Yang et al. 2003). However, the Baynes hypothesis appears to be inconsistent with the multiyear long collagen turnover times calculated by independent labs (Sell et al. 2005), and the rapid in vivo benefits observed with alagebrium (Asif et al. 2000; Kass et al. 2001; Vaitkevicius et al. 2001).

Jerums and colleagues report that alagebrium treatment reduced kidney damage (Jerums et al. 2003). There are also reports that alagebrium treatment reverses the AGE-stimulated progression of several pathologic markers in the hearts of diabetic rats, including collagen solubility and expression of the AGE receptors RAGE and R3 (Candido et al. 2003; Kass 2003; Tikellis et al. 2008).

Phase 2 clinical trials of alagebrium began in 1998 (Vasan et al. 2003). As of mid-2009, several phase 2 trials had been completed, but Synvista had stopped further trials citing lack of funds. By 2007, about 1000 people had taken alagebrium in various phase 2b clinical trials (Haimes 2007). So far, the safety profile of the drug appears to be excellent in human subjects. Concerns arose in December 2004 regarding liver cell irregularities in male Sprague-Dawley rats that had been given alagebrium throughout their whole lives. After investigating, FDA allowed continuation of clinical trials. Apparently, Sprague-Dawley rats have exhibited similar changes in response to other approved drugs, such as statins. It appears that this breed of lab rat is not a reliable model for long-term human drug safety tests, although it was long been used because it is easy to handle (Creel 2008).

Alagebrium treatments have produced improvements in DHF patients, for whom ventricular hypertrophy was reduced and heart function was improved (Little et al. 2005). Other patients with systolic hypertension showed improvement in arterial pulse pressure and arterial compliance (Kass et al. 2001). Endothelial function was also improved, probably because removal of AGE crosslinks allowed better stretch-mediated release of NO (Zieman et al. 2007).

Preliminary results indicate that alagebrium is able to repair erectile dysfunction, probably due to improved vascular compliance, NO signaling, and endothelial function (Coughlan et al. 2007). This was first reported in studies of diabetic rats (Usta et al. 2004, 2006). This author has heard firsthand reports from several men remarking on their improvement after several weeks or months of oral alagebrium (100–300 mg per day).

There appears to be a synergistic benefit of combining exercise (see Section 19.5.2) and alagebrium therapy. To the extent that alagebrium breaks LESP crosslinks and improves flexibility, exercise would be easier and tissue remodeling would be facilitated (Haimes 2007). This author has heard firsthand reports from several people remarking on their improved exercise tolerance after several weeks of oral alagebrium (100–300 mg per day). Two people noted that reduced arthritis allowed them to hike longer in the hills.

In June 2005, Alteon announced that it had granted a nonexclusive worldwide license to Avon Products, Inc. for the use of 2-amino-4,5-dimethylthiazole HBr to improve skin wrinkles and elasticity. Very soon after, Avon brought out its "Age Intensive" skin cream, containing this substance as a minor ingredient. The product is popular, although clinical comparisons with common moisturizers have not been published.

Anecdotally, several longtime users of alagebrium have told the author that they noticed improvements in bladder capacity, peripheral neuropathy, erectile function, kidney function, angina pectoris, or joint pain after several months of usage. Each was taking 100–400 mg per day, orally.

Several people have been giving alagebrium to their elderly dogs (ages 10–16 years), mixed with food or water. They told the author that their dogs had previously been exhibiting arthritis, low energy, and restricted movement. After about a month on alagebrium, their dogs were running and jumping as though they were several years younger. Their subjective assessment was that the alagebrium treatments had given their pets two additional years of quality life. Dosage was approximately 1–2 mg/kg per day.

19.5.4.4 Glucosepane Crosslink Breakers

So far, no small molecule has been identified that breaks glucosepane crosslinks. However, because an assay has not yet been implemented to test for glucosepane breakers, it is possible that some of the small-molecule breakers described in Section 19.5.4.3 might actually break glucosepane, yet we would not know it.

A drug discovery effort targeted at breaking glucosepane crosslinks might yield therapeutic leads. The isoimidizole structure at its core may be unique enough that

a chemical agent could cleave it while not harming other essential extracellular structures.

Besides small molecule drugs, it is also possible that enzymes might be discovered or designed that could break glucosepane. However, there is not much space within the tightly packed collagen matrix where the crosslink is located, so enzymes might not fit. Nevertheless, we cannot rule out the possibility that a small enzyme might slip in, first breaking the most exposed crosslinks, and thereby opening the collagen matrix to access the more cryptic crosslinks. Perhaps in combination with alagebrium, other small molecules, and exercise, glucosepane-breaking enzymes might be even more effective.

As noted in Section 19.5.3, a couple of compounds have been found to inhibit glucosepane formation in vitro. Development of a drug to inhibit glucosepane formation in vivo could be beneficial until a therapy to remove glucosepane is developed.

19.5.5 Tuned Electromagnetic Energy

It is attractive to speculate that laser frequencies might exist that would safely penetrate tissues, while coupling energetically enough with crosslink structures to break them. Experiments with tunable lasers could explore frequencies in search of effective ones. There is no assurance of success. Even if cleaved, the crosslinks might quickly reform by the reverse reaction. Nevertheless, I predict that the costs of preliminary experiments on pieces of meat could be low and the potential payoff high. A physics lab that has a tunable laser, in collaboration with a biochemist who can assay crosslinks in animal tissue, could yield answers in a very short time.

19.5.6 Removing β-Amyloid Plaques

Considerable work is underway to find treatments for Alzheimer's disease. A promising approach is directed at solubilising and flushing out the extracellular β-amyloid plaques, by removing the metals around which they aggregate. An 8-hydroxyquinoline agent, PBT2, in clinical trials sponsored by Prana Biotechnology, is showing early success (Adlard et al. 2008).

19.5.7 Enhancing Turnover of ECM by FLCs

Human FLCs have the means to digest LESPs, and to replace them with newly synthesized fibers (Bucala and Cerami 1992; Murphy and Reynolds 2002). Unlike crosslink-breaking enzymes, which might be unable squeeze between collagen fibrils to reach crosslinks, enzymes secreted by FLCs to digest ECM start at the outside of the collagen fiber and chew their way in, so steric hindrance is not a problem.

Even cartilage and bone can be remodeled by appropriate cell types. Future developments might stimulate or reprogram FLCs to more quickly digest and replace age-damaged ECM in a controlled fashion. We might speculate that future bioengineers could integrate AGE receptors into signaling systems in FLCs to target these activated FLCs to turn over glycated ECM.

An important challenge will be to ensure that the turnover is well regulated, to prevent either thinning and loss of ECM or excess, disorganized fibrosis and cicatrix formation. Obviously, inducing widespread scar formation would not be a desirable fix for AGE accumulation. Ideally, working fiber-by-fiber, even the strands reinforcing blood vessels might be replaced without catastrophic system failure.

With advancing age, the population of FLCs declines and becomes less active at turning over LESPs (Campisi 2005). It is reasonable to foresee that a successful therapy would expand the numbers of FLCs, and also stimulate their activity of turning over LESPs. For example, platelet-derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1) have long been known to promote growth and mitosis of mesenchymal/fibroblast lineage cells (Bucala and Cerami 1992). Recent work at the University of Glasgow has shown that inserting an extra copy of the TERT gene into chondrocytes from articular cartilage results in longer telomeres and increased replicative lifespan, without neoplastic transformation. So far, the Glasgow results have been reported only for cell cultures of chondrocytes from young dogs (Nicholson et al. 2007). More work is needed to reveal whether altered integrin binding in old cartilage (Section 19.4.6) would harm the transgenic chondrocytes, or whether the activated FLCs could turn over the old ECM before it could harm them. Careful work could refine the optimal dosage, timing, and combinations of factors to expand cell numbers and induce differentiation into cell types best able to turn over ECM.

FLC stimulation might be done either in the body or in cell culture. In the body, biological response modifiers such as signaling molecules could be administered or gene therapy vectors might be injected. These agents might be designed to act directly on FLCs or they might work indirectly through other cells, which would signal to the FLCs. However, dosing of the target cells could not be uniform or precise, or responsively tailored to observed progress on the differentiation path. Furthermore, it might be difficult to prevent unintended cell populations from proliferating in response to systemically administered therapies. These issues might not be problematical if the treatment could be something like restoring youthful levels of hormones and other signals. There is still much to be learned.

An alternative method would be to extract and treat FLCs in culture. Fibroblasts, bone marrow stem cells, or MSCs could be treated *ex vivo* to increase their numbers. Then they could be monitored while differentiation agents are used to enhance their activity. Finally, the activated autologous cells would be injected into the patient to increase regeneration of the ECM (see also the Chapter 14).

As noted in Section 19.3, exercise and mechanical force can increase the rate of collagen turnover and ECM remodeling by fibroblasts in various human tissues. Close examination of the signaling pathways and cytoskeletal responses to

exercise and force could reveal clues to developing more general ECM rejuvenation therapies.

Useful lessons about enhancing human ECM turnover may also be learned by studying the regeneration of amphibians, such as the axolotl (*Ambystoma mexicanum*). Some amphibians and invertebrates are able to replace whole body parts after amputation. As Muneoka and colleagues note in their review, axolotls repair wounds and amputations perfectly, without scar formation. For example, axolotl limb regeneration results in a perfectly formed new limb, with new bone, new joints, new ECM, and new cells, all in exactly the correct pattern (Muneoka et al. 2008). Importantly, in the early phase of regeneration, the ECM at the wound site is extensively remodeled by migrating dermal fibroblasts, which have positional information to correctly rebuild the regenerating structure (Rinn et al. 2006). Collagen in the stump is first digested and then new collagen is created as the wound site is remodeled. Subsequently, additional ECM is built and populated by cells to rebuild the entire limb (Gardiner 2005).

It is encouraging that in humans, repair of oral mucosa wounds inside the mouth does not involve scar formation; it somewhat resembles amphibian regeneration (Schrementi et al. 2008). Furthermore, Muneoka, Han, and Gardiner point out, "wounds in [human] fetal skin heal without forming scars–yielding perfect skin regeneration and indicating that the switch to a fibrotic [scar-forming] response arises with the developmental maturation of the skin." This suggests that the human genome still possesses the ancient genes needed to accomplish regeneration (Muneoka et al. 2008). An important challenge will be to learn how to activate those inherent abilities, in a controlled manner, to remodel ECM that has become aged and glycated. Furthermore, of course, activation presumably would need to occur without prior wounding, in order to safely remodel critical structures, such as arterial walls and lung alveoli. Scheid and colleagues have observed that transforming growth factor β3 (TGFβ3) is expressed in regenerating fetal wounds, and that it promotes epithelial and mesenchymal cell migrations and cell-ECM interactions (Tredget and Ding 2009; Scheid et al. 2002). Subsequently Ferguson and colleagues demonstrated reduced scar formation during adult human wound healing treated with TGFβ3 (Ferguson et al. 2009). This suggests that factors might be found to induce adult FLCs to regenerate and repair age-damaged tissues.

19.5.8 General Therapy Design Considerations

"Rational drug design" (RDD) looks at a target structure (crosslink or adduct) to figure out what sort of molecule would effectively break it or remove it. Interactive molecular models in silico (in computers) are very helpful in these studies. Designers must bring the active sites of the agent and the target molecules close enough to interact. If the agent is not properly shaped, steric hindrance can prevent active site contact. Large molecules such as proteins may have particular problems squeezing among collagen fibrils to reach crosslinks or adducts. Electrostatic

interactions can also affect apposition of active sites. Furthermore, reactions must be energetically favored. Local chemistry predicts whether the reaction will move forward. If the target bonds are not sufficiently energetic to be catalytically broken, then the agent, or nearby reactants such as oxygen, must provide some of the energy to move the reaction forward. We would also like some small products to move away quickly, to decrease the reverse reaction rate. There is some evidence that crosslinks broken by alagebrium might relink within a few weeks. This would suggest that alagebrium leaves reactive pieces in place, which can reassemble.

"High throughput screening" (HTS) creates a standardized chemical version of the target structure inside thousands of tiny reaction vessels. With a standardized assay, thousands of compounds are tested for any that show effectiveness. When promising lead compounds are discovered, variations on the structure are tested to find those with the best performance.

The best leads from RDD and HTS are used as starting points for creating families of similar structures, which are extensively tested in vitro. Compounds that look promising in vitro are next tested in animals for efficacy, side effects, and toxicity, as well as for the pharmacokinetics of absorption, distribution, metabolism, and excretion (ADME). RDD modeling can also be helpful in predicting whether problems such as collateral molecular damage might be caused by candidate breakers, and in determining whether such damage might be reparable. The structures of biomolecules can be compared with glucosepane to determine whether they share any structural motifs that might be damaged by the candidate agent.

Perhaps in the distant future, engineers will compete with biologists to see if they can repair aging ECM better with tiny, nonliving *nanobot* machines (see Chapter 23).

19.5.9 Therapy Usage and Frequency

If the therapeutic agent is a large molecule, such as a protein or enzyme, it might be injected or implemented through gene therapy because proteins get digested when taken orally, and they are not well absorbed from the GI tract. Small molecule agents can often be made in an orally bioavailable form. (See Section 19.5.7 for a discussion of FLC therapy administration.)

An effective therapy might repair the ECM so well that it need be repeated only at multiyear intervals. Less effective therapies might leave reactive residues or require more frequent re-treatments, perhaps even daily. If glycation inhibitors are used instead of repair therapies, continual use would be required for maximum effect, and even then glycation could probably not be completely halted. Perhaps some combination of therapies will prove to be the best treatment.

Large-molecule therapies might stimulate dangerous antigenic responses, especially if they are administered repeatedly. However, in the future, techniques might be developed to control antigenic responses to large molecule therapies. That problem is under intense study by many labs that are developing protein therapies for a variety of conditions.

19.6 Summary and Conclusions

Damage to extracellular proteins, including strand breaks, crosslinks, and AGE/ALE adducts impair the structure and function of the ECM, causing or contributing to many diseases of aging. Furthermore, with increasing age, the rate of turnover and repair of the damaged ECM declines, and damage accumulates faster. Good diet and glycation inhibitors can slow the accumulation of damage. Weight-bearing exercise stimulates natural turnover and remodeling of ECM in tendons and skeletal muscles. Thiazolium compounds can repair a portion of the AGE crosslinks, and provide clinical improvements of several age-associated pathologies. Perhaps a series of future drug discoveries will remove the entire menagerie of pathogenic crosslinks and adducts. Alternatively, a straightforward, complete therapy for extracellular aging might involve stimulating fibroblast lineage cells to more rapidly replace and regenerate the damaged ECM with newly synthesized ECM, as they move through it.

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