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Glycation and Diabetic Complications

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Less than 25 years ago, medical students were commonly taught that there was no relationship between hyperglycemia and diabetic complications. However, subsequent epidemiological, animal, and biochemical studies strongly suggested that a relationship did indeed exist (1–3). The recent results of the Diabetes Control and Complications Trial have provided the final conclusive confirmatory evidence that this is so (4). Although we know now that the degree of hyperglycemia is directly correlated with the extent of diabetic complications, we are just beginning to understand the mechanisms linking the two.

During the past 15 years, my own work, and that of many others, has suggested that advanced glycation end products (AGEs) play a central role in the pathogenesis of diabetic complications. This occurs by three general mechanisms, shown schematically in Fig. 1. First, AGEs alter signal transduction pathways involving ligands on extracellular matrix. Second, AGEs alter the level of soluble signals such as cytokines, hormones, and free radicals, through interactions with AGE-specific cellular receptors. Third, intracellular glycation by glucose, fructose, and metabolic pathway intermediates can directly alter protein function in target tissues. In this review, I discuss each of these mechanisms individually, after first presenting an overview of AGE formation. I conclude with a summary of AGE inhibition and its effects on diabetic pathology.

OVERVIEW OF AGE FORMATION

How do AGEs form? What is the effect of diabetic hyperglycemia on their accumulation? Finally, why might some poorly controlled patients escape complications, while some well-controlled patients don't? Irreversible AGEs originate from the well-known glycohemoglobin modification, the 1-amino-1-deoxyketose product (Fig. 2). This product forms at a rate that's directly proportional to the glucose concentration. Analagous adducts form from highly reactive sugars found intracellularly, such as triose phosphates and other glycolytic intermediates (5).

Glycohemoglobin-type products go on to generate a variety of fragmentation products, particularly highly reactive carbonyl compounds such as 3-deoxyglucosone (6–11). This compound reacts again with free amino groups to form a variety of intermediate and AGEs. Studies with antibodies to AGEs suggest that immunologically similar structures form from the incubation of different sugars with different proteins (12–15). Alternatively, compounds such as 3-deoxyglucosone can be reduced to more inactive metabolites such as 3-deoxyfructose by the activity of specific reductase enzymes. The nature and efficiency of such enzymes could be an important determinant of the amount of AGEs that form at a given level of hyperglycemia.

To determine whether the AGE-intermediate 3-deoxyglucosone is actually produced in significant quantities in normal subjects, Knecht et al. (16) measured urinary and plasma levels of the relatively inactive 3-deoxyglucosone reduction product, 3-deoxyfructose. In this study, the concentration of urinary 3-deoxyfructose averaged 5.3 μg/mg creatinine, and the plasma concentration averaged 6.7 μg/dl. The plasma

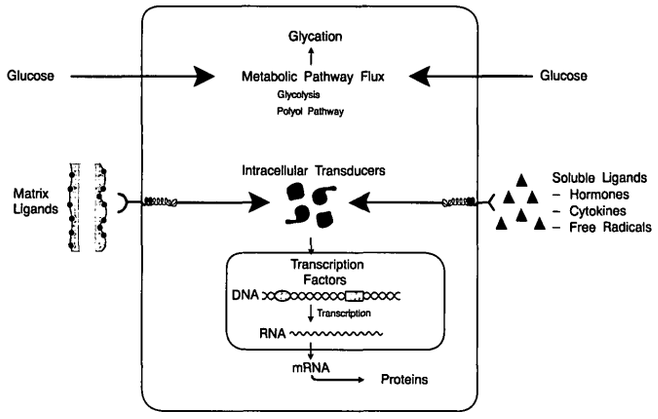

FIG. 1. Schematic cell showing three general mechanisms by which AGEs may cause pathological changes in diabetes (see text for details).

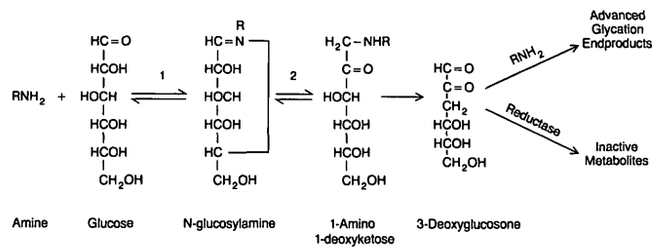

FIG. 2. Formation of AGEs from glucose. Reversible early products give rise to irreversible advanced products through generation of highly reactive carbonyl compounds such as 3-deoxyglucosone. AGE formation in vivo may be retarded by the action of reductase enzymes. (Data from Brownlee M: Glycation products and the pathogenesis of complications. *Diabetes Care* 15:1835–1843, 1992).

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AGE, advanced glycation end product; ELISA, enzyme-linked immunosorbent assay; HSPG, heparin sulfate proteoglycan; bFGF, basic fibroblast growth factor; MEAG, morpholino-ethyl-aminoguanidine; GFR, glomerular filtration rate.

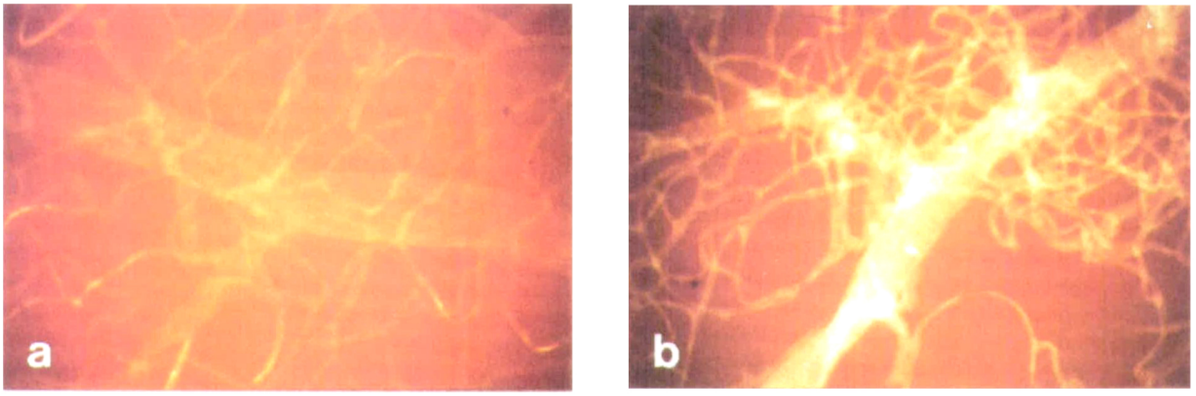

FIG. 3. AGE-specific fluorescence in photomicrographs of retinal vessels prepared from (*a*) nondiabetic and (*b*) diabetic rats (61).

concentration of 3-deoxyglucosone averaged 1.0 μg/dl. These results suggest that several milligrams of 3-deoxyglucosone are formed in the nondiabetic body per day and detoxified by reduction to 3-deoxyfructose. The amount of 3-deoxyglucosone that goes on to form irreversible AGEs is unknown.

The qualitative relationship between blood glucose level and tissue accumulation of AGEs is illustrated in Fig. 3. In retinal vessel preparations from nondiabetic and diabetic rats, AGE-specific fluorescence increased 2.6-fold after 26 weeks of diabetes (17). A similar magnitude of change in AGE-specific fluorescence has been observed in diabetic lens proteins (18) and renal cortex (19). However, these fluorescent measurements of AGE accumulation in hyperglycemic tissues appear to grossly underestimate the total quantity of AGEs. Enzyme-linked immunosorbent assays (ELISAs) using AGE-specific antibodies show that these same diabetic samples have 10–45 times more AGEs than nondiabetic samples after 5–20 weeks of diabetes. These results suggest that nonfluorescent AGEs predominant over fluorescent AGEs in diabetic tissues and show that AGE formation increases disproportionately to the increase in blood glucose.

Clinically, we know that patient groups with higher levels of mean blood glucose have a higher prevalence of diabetic complications. There are some individual patients with poor control who escape complications, however, and others with excellent control who develop severe complications. Inherited differences in the ability to enzymatically detoxify AGE intermediates, such as 3-deoxyglucosone, may be one important genetic factor responsible for determining the impact of a given level of hyperglycemia on diabetic complications.

AGES AND MATRIX FUNCTION

One major mechanism by which hyperglycemia-induced AGE formation contributes to the development of diabetic complications involves changes in the structure and function of extracellular matrix. AGE formation alters the functional properties of several important matrix components (summarized in Table 1).

Collagen was the first matrix protein used to demonstrate that glucose-derived AGEs form covalent, intermolecular bonds (20,21). On type IV collagen from basement membrane, AGE formation decreases binding of the noncollagenous NC1 domain to the helix-rich domain, inhibiting lateral association of these molecules into a normal network-like structure (22). On type I collagen, AGEs induce an expansion of the molecular packing (23). In addition, AGE formation on collagen covalently traps soluble plasma proteins such as low-density lipoprotein and immunoglobulin G, which may contribute to occlusion of diabetic vessels (24–26).

Function of the adhesive matrix molecule vitronectin is also profoundly affected by AGE formation. Site-specific binding of both heparin and collagen is markedly reduced (H.P. Hammes, K. Preissner, M. Eppinger-Albrecht, K. Benner, A. Weiss, M.B., unpublished observations). These observations are consistent with the decreased binding of heparin sulfate proteoglycan (HSPG) to diabetic basement membrane (27) and the loss of vitronectin and HSPG immunoreactivity in diabetic retinas (28). Such diabetes-induced loss of matrix-bound HSPG is thought to stimulate a compensatory overproduction of other matrix components in the vessel wall (29,30). AGE formation on laminin causes decreased polymer self-assembly, decreased binding to type IV collagen, and decreased binding of HSPG (31).

In addition to affecting target-tissue integrity by altering matrix-matrix interactions, AGE formation also interferes with important matrix-cell interactions. Recent studies have defined a lysine-containing amino acid sequence within the A-chain of the laminin molecule that promotes neurite outgrowth (32). Modification of this sequence by AGE formation inhibits neurite outgrowth by 55–65% (33). Similarly, modification of the cell-binding domains of type IV collagen causes decreased endothelial cell adhesion (34).

AGE formation on intact matrix also affects biological functions important to normal vascular tissue integrity. The endothelium-derived relaxing factor and antiproliferative

TABLE 1
Effects of AGEs on matrix function

Collagen
Type IV ultrastructural assembly (↓)
Type I intermolecular spacing (↑)
Type I immobilization of soluble proteins (↑)
Type IV endothelial cell adhesion (↓)
Vitronectin
Binding of heparin (↓)
Binding of type IV collagen (↓)
Laminin
Polymerization/self-assembly (↓)
Binding of type IV collagen (↓)
Binding of heparan sulfate (↓)
Stimulation of neurite outgrowth (↓)
Matrix
Quenching of nitric oxide (↑)
Arterial wall elasticity (↓)
Arterial wall fluid filtration (↑)

factor nitric oxide is quenched by AGEs in a dose-dependent fashion. In diabetic animals, defects in the vasodilatory response to nitric oxide correlate with the level of accumulated AGEs and are prevented by inhibition of AGE formation (35). In cell culture, AGEs block the cytostatic effect of nitric oxide on aortic smooth muscle cells and mesangial cells (36). In large arteries from diabetic rats, AGEs decrease elasticity even after abolition of vascular tone and increase fluid filtration across the carotid artery significantly (37).

AGE-SPECIFIC CELLULAR RECEPTORS

A second major mechanism by which hyperglycemia-induced AGE formation contributes to the development of diabetic complications involves alterations in the level of soluble signals such as cytokines, hormones, and free radicals, through interactions with AGE-specific cellular receptors.

The first high-affinity receptor was identified on monocytes and macrophages (38). There are 1.5×10^{-5} macrophage receptors for AGE-modified proteins per cell, with a binding affinity of 1.75×10^{-7} M^{-1}. This receptor has a unique biological significance, since it is the first receptor that recognizes a posttranslational protein modification known to occur extensively in vivo. A 60-kD and a 90-kD AGE-binding protein have been isolated from rat liver (39). Both proteins are present on monocyte/macrophages, and antisera to either protein block AGE binding to macrophages. AGE receptors have been identified on glomerular mesangial cells using antisera against these two proteins, and interaction with AGE proteins increases platelet-derived growth factor–mediated mesangial cell production of type IV collagen, laminin, and HSPG (40,41).

When AGE protein binds to its macrophage receptor, it induces production of interleukin–1 and insulin-like growth factor I, in addition to tumor necrosis factor α. The concentrations of these induced cytokines have been shown to be sufficient to stimulate proliferation of glomerular mesangial cells and arterial smooth muscle cells and to increase glomerular synthesis of type IV collagen (42,43).

Endothelial cells also express AGE-specific receptors. Ligand binding to this receptor on macrovascular endothelial cells induces two additive procoagulatory changes in the endothelial surface (44). One procoagulatory change is a rapid reduction in thrombomodulin activity. This prevents activation of the anticoagulant protein C pathway. The other procoagulatory change induced by AGE receptor binding is an increase in tissue factor activity. This increase activates coagulation factors IX and X through factor VIIa binding. In addition to these procoagulatory changes, AGE protein binding to the endothelial cell AGE receptor also induces increased production of the potent vasoconstrictor peptide endothelin-1 (45). The consequences of these AGE-induced changes in endothelial function are focal thrombosis and excessive vasoconstriction.

Two endothelial cell AGE-binding proteins have recently been isolated and characterized (46–48). A 35-kD and a 46-kD protein were purified to homogeneity. The NH_2-terminal sequence of the 35-kD protein was identical to lactoferrin, while the 46-kD protein was novel. A full-length 1.5-kb cDNA for the 46-kD protein was cloned. This integral membrane protein appears to be a new member of the superfamily of immunoglobulin-related proteins, with three disulfide-bonded immunoglobulin homology units.

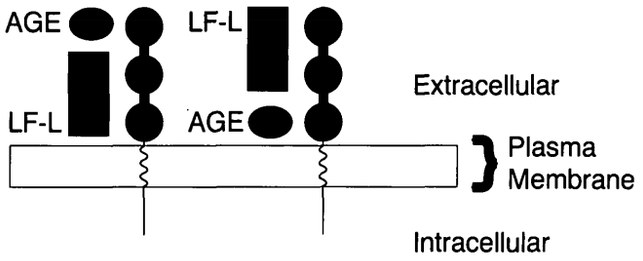

FIG. 4. Model of AGE binding to a receptor composed of an integral membrane protein and its non-covalently associated lactoferrin-like polypeptide (see text for details).

Antibodies to either protein block binding of AGEs, and immunoelectron microscopy suggests that the two proteins are closely associated on the cell surface. In vitro, the two purified proteins bind together with high affinity (K_d = 100 pM), and cross-linking studies with endothelial cells show formation of a new higher molecular weight band that reacts with antibodies to both proteins. Human monocytes have two AGE-binding proteins that are immunochemically related to the endothelial cell receptor (49). Antibodies to either protein block AGE-induced chemotaxis. Figure 4 shows a model of AGE binding to a receptor composed of the integral membrane protein and its non-covalently associated lactoferrin-like polypeptide.

Signal transduction by the AGE receptor appears to involve generation of oxygen free radicals. AGE binding to endothelial cells results in oxidant stress that is blocked by antibodies to either of the AGE receptor components (50). Receptor-mediated oxidant stress is also blocked by antibodies to AGEs themselves.

Infusion of AGE albumin caused activation of the transcription factor NF-KB, a pleiotropic regulator of many "response-to-injury" genes. In endothelial cells, these include AGE-induced genes such as tissue factor and endothelin-1. This activation was inhibited by pretreatment of animals with antibodies to the AGE receptor. These data suggest that interaction of AGEs with their cellular receptor leads to oxidant stress that results in potentially damaging changes in gene expression.

INTRACELLULAR FORMATION OF AGES

AGEs on extracellular molecules are exclusively glucose-derived. Inside cells, however, there are a number of other glycating sugars that react much more quickly than glucose to form AGEs. Because intracellular levels of fructose are elevated in many target tissues of diabetic complications, McPherson et al. (51) measured the in vitro rate of AGE formation with fructose. At 5 days, the level of fructose-derived AGEs was 10 times greater than the level of glucose-derived AGEs (51). Elevated levels of glycolytic intermediates, such as glyceraldehyde-3-phosphate, form intracellular AGEs even more quickly (52; I. Giardino, M.B., unpublished data). AGEs form on prokaryotic DNA in vitro and cause mutations and DNA transposition in bacteria and mammalian cells (53–57). If AGEs also form on DNA in vivo, deleterious effects on gene expression may occur.

AGEs do form on proteins in vivo. In erythrocytes, AGE hemoglobin accounts for 0.42% of circulating hemoglobin in normal subjects and 0.75% in diabetic subjects (58). When endothelial cells are cultured in high glucose–containing media for only 1 week, intracellular AGE content increases

TABLE 2
Effects of aminoguanidine treatment of diabetic target tissues

	Nondiabetic	Diabetic	Diabetic Rx	Reference
Retinal acellular capillaries (mm^2)	9 ± 2	167 ± 27	33 ± 11	61
Retinal microaneurysms (% pos.)	0	37.5	0	61
Urinary albumin excretion mg/24 h	2.4 ± 1.3	38.9 ± 1.4	5.1 ± 1.5	62
Mesangial volume fraction (%)	12.5 ± 2.5	18.8 ± 2.5	13.7 ± 0.6	62
Motor nerve conduction velocity (m/s)	65.5 ± 2	52.4 ± 3	64 ± 2	67
Nerve action-potential amplitude (%)	100	63	97	68
Arterial elasticity (nl/mmHg/mm)	—	7.5 ± 1.5	10.8 ± 3	37
Arterial fluid filtration (nl/sec/mm)	—	0.9	0.45	37

more than fivefold (I. Giardino, M.B., unpublished data). These data suggest that intracellular AGE formation occurs at a much faster rate than does glucose-derived extracellular AGE formation.

The consequences of intracellular AGE formation have been studied using cultured endothelial cells. In 30 mM glucose, the rate of glucose transport increased 11.6-fold, and the intracellular glucose concentration increased 6-fold at 24 h. The level of total AGE-modified protein in the cytosol was increased 7-fold at 168 h. The major AGE-modified protein was basic fibroblast growth factor (bFGF). Anti-bFGF antibody completely neutralized cytosolic mitogenic activity at both 5 and 30 mM glucose, demonstrating that all the mitogenic activity was caused by bFGF. At 30 mM glucose, mitogenic activity of endothelial cell cytosol was reduced 70%. Quantitation by ELISA showed that 30 mM glucose did not decrease the level of bFGF protein, suggesting that the marked decrease in bFGF mitogenic activity resulted from posttranslational modification of bFGF by AGEs. In vitro AGE modification of recombinant bFGF using different sugars reduced mitogenic activity 50–90% (I. Giardino, D. Edelstein, M.B., *J Clin Invest.* In press).

AGE INHIBITION AND ITS EFFECTS ON DIABETIC PATHOLOGY

To test the hypothesis that AGE formation and its consequences play an important role in the pathogenesis of diabetic complications in vivo, it was necessary to have pharmacological agents that inhibit AGE formation. Aminoguanidine was investigated because it has a structure similar to α-hydrazinohistidine, a compound that reduces diabetes-induced vascular leakage, while having opposite effects on histamine levels. These experiments established aminoguanidine as the prototype inhibitor of AGE formation (20).

At first it was thought that aminoguanidine prevented AGE formation by reacting with the glycohemoglobin-like product on proteins. Later, mass spectroscopy data showed that aminoguanidine was reacting mainly with non-protein-bound derivatives of early glycation products such as 3-deoxyglucosone (59). This was important to know, because it meant that potentially antigenic aminoguanidine adducts were not forming on proteins. Milton Feather (60) has subsequently used nuclear magnetic resonance, mass spectroscopy, and X-ray diffraction to show that aminoguanidine reacts with the AGE precursor 3-deoxyglucosone to form 3-amino–5- and 3-amino–6-substituted triazines. These triazines are produced as a result of initial hydrazone formation at either C-1 or C-2.

The effects of aminoguanidine on diabetic pathology have been investigated in retina, kidney, nerve, and artery (Table 2). In the retina, excess AGE formation in diabetic microvessels is prevented by aminoguanidine treatment (Fig. 3). The effect of AGE inhibition on retinal pathology is summarized in Table 2. In this model, diabetes causes a 19-fold increase in the number of acellular capillaries. Treatment of diabetic subjects with aminoguanidine reduced the number of acellular capillaries by 80%. A similar effect of diabetes and aminoguanidine treatment on the number of eyes positive for microaneurysms was also observed. In addition, aminoguanidine treatment markedly reduced pericyte dropout (61).

Similar results have been obtained in animal models of diabetic kidney disease (62–64). Diabetes increases AGEs in the renal glomerulus, and aminoguanidine treatment prevents this diabetes-induced increase. Untreated diabetic animals developed the characteristic structural feature of human diabetic nephropathy, increased fractional mesangial volume. It is this increase in mesangial volume that is thought to obliterate glomerular capillaries and cause glomerular filtration rate (GFR) to decline (65,66). When diabetic animals were treated with aminoguanidine, this increase in fractional mesangial volume was completely prevented (Table 2). Untreated diabetic animals also developed albuminuria that averaged 30 mg/24 h by 32 weeks. Compared to controls, this was more than a 10-fold increase. The level of albumin excretion was reduced nearly 90% in aminoguanidine-treated animals (62). Aminoguanidine also prevented albuminuria in hypertensive diabetic rats without affecting blood pressure (63). Abnormalities of diabetic peripheral nerve are also improved by aminoguanidine treatment. In one study, both motor nerve and sensory nerve conduction velocity were decreased by 8 weeks of diabetes (67). These decreases were prevented by aminoguanidine treatment. In another study, 24 weeks of diabetes decreased nerve action potential amplitude by 37%, and peripheral nerve blood flow by 57%. Both were normalized by aminoguanidine treatment (68).

In large arteries from diabetic rats, aminoguanidine treatment increased elasticity as measured by static compliance, aortic input impedence, and left ventricular power output. In addition, fluid filtration across the carotid wall was decreased significantly (37).

Recently, it has been suggested that some of the in vivo effects of aminoguanidine might be caused by inhibition of the inducible isoform of the enzyme nitric oxide synthase (69). To evaluate this, the novel aminoguanidine derivative morpholino-ethyl-aminoguanidine (MEAG) was synthesized (70). MEAG does not inhibit either the constitutive or inducible forms of nitric oxide synthase. Furthermore, MEAG is 1,000-fold less potent an inhibitor of diamine oxidase than is aminoguanidine. However, it still retains aminoguanidine's ability to inhibit AGE formation in vitro. When studies of aminoguanidine-responsive pathologies are

repeated with MEAG, the results are identical to those obtained with aminoguanidine. In vivo, then, inhibition of AGE formation appears to be the predominant mechanism by which diabetic pathology is prevented by aminoguanidine treatment.

The prevention of diabetic retinopathy, nephropathy, and neuropathy by pharmacologic inhibition of AGE formation in animal models suggests that aminoguanidine and other AGE inhibitors have a potential therapeutic role in the treatment of diabetic patients. Currently, a multi-centered, randomized, double-blind study is recruiting patients to evaluate the effects of aminoguanidine on various endpoints in different stages of diabetic nephropathy. The study has two major components.

In the overt diabetic nephropathy protocol, adult insulin-dependent patients with diabetes onset before age 25, proteinuria >500 mg/day, and creatinine clearances between 40 and 90 ml/min, will be randomized to either placebo or one of two different aminoguanidine dosage treatment groups. Decline in GFR and changes in urinary protein excretion will be sequentially evaluated. In the end-stage renal disease protocol, diabetic patients with end-stage renal failure who have been on chronic hemodialysis <3 months will be randomized to either placebo or one of two aminoguanidine dosage treatment groups. In this protocol, the primary end points are cardiovascular morbidity and mortality.

Clinical studies of aminoguanidine build on the knowledge summarized in this review. We know that AGEs accumulate as a function of the level of chronic hyperglycemia. We know that AGE accumulation causes dysfunctional changes in extracellular matrix, abnormal receptor-mediated production of cytokines, and altered function of intracellular proteins. Most importantly, we know that pharmacologic inhibition of AGEs prevents diabetic complications in animal models. What we don't know yet is how many of these same effects will occur in people with diabetes. The answer to that question must await the completion of definitive clinical trials.

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