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Role of Nonenzymatic Glycosylation in Atherogenesis

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This review summarizes progress in nonenzymatic glycosylation research of potential relevance to atherosclerosis using a hypothetical model based on current concepts of atherogenesis. Recently, new information has been presented showing that the initial Amadori product undergoes a series of further reactions and rearrangements to form adducts, called advanced glycosylation end products (AGE). These products are irreversible and accumulate indefinitely on long-lived molecules. These AGE covalently trap soluble plasma proteins, act as signals for macrophage recognition and uptake, and induce mutations in double-stranded plasmid DNA. Covalent trapping of low-density lipoprotein (LDL) by AGE on collagen or elastin could promote lipid accumulation in the arterial wall, whereas AGE trapping of von Willebrand factor would increse platelet adhesion and aggregation leading to intimal smooth muscle cell proliferation. Recognition and uptake of AGE-proteins by scavenging macrophages could further contribute to the process of atherogenesis by stimulating release of macrophage secretory products such as macrophage-derived growth factor. Accumulation of AGE on smooth muscle cell DNA might also enhance arterial smooth muscle cell proliferation by increasing the rate of mutations affecting growth controls. This model should provide the basis for future experiments.

Key words: advanced glycosylation end products (AGE), macrophage recognition, MDGF, PDGF, von Willebrand factor, DNA, cross-linking of proteins, low-density lipoprotein, 2-furoyl-4(5)-(2-furanyl)-1*H*-imidazole (FFI)

Glucose and other reducing sugars can react with proteins and nucleic acids without the aid of enzymes to form stable covalent adducts. Although these reactions have been studied for a long time by food chemists, in vivo formation of nonenzymatic glycosylation products has only recently been found to occur. This finding has led to the hypothesis that the accumulation of slowly formed, irreversible advanced glycosylation end products (AGE) plays an important role in the aging of long-lived proteins and nucleic acids. The present review summarizes progress in glycosylation research of relevance to atherosclerosis using a hypothetical model based on current concepts of atherogenesis.

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The concept that glucose reacts with cellular proteins in vivo originated with the observation that a glycohemoglobin present in the red cell, hemoglobin A_{1c} (HbA_{1c}), was formed as a posttranslational modification of hemoglobin A. The amount formed was dependent on time and the glycemic state; diabetic animals formed HbA_{1c} at three times the rate of normal animals [1]. Subsequent work [2,3] showed that HbA_{1c} was formed by the nonenzymatic addition of glucose to the amino groups of hemoglobin, forming a Schiff base, which could then slowly rearrange to form an Amadori product (Fig. 1). This reaction is not unique to hemoglobin. At present, more than 20 proteins have been demonstrated to have attached Amadori products in vivo [see 4, for review]. In all cases examined, patients with poorly controlled diabetes have increased amounts of the Amadori product on proteins. In fact, measurement of the Amadori product attached to hemoglobin or albumin is widely used as a clinical assessment of the control of diabetes mellitus [5,6]. The amounts of hemoglobin A_{1c} and glycosylated albumin give integrals of the time-averaged blood glucose concentration for approximately 28 days and 14 days, respectively. The quantity of Amadori product found on a protein reflects the time-averaged blood glucose concentration and the half-life of the protein, hence the difference between hemoglobin and albumin.

It is important to note that the Amadori product is an equilibrium product, which reaches equilibrium in approximately 28 days. This accounts for the shorter than anticipated integral of ambient glucose concentration found on hemoglobin, which in the red cell has a half-life of 60 days. Once equilibrium is attained, the amount of Amadori product remains constant whether the protein has a half-life of 2 months or 20 years.

The Amadori product can, however, proceed very slowly to undergo a number of further dehydrations and rearrangements to form advanced glycosylation end products (AGE) on proteins (Fig. 1). The AGE moieties are characterized by being brown, fluorescent chromophores that can cross-link proteins [7]. In contrast to the Amadori product, AGE are irreversible and accumulate on long-lived proteins (eg, collagen, lens crystallins) for many years.

Figure 2 illustrates this phenomenon. Measurement of either fluorescence or absorbance at 340 nm of dura collagen samples from normal individuals revealed a linear increase with subject age: diabetics, on the other hand, did not fall in the normal range but had significantly more absorbance or fluorescence than age-matched

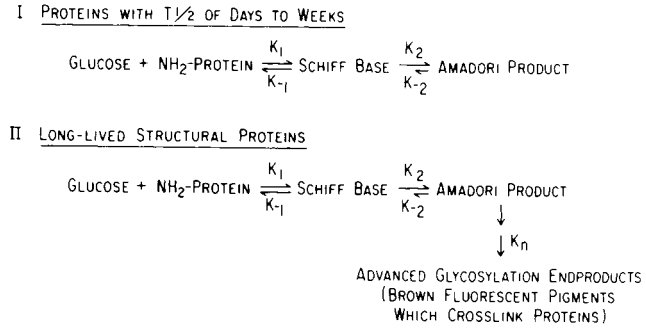

Fig. 1. Reaction scheme for nonenzymatic glycosylation.

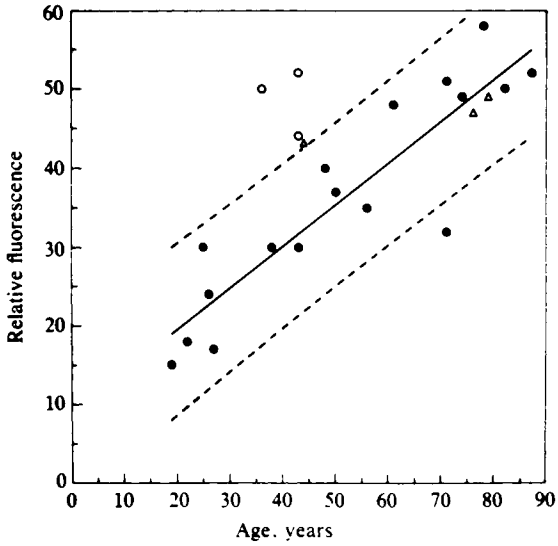

Fig. 2. Fluorescence at 440 nm on excitation at 370 nm per mg of hydroxyproline in insoluble dura collagen solubilized by collagenase digestion. The solid line represents the regression equation $y = 0.53x + 8.9$ ($r = 0.90$, $P < 0.001$). (○), patients with juvenile diabetes; (△), patients with maturity onset diabetes; (●), normal subjects.

controls [8]. This would be anticipated; with higher blood glucose, more Amadori products would occur and subsequently rearrange to form AGE.

The chemical nature of AGE-proteins has been difficult to ascertain; the Amadori product could theoretically undergo a myriad of potential rearrangements. We have recently been able to determine the structure of one of the AGE products, 2 furoyl-4(5)-(2-furanyl)-1*H*-imidazole (FFI), formed by incubating albumin and polylysine with glucose [9]. This compound is yellow-brown in color and has the characteristic fluorescence noted in AGE proteins. The structure is of particular interest; it gives insight into how glucose can cross-link proteins together. As is shown in Figure 3, two glucose molecules can condense by several routes to form the unsaturated, stable FFI. Recently, we have developed a radioimmunoassay for FFI and found it to be present in a number of long-lived proteins that had been digested enzymatically [10]. This rules out the possibility that FFI forms only during the isolation procedure. Clinically, measurement of FFI on long-lived proteins should make possible the assessment of average blood glucose over time intervals measured in years.

Besides imparting a brown color to proteins, glucose-derived AGE such as FFI can effectively cross-link proteins intra- and intermolecularly. This has been studied with several proteins (eg, lens crystallins, RNase, collagen). Lens crystallins incubated with glucose and glucose-6-phosphate have brown fluorescent pigments that cross-link the crystallin proteins [11]. These aggregates resemble the nonsulfhydryl-reducible aggregates noted in senile cataracts [12]. Studies by Eble et al [13] point to the formation of cross-links of RNase molecules even if free glucose is removed. Presumably, the Amadori product, once formed, gives rise to reactive products that

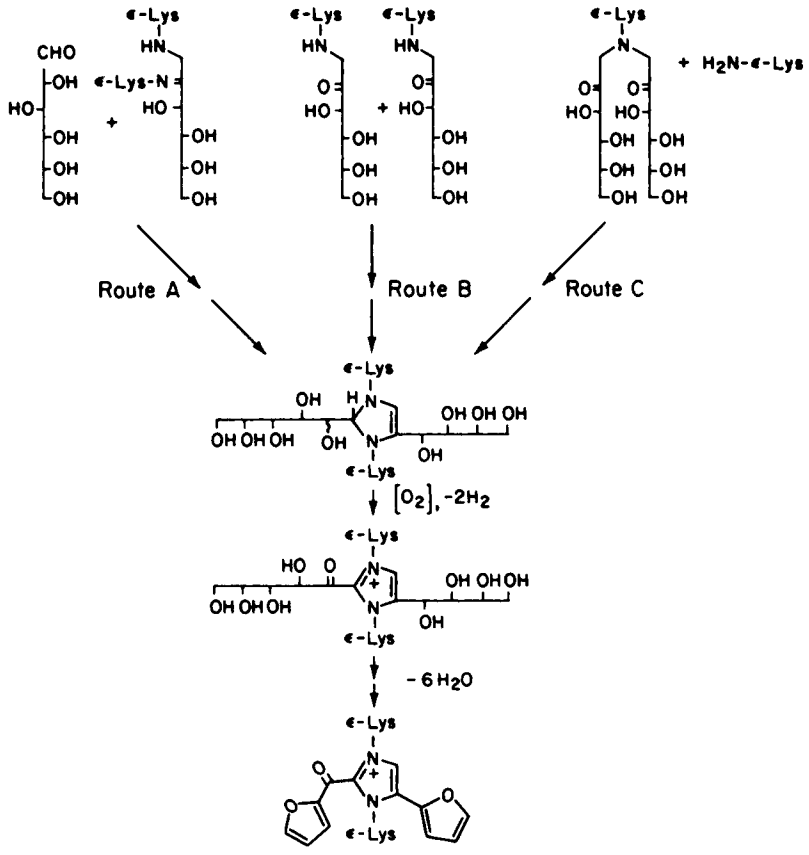

Fig. 3. Possible routes for the formation of FFI-like protein cross-links from initial Amadori adducts of glucose and ε-lysyl residues.

can react subsequently with other proteins. This has been shown convincingly in the case of collagen attached to beads [14]. Formation of AGE-collagen can subsequently trap bystander protein molecules (eg, IgG, albumin) that had not been exposed to glucose.

Another example of sugars cross-linking proteins occurs when collagen from rat tail tendons is incubated with reducing sugars. The tendon fibers, attached to weights and suspended in a 7 M urea solution, eventually break. The time necessary for breakage of the fiber is a function of the degree of cross-linking. Fibers accumulating AGE products have a greatly increased time for breaking, indicative of intermolecular cross-links [15]. Previous studies of rat tail tendons have demonstrated an increased rupture time as a function of the animals age [16]. A loss of flexibility of collagen is also one of the hallmarks of human aging.

Another effect of accumulated AGE on proteins is a decreased ability to be degraded by proteolytic enzymes. For example, there is an age-related linear decrease of collagen digestibility by bacterial collagenase or pepsin [17]. Fibrin degradation by plasmin is also reduced by AGE accumulation [18]. This important consequence of

nonenzymatic glycosylation could account for collagen accumulation and fibrin deposits noted in arterial walls of aging individuals.

From the information presented above, it should be apparent that proteins will react with glucose in vitro and in vivo to form various covalent adducts that continue to form and accumulate as a function of time. An obvious question is whether there is also an in vivo removal system for these modified proteins, which might reduce the rate at which they accumulate. Recently, the macrophage has been shown to possess a specific uptake and degradation system for AGE-proteins [19]. The senescent protein removal system of the macrophage was first noted with peripheral nerve proteins that had undergone long-term exposure to glucose in vitro or in vivo [20]. As can be seen in Figure 4A, when radiolabeled myelin was incubated with glucose or glucose-6-phosphate for 8 weeks and then exposed to macrophages, there was increased uptake compared to myelin proteins incubated in the absence of reducing sugars. (We have frequently utilized glucose-6-phosphate as a model sugar in our studies, because it reacts with proteins at a more rapid rate than glucose). Similarly, myelin exposed to glucose in vivo was also more avidly taken up by the macrophages. The signal for uptake is not the Amadori product but rather AGE. Figure 4B shows that myelin proteins of diabetic rats exposed to high glucose for 4–6 weeks had uptake similar to that observed with myelin from age-matched normal animals despite a threefold increase in Amadori product. In contrast, after AGE products formed on myelin exposed to high glucose in vivo for longer periods of time, a fourfold increase in macrophage uptake occurred (Fig. 4C). Increased uptake of myelin from old rats was also increased compared to macrophage uptake of myelin from young rats. A similar age-related increase in upatke of peripheral myelin from normal humans by human monocytes has also been observed [21]. The uptake of AGE-myelin by macrophages could be competitively inhibited by adding other AGE-containing proteins (eg, AGE-albumin) but not by unmodified proteins or mannan.

Utilizing AGE-albumin as a model system, it has been possible to demonstrate the presence of approximately 100,000 high-affinity receptors ($Ka = 1.75 \times 10^7$ M^{-1}) for AGE-albumin on mouse macrophages [19]. In these experiments, it was observed that a small amount of normal albumin also bound to macrophages and was subsequently degraded. This appeared to represent small amounts of AGE in the normal albumin in that it could be competed by AGE-albumin. Subsequent analysis of freshly isolated human albumin [10] confirmed this, showing 9.2 pmoles/mg protein of the specific AGE marker FFI. The half-life of albumin in the circulation (14 days) is thus a sufficient amount of time to permit condensation of two Amadori products to form FFI. The ligand specificity of the AGE-protein receptor is under investigation. Chemical attachment of FFI to albumin (FFI-albumin) leads to specific binding and endocytosis by the macrophage. Whether FFI is the specific recognition signal or representative of a family of AGE products remains to be ascertained.

Covalent modification of DNA can also occur readily by simply incubating DNA with reducing sugars [22]. As in the case of proteins, yellow-brown fluorescent pigments form on the DNA to form AGE-DNA. Because the amino groups of nucleotides are less reactive than amino groups of proteins, however, the amount of AGE-DNA formed is significantly less. The accumulation of AGE on DNA of the f1 bacteriophage reduced the ability of the DNA to transfect Escherichia coli at a rate proportional to both incubation time and sugar concentration (Fig. 5). Of considerable importance is the observation that addition of lysine to the incubation mixture of f1

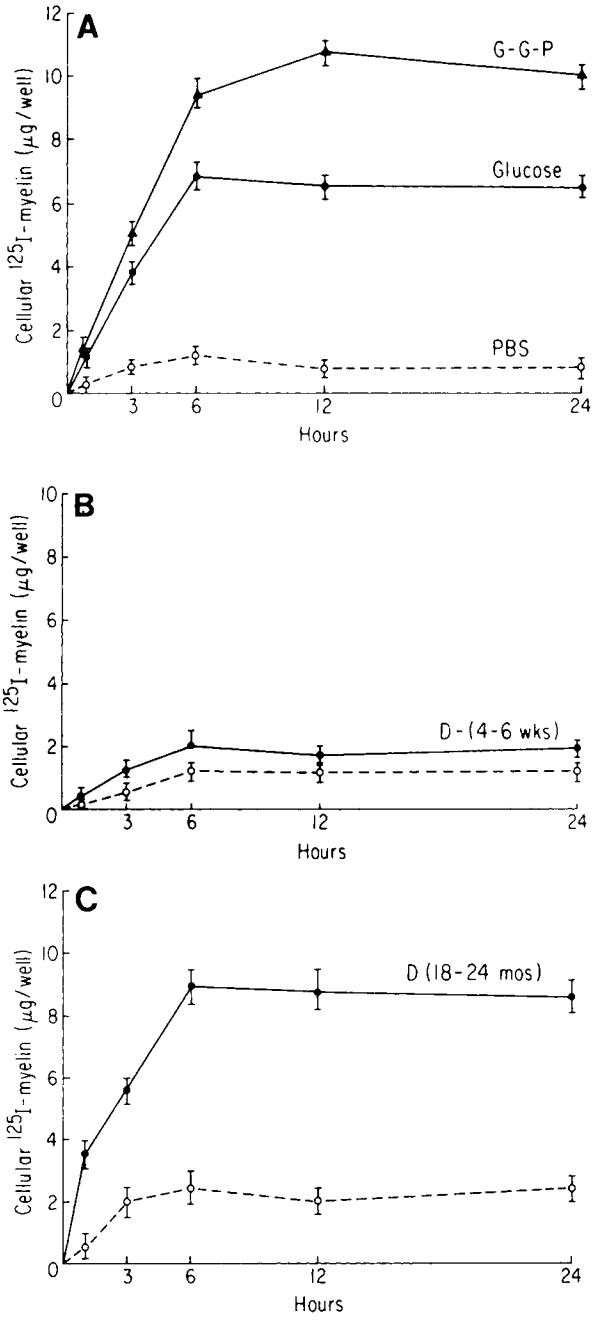

Fig. 4. Accumulation of ^{125}I-myelin by mouse peritoneal macrophages as a function of time (hr). Normal rat peripheral nerve myelin was previously incubated in 50 mM glucose (●) 50 mM G6P (▲), or PBS (○) at 37°C for 8 weeks. B, accumulation of ^{125}I-myelin from short-term diabetic (●) and age-matched normal rats (○) by mouse peritoneal macrophages as a function of time (hr). C, accumulation of ^{125}I-myelin from long-term diabetic (●) and aged-matched normal rats (○) by mouse peritoneal macrophages as a function of time (hr).

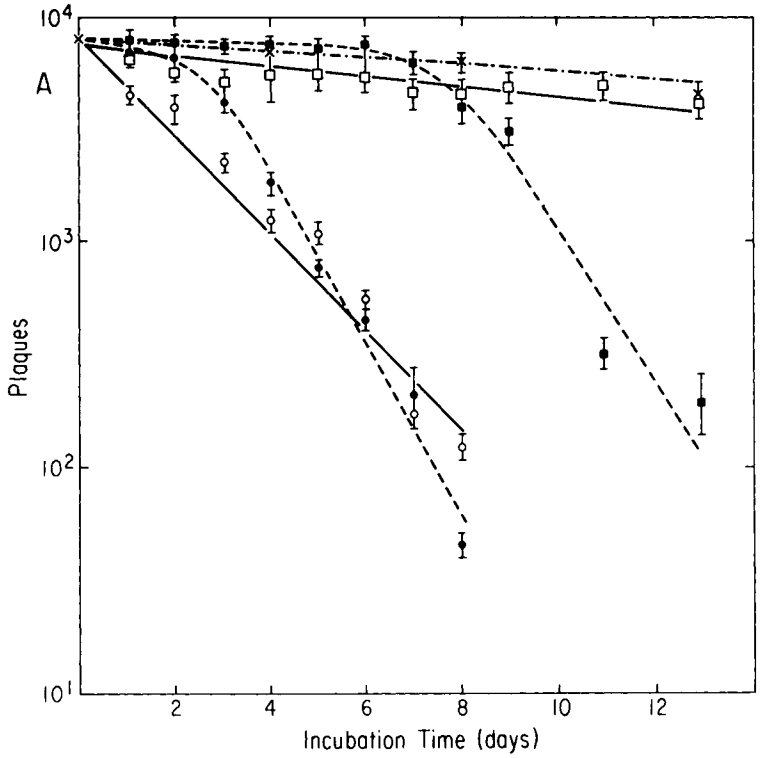

Fig. 5. (A) rate of f1 DNA inactivation by 25 mM G6P (○). 25 mM glucose (□), 25 mM G6P‖5 mM Boc-lysine (●), and 25 mM glucose‖5 M Boc-lysine (■). X, control DNA incubated with Boc-lysine alone.

DNA and glucose or glucose-6-phosphate resulted in an early preservation of biological activity, followed by a very rapid loss of activity. Presumably, this reflects the reaction of the sugar with the amino acid to form an AGE-lysine, which can react rapidly with the DNA. The covalent attachment of protein to DNA that has been noted to occur in aged cells [23] could arise by this mechanism.

The reaction of reducing sugars with DNA also has been found to be mutagenic [24]. Incubation of glucose-6-phosphate with the plasmid BR322 containing ampicillin- and tetracycline-resistance gene resulted in the occurrence of mutants that had lost the tetracycline gene when the plasmid was introduced to E coli. Most of these mutants proved to be the result of deletions or insertions of DNA in the tetracycline gene. These DNA alterations are believed to arise during the attempt by the bacteria to repair the AGE-DNA; bacterial mutants lacking the DNA repair enzyme (uvrc$^-$) did not produce mutations in AGE-PBR322.

Calculations of the rate of AGE formation in a human cell at physiological concentrations of G6P and DNA predict a rate of three hits per day. The amount of potential damage increases if one considers the effect of other reducing sugars as well. Whether those reactions occur in vivo has yet to be established. Future studies will require new methods for measuring the amounts of AGE-DNA and protein linked

to DNA by AGE. In addition, potential mechanisms for repair of the AGE-DNA need to be explored.

In that nucleic acids are long-lived molecules in the resting cell, AGE-DNA could accumulate progressively over time. Such accumulation might be responsible for age-dependent changes in the genetic material that include chromosomal alterations [25], DNA strand breaks [26], and a decline in DNA repair, replication, and transcription [27–29]. A possible role for AGE-DNA in the senescence of cells remains an attractive hypothesis to explain the age-dependent loss of human cell viability in culture.

Figure 6 outlines a working hypothesis relating AGE formation to accelerated development of atherosclerosis. This hypothesis is based on current concepts of atherogenesis, which hold that the development of atherosclerotic lesions results from two major processes: accumulation of plasma lipids in the vessel wall and the proliferation of arterial smooth muscle cells [30,31]. The rate at which atherosclerosis develops in a given individual might reflect independent contributions from each of these causal factors.

Normally, arterial intima lipoprotein cholesterol concentration is strongly correlated with plasma levels of low-density lipoprotein (LDL) [32]. Elevation of plasma LDL increases the rate of LDL infiltration, and a correspondingly larger amount of LDL is deposited in the arterial wall. As noted above, advanced glycosylation end products on connective tissue components can promote the extracellular trapping of plasma lipoproteins, which could lead to excessive lipid accumulation (Fig. 6). In

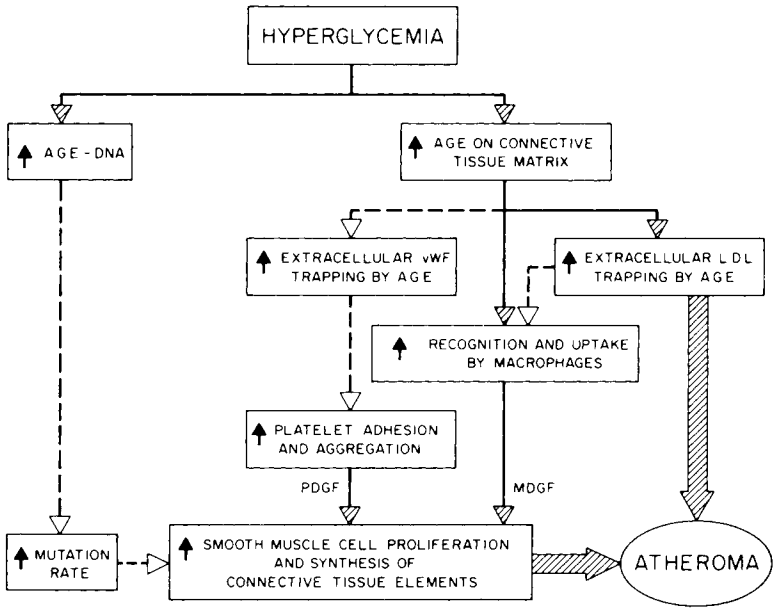

Fig. 6. Schematic representation of potential mechanisms by which excessive nonenzymatic glycosylation might contribute to the accelerated development of atherosclerosis in patients with diabetes mellitus. Solid lines indicate components for which experimental evidence has been obtained; dashed lines indicate postulated components.

addition, AGE trapping, by preventing LDL diffusions out of the intima, might also promote formation of AGE on the LDL particle itself. Subsequent recognition and uptake of this AGE-LDL by scavenging macrophages could further contribute to the process of atherogenesis by increasing secretion of the smooth muscle cell mitogen, macrophage-derived growth factor (MDGF) [33], and/or other macrophage secretory products such as enzymes.

Platelet-derived growth factor (PDGF) appears to be the primary stimulus for smooth muscle cell proliferation in vivo [31]. PDGF is released by platelets that have been stimulated to aggregate by adhesion to connective tissue-bound von Willebrand factor (VWF) [34]. The accumulation of AGE on connective tissue matrix should enhance extracellular trapping of VWF in a manner exactly analogous to that occurring with LDL and induce platelet aggregation.

Arterial smooth muscle cells might also arise from mutations affecting intrinsic growth controls, as is suggested by the observation that cells of some human atherosclerotic lesions appear to have a monoclonal origin [35]. The atherosclerosis induced in normolipidemic chickens by Marek disease herpes virus might be one example of this [36], and atherogenesis in humans has been postulated to involve other viral and chemical mutagens [37]. Accumulation of AGE on smooth muscle cell DNA could eventually result in an increased mutation rate, affecting cellular growth and proliferation, by mechanisms similar to those responsible for the sugar-induced mutations of plasmid DNA.

Formation of advanced glycosylation end products on long-lived molecules in the arterial wall of diabetics undoubtedly acts in synergy with other pathogenetic mechanisms to produce large-vessel disease [38]. Further understanding of each of these components will provide the basis for the design and development of therapeutic advances in the future.

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