ADVANCED PROTEIN GLYCOSYLATION IN DIABETES AND AGING

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ABSTRACT

Products of advanced protein glycosylation (advanced glycation end products, or AGEs) accumulate in tissues as a function of time and sugar concentration. AGEs induce permanent abnormalities in extracellular matrix component function, stimulate cytokine and reactive oxygen species production through AGE-specific receptors, and modify intracellular proteins. Pharmacologic inhibition of AGE formation in long-term diabetic animals prevents diabetic retinopathy, nephropathy, neuropathy, and arterial abnormalities in animal models. Clinical trials in humans are currently in progress.

INTRODUCTION

Advanced protein glycosylation, a process involving the nonenzymatic modification of tissue proteins by physiologic sugars in vivo, appears to play a central role in the pathogenesis of diabetic complications. These sugar-derived modifications, called advanced glycation end products (AGEs), may also figure prominently in the pathogenesis of age-related disorders affecting connective tissue, lens, blood vessels, and nerves. Nearly all in vivo studies of AGEs have focused on diabetes rather than on age-associated disorders because in diabetes, tissue damage develops over a much shorter experimental observa-

tion period. Nevertheless, AGEs do accumulate on long-lived human proteins as a function of age (1) and would be expected to damage tissue by the same mechanisms operative in diabetes. As described below, accumulation of AGEs depends on both sugar concentration and the rate of protein turnover. Thus, some proteins that reach critical levels of AGE modification in sites where diabetic complications occur may turn over too quickly for normal levels of blood glucose to cause functional alterations, while proteins with a longer half-life would continue to be modified over a longer period of time.

BIOCHEMISTRY OF AGE FORMATION

AGEs are generated from the so-called Amadori product, a 1-amino-1-deoxyketose produced by the reaction of glucose with protein amino groups. The rate of formation of Amadori products is directly proportional to the glucose concentration. Analogous products are formed from the reaction of other aldoses with proteins (2). Studies with antibodies to AGEs suggest that immunologically similar structures form from the reaction of a number of different sugars with proteins (1, 3–5).

Highly reactive carbonyl compounds, such as 3-deoxyglucosone, and other sugar fragmentation products are formed by processes requiring reactive oxygen species (6–13). These compounds then react again with protein amino groups to form a variety of intermediate and advanced glycation end products. Urinary and plasma levels of the relatively inactive 3-deoxyglucosone reduction product, deoxyfructose, have been measured to ascertain whether the AGE intermediate 3-deoxyglucosone is actually produced in significant quantities in normal humans (14). From the results obtained, one can calculate that several milligrams of 3-deoxyglucosone are formed in the nondiabetic body per day and detoxified by reduction to 3-deoxyfructose.

The prominence of 3-deoxyfructose in plasma and urine strongly suggests that the body contains specific reductase enzymes that detoxify AGE precursors and prevent AGE formation. The nature and efficiency of such enzymes could be an important determinant of the amount of AGEs that form at any given level of blood glucose in both diabetics and normals.

The qualitative relationship between blood glucose level, tissue accumulation of AGEs, and extent of tissue pathology has been most extensively studied in animals. In retinal vessel preparations from nondiabetic and diabetic rats, AGE-specific fluorescence increased 2.6-fold after 26 weeks of diabetes (15). A similar magnitude of change in AGE-specific fluorescence has been observed in diabetic lens proteins (16) and renal cortex (17). These increases in AGE accumulation preceded and were accompanied by histologic evidence of diabetic tissue damage. More recently, enzyme-linked immunosorbent assays (ELISAs) using AGE-specific antibodies have shown that these same

diabetic samples contain 10–45 times more AGEs than do nondiabetic samples after 5–20 weeks of diabetes. These results suggest that nonfluorescent AGEs predominante over fluorescent AGEs in diabetic tissues and show that AGE formation increases at a much greater rate than blood glucose. This relationship suggests that even modest elevations in diabetic blood glucose levels result in substantial increases in AGE accumulation.

Clinical studies such as the Diabetes Control and Complications Trial (18) confirm that in the aggregate, patients with higher levels of mean blood glucose, like diabetic animals, have a higher prevalence of diabetic complications. Among individual patients, however, some with poor control escape complications and others with excellent control develop severe complications. Similar variation is observed in the severity of age-associated degenerative processes among individuals of comparable age. 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 glycemia on both diabetic complications and age-related degenerative processes.

AGE formation causes pathological changes via three general mechanisms. 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 AGE formation by glucose, fructose, and more highly reactive metabolic pathway intermediates can directly alter protein function in target tissues. Each of these mechanisms is considered individually below.

EXTRACELLULAR AGEs AND MATRIX DYSFUNCTION

The functional properties of several important matrix components are altered by AGE formation. The first matrix protein used to demonstrate that glucose-derived AGEs form covalent, intermolecular bonds was collagen (19, 20). On type I collagen, this cross-linking induces an expansion of the molecular packing (21). AGE formation on collagen also covalently cross-links soluble plasma proteins such as low-density lipoprotein (LDL) and immunoglobulin G (22–24). These trapped plasma proteins contribute to the characteristic narrowing of the vascular lumen in diabetes. On type IV collagen from basement membrane, AGE formation inhibits lateral association of these molecules into a normal network-like structure by interfering with binding of the noncollagenous NC1 domain to the helix-rich domain (25). On laminin, AGE formation causes decreased polymer self-assembly, decreased binding to type IV collagen, and decreased binding of heparan sulfate proteoglycan (HSPG) (26).

Decreased site-specific binding of heparan and collagen to the adhesive matrix molecule vitronectin are also markedly reduced by AGE formation (HP Hammes, K Preissner, M Eppinger-Albrecht, K Benner, A Weiss, M Brownlee, unpublished data). Diabetes-induced loss of matrix-bound heparan-sulfate proteoglycan due to AGE modificiation of vitronectin and laminin may explain the observed decrease in binding of HSPG to diabetic basement membrane (27), which is thought to stimulate a compensatory overproduction of other matrix components in the vessel wall (28, 29).

AGE formation on extracellular matrix interferes not only with matrix-matrix interactions but also with matrix-cell interactions. For example, decreased endothelial cell adhesion to type IV collagen results from AGE modification of the collagen's cell-binding domains (30), and decreased neurite outgrowth results from AGE modification of a six–amino acid growth-promoting sequence in the A chain of the laminin molecule (31).

The structure and function of intact vessels appear to be altered by these AGE-induced abnormalities in extracellular matrix. AGEs decrease elasticity in large vessels from diabetic rats, even after vascular tone has been abolished, and increase fluid filtration across the carotid artery (32). 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 (33) as a result of dose-dependent quenching by AGEs. This quenching of nitric oxide by AGEs may also contribute to accelerated atherosclerosis, since AGEs block the cytostatic effect of nitric oxide on aortic smooth-muscle cells (34).

CELLULAR RECEPTORS FOR AGEs

Monocytes and macrophages were the first cells on which a high-affinity receptor for AGEs was identified (35). Macrophages have 1.5×10^5 receptors for AGE-modified proteins per cell, with a binding affinity of $1.75 \times 10^{-7} \, M^{-1}$. AGE binding proteins with molecular weights of 60 and 90 kDa have been isolated from rat liver (36). Both proteins are also present on monocyte/macrophages, and antisera to either protein block AGE binding to macrophages.

Macrophages produce interleukin-1, insulin-like growth factor I (IGF-I), tumor necrosis factor α (TNF-α), and granulocyte/macrophage colony–simulating factor in response to receptor binding by AGE protein (37–39). These cytokines are produced at levels that have been shown to increase glomerular synthesis of type IV collagen and to stimulate proliferation of both arterial smooth-muscle cells and macrophages. Using antisera against the two macrophage proteins, AGE receptors have also been identified on glomerular mesangial cells. Platelet-derived growth factor (PDGF)–mediated mesangial cell production of type IV collagen, laminin, and HSPG is stimulated by receptor binding to AGE proteins (40, 41).

AGE-specific receptors are also expressed on vascular endothelial cells. A 35- and a 46-kDa AGE binding protein have been purified to homogeneity from endothelial cells (42–44). The N-terminal sequence of the 46-kDa protein was novel, while the 35-kDa protein was identical to lactoferrin. A full-length, 1.5-kb cDNA for the 46-kDa protein was cloned. From its sequence, this AGE receptor protein appears to be an integral membrane protein with three disulfide-bonded immunoglobulin homology units. Immunoelectron microscopy suggests that the two proteins are closely associated on the cell surface, and AGE binding is blocked by antibodies to either protein. Cross-linking studies with endothelial cells show formation of a new band of higher molecular weight that reacts with antibodies to both proteins. In vitro, the two purified proteins bind together with high affinity [k_d = 100 picomolar (pM)]. Human monocytes have two AGE binding proteins that are immunochemically related to the endothelial cell receptor (45), and monocyte chemotaxis is blocked by antibodies to either protein.

The AGE receptor is not a tyrosine kinase. Rather, it appears to mediate signal transduction through the generation of oxygen free radicals. Oxidant stress is induced by AGE binding to endothelial cells. Free radical generation can be blocked by antibodies to either of the AGE receptor components and by antibodies to AGEs themselves (46). The free radical–sensitive transcription factor NF-κB is activated by infusion of AGE albumin. This activation is inhibited by pretreatment of animals with antibodies to the AGE receptor. Since NF-κB is a pleiotropic regulator of many "response-to-injury" genes, these data suggest that interaction of AGEs with their cellular receptor leads to oxidant stress that results in potentially damaging changes in gene expression.

In endothelial cells, such AGE-induced changes in gene expression include alterations in thrombomodulin, tissue factor, and endothelin-1 (47, 48). These changes induce two additive procoagulatory changes in the endothelial surface: (*a*) a rapid reduction in thrombomodulin activity, which prevents activation of the anticoagulant protein C pathway, and (*b*) an increase in tissue factor activity, which activates coagulation factors IX and X through factor VIIa binding. AGE protein binding to the endothelial cell AGE receptor also induces increased production of the potent vasoconstrictor peptide endothelin-1 (48). The predicted consequences of these AGE-induced changes in endothelial function are focal thrombosis and vasoconstriction.

INTRACELLULAR AGEs

Hemoglobin was the first protein for which nonenzymatic glycosylation was demonstrated to reflect time-integrated glucose concentration; liver alcohol dehydrogenase has also been shown to be glycosylated in vivo (49). However, these modifications involved the Amadori product, not AGEs. It had been

thought that AGEs formed only on long-lived extracellular macromolecules, since the rate of nonenzymatic glycosylation is a function of both sugar concentration and time. Recently, it was found that glucose has the slowest rate of glycosylation product formation of any naturally occurring sugar. Thus, the rate of AGE formation by such intracellular sugars as fructose, glucose-6-phosphate, and glyceraldehyde-3-phosphate is considerably faster than the rate of AGE formation by glucose (50). In vitro, the level of fructose-derived AGEs after 5 days is 10 times greater than that of glucose-derived AGEs (51).

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 diabetics (52). More striking is the observation that in endothelial cells cultured in high glucose–containing media for only one week, intracellular AGE content increases 13.8-fold (53). The major AGE-modified protein is basic fibroblast growth factor (bFGF). Anti-bFGF antibody completely neutralizes cytosolic mitogenic activity at both 5 and 30 mM glucose, which demonstrates that all mitogenic activity is due to bFGF. At 30 mM glucose, mitogenic activity of endothelial cell cytosol is 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. Cytosolic AGE-bFGF was increased 6.1-fold. These data are consistent with the hypothesis that AGE modification of intracellular proteins can alter vascular cell function.

Intracellular AGE formation may also affect DNA function. AGEs form on prokaryotic DNA in vitro and cause mutations and DNA transposition in bacteria and mammalian cells (54–58). If AGEs also form on DNA in vivo, deleterious effects on gene expression may occur.

EFFECT OF AGE INHIBITORS ON DIABETIC PATHOLOGY

Pharmacologic agents that specifically inhibit AGE formation have allowed the effects of AGEs on organ pathology to be investigated in vivo. Initial experiments established the hydrazine compound aminoguanidine as the prototype inhibitor of AGE formation (19). The mechanism by which aminoguanidine prevents AGE formation does not involve adduct formation with Amadori products on proteins. Rather, aminoguanidine reacts mainly with non-protein-bound derivatives of early glycation products such as 3-deoxyglucosone (59). Thus, potentially antigenic aminoguanidine adducts do not appear to form on proteins. More detailed mechanistic studies using nuclear magnetic resonance (NMR), mass spectroscopy, and X-ray diffraction have shown that aminoguanidine reacts with the AGE precursor 3-deoxyglucosone to form 3-amino-5- and 3-amino-6-substituted triazines (60). 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. In the retina, excess AGE formation in diabetic microvessels can be prevented by aminoguanidine treatment. In this model, diabetes causes a 19-fold increase in the number of acellular capillaries, but aminoguanidine treatment of diabetics reduced the number of acellular capillaries by 80%. Aminoguanidine treatment had a similar effect on the number of diabetic eyes positive for microaneurysms. Diabetes-induced pericyte dropout was also markedly reduced by aminoguanidine treatment (15). Additionally, aminoguanidine treatment inhibits the development of accelerated diabetic retinopathy in the spontaneous hypertensive rat model, suggesting that hypertension-induced deposition of AGEs in the retinal vasculature plays an important role in the acceleration of diabetic retinopathy by hypertension (61).

In animal models of diabetic kidney disease, similar results have been obtained (62–64). Diabetes increases AGEs in the renal glomerulus, and aminoguanidine treatment prevents this increase. Untreated diabetic animals develop the characteristic structural feature of human diabetic nephropathy, increased fractional mesangial volume. However, when diabetic animals are treated with aminoguanidine, this increase is completely prevented. Untreated diabetic animals also developed albuminuria that averaged 30 mg/24 h by 32 weeks, more than a 10-fold increase above control levels. Conversely, in aminoguanidine-treated diabetics the level of albumin excretion was reduced nearly 90% (62). In hypertensive diabetic rats, aminoguanidine treatment also prevented albuminuria without affecting blood pressure (64).

Aminoguanidine treatment also improves abnormalities of diabetic peripheral nerve. After 8 weeks of diabetes, both motor nerve and sensory nerve conduction velocity are decreased (65), but these decreases can be prevented by aminoguanidine treatment. After 24 weeks of diabetes, nerve action potential amplitude was decreased by 37% and peripheral nerve blood flow by 57%. Aminoguanidine treatment normalized both (66). Inhibition of AGE formation by aminoguanidine treatment also ameliorates the effects of diabetes on large arteries. In animal models, aminoguanidine treatment increased elasticity as measured by static compliance, aortic input impedence, and left ventricular power output. Abnormal increases in fluid filtration across the carotid wall were also significantly reduced (32).

In vivo, inhibition of AGE formation appears to be the predominant mechanism by which diabetic pathology is prevented by aminoguanidine treatment, but an additional in vivo inhibitory effect on the inducible form of nitric oxide synthase has been hypothesized (67). An effect of aminoguanidine on this enzyme is unlikely to play a role in the prevention of diabetic pathology, however, since an aminoguanidine derivative with no in vitro effect on nitric

oxide synthase activity (morpholino-ethyl-aminoguanidine) inhibits both AGE formation and the development of diabetic pathology (68).

CLINICAL STUDIES

From the biochemical, cell, and animal studies described in this chapter we can conclude that AGEs accumulate as a function of the level of chronic hyperglycemia and that this accumulation causes dysfunctional changes in extracellular matrix, abnormal receptor-mediated production of cytokines, and altered function of intracellular proteins. Most importantly, pharmacologic inhibition of AGEs prevents diabetic complications in animal models. However, whether AGE inhibition will prevent diabetic complications in humans remains unknown. To answer this question, a multicenter, randomized, double-blind study is currently in progress to examine the effects of aminoguanidine on various endpoints in different stages of diabetic nephropathy.

This study involves two major components. The first is an overt diabetic nephropathy protocol, in which adult insulin-dependent patients will be randomized to either placebo or one of two different aminoguanidine dosage treatment groups. Entry criteria include diabetes onset prior to age 25, proteinuria >500 mg/day, and creatinine clearances between 40 and 90 ml/min. Decline in glomerular filtration rate (GFR) and changes in urinary protein excretion will be sequentially evaluated. The other component is an end-stage renal disease (ESRD) protocol, in which diabetic patients with end-stage renal failure who have been on chronic hemodialysis less than three months will be randomized to either placebo or one of two aminoguanidine dosage treatment groups. The primary end points in this portion of the study are cardiovascular morbidity and mortality. Additional clinical studies focused on nonrenal end points will follow in the near future. The results of these definitive clinical trials will define the place of AGE inhibitors in the prevention and treatment of diabetic complications.

AGEs AND ALZHEIMER'S DISEASE

Two recent reports now provide evidence that advanced protein glycosylation may be involved in the development of Alzheimer's disease (69, 70). When amyloid β-protein, the precursor of Alzheimer's senile plaques, is modified by AGEs, it causes accelerated aggregation of soluble amyloid β-protein. Furthermore, Alzheimer's plaques have three times more immunoreactive AGE than does normal brain. AGE immunoreactivity colocalizes with both histological features of Alzheimer's, neurofibrillary tangles and plaques. Similarly, glycation of tau protein, the precursor of Alzheimer's neurofibrillary tangles, appears to stabilize the formation of paired helical filaments within tangles (71).

Although this work does not establish a causal role for AGEs in Alzheimer's disease, it is consistent with such a role. Further investigation will define the exact relationships between aging, AGE accumulation rate, and Alzheimer's disease.

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