# Hemoglobin-AGE: A Circulating Marker of Advanced Glycosylation

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Advanced glycosylation end products (AGEs) form spontaneously from glucose-derived Amadori products and accumulate on long-lived tissue proteins. AGEs have been implicated in the pathogenesis of several of the complications of aging and diabetes, including atherosclerosis and renal disease. With the use of recently developed AGE-specific antibodies, an AGE-modified form of human hemoglobin has been identified. Termed hemoglobin-AGE (Hb-AGE), this modified species accounts for 0.42 percent of circulating hemoglobin in normal individuals but increases to 0.75 percent in patients with diabetes-induced hyperglycemia. In a group of diabetic patients treated with the advanced glycosylation inhibitor aminoguanidine, Hb-AGE levels decreased significantly over a 1-month period. Hemoglobin-AGE measurements may provide an index of long-term tissue modification by AGEs and prove useful in assessing the contribution of advanced glycosylation to a variety of diabetic and age-related complications.

Glucose reacts nonenzymatically with protein amino groups to initiate a posttranslational modification process known as nonenzymatic glycosylation or glycation (*1*). This process begins with the conversion of reversible Schiff base adducts to stable, covalently bound Amadori rearrangement products. The formation of the minor hemoglobin species $A_{1c}$ ($HbA_{1c}$) is the best-studied example of this stage of glycation (*2*). Hemoglobin $A_{1c}$ results from the addition of glucose to the $NH_2$-terminal valine of the hemoglobin β chain and accumulates slowly over the life of each red cell. Because the amount of Amadori product that forms reflects the average blood glucose concentration, measurement of $HbA_{1c}$ has become an important indicator of long-term (3- to 4-week) glucose control in patients with diabetes mellitus (*3*).

Over a course of weeks to months, Amadori products undergo further rearrangement reactions to form fluorescent, cross-linking moieties called advanced glycosylation end products or AGEs (*1*). These products remain irreversibly bound to long-lived proteins such as collagen and accumulate as a function of age. Hyperglycemia accelerates the formation of protein-bound AGEs. Consequently, tissue AGE amounts increase rapidly in patients with diabetes mellitus.

Recent studies indicate that a number of age- and diabetes-related complications result in part from the pathological effects of protein-bound AGEs. For example, AGEs progressively cross-link connective tissue collagen. This leads to an age-related increase in connective tissue and arterial rigidity that occurs at an accelerated rate in patients with diabetes (*4*). Scavenger receptors specific for the uptake of AGE-modified proteins are present on monocytes, endothelial cells, and renal mesangial cells (*5, 6*). AGEs are chemotactic for monocytes, and the receptor-mediated uptake of AGE-modified proteins by monocytes initiates cytokine-mediated processes that promote tissue remodeling (*7*). Occupancy of endothelial cell receptors by AGEs leads to vascular permeability, down-regulation of the anticoagulant factor thrombomodulin, and increased synthesis and cell surface expression of the procoagulant tissue factor (*5, 6*). Protein-bound AGEs also act directly to chemically inactivate endothelial-derived relaxing factor (nitric oxide) and may contribute to diabetes- and age-related vascular dysfunction (*8*). Thus, a variety of in vitro and in vivo studies indicate that AGEs contribute significantly to the vascular, renal, and retinal complications of diabetes mellitus. Additional studies suggest that the slow but progressive increase in tissue AGEs that accompanies normal aging also affects the age-related prevalence of atherosclerosis and hypertension (*1, 4–8*).

Interest in the pathological role of AGEs has been motivated further by the development of aminoguanidine, a pharmacological inhibitor of the advanced glycosylation pathway (*9*). Aminoguanidine has been shown to ameliorate a variety of diabetic complications in experimental animal systems (*10*) and is presently undergoing efficacy trials for the prevention of diabetic complications in human patients. The investigation of AGE formation in vivo has been hampered by a lack of specific assay methods and by the general inaccessibility of tissue AGEs to in vivo analysis (*1*). The realization that Amadori products such as $HbA_{1c}$ are AGE precursors led us to consider the possibility that hemoglobin also might acquire AGE modifications that could be measured with recently developed AGE-specific enzyme-linked immunosorbent assay (ELISA) techniques (*11*).

Antibodies against AGEs (anti-AGEs) developed for the detection of in vivo–formed AGEs were used in a competitive ELISA (*12*) to measure hemoglobin-linked AGEs in red cell hemolysates (Fig. 1). Hemoglobin AGEs were detected in diabetic (DM) and nondiabetic, normoglycemic (NL) individuals, but significantly higher amounts were present in the diabetic group [NL ($n$ = 9): 4.3 ± 0.3 units of AGE per milligram of hemoglobin (units AGE/mg Hb); DM ($n$ = 23): 7.7 ± 0.6 units AGE/mg Hb (mean ± SE), $P < 0.001$ by Student's unpaired $t$ test]. Antibody reactivity, expressed in AGE units, was calculated relative to a synthesized AGE-albumin standard (*11*). Additional experiments showed that the Hb-AGE modification was stable to dialysis, acid precipitation, and proteolysis and was unaffected by borohydride reduction. Previous studies have shown that alkaline neutralization of early glycation products may catalyze the formation of an AGE called FFI [2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole)] (*13, 14*). The possibility that FFI might account for hemoglobin-linked AGEs appeared unlike-

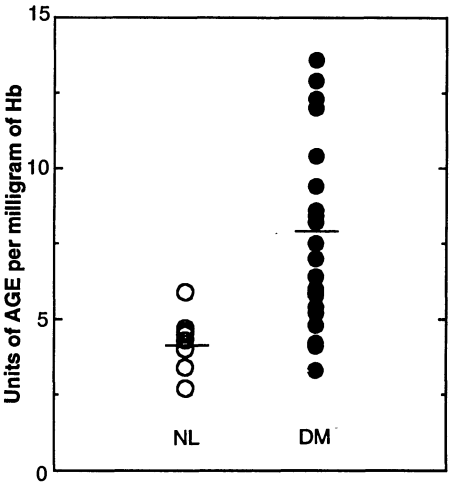


**Fig. 1.** Hemoglonin-AGE levels as measured by AGE-specific ELISA in 23 diabetic individuals (DM) and 9 nondiabetic, normoglycemic individuals (NL). Each value represents the mean of triplicate determinations assayed at three to four hemoglobin dilutions to ensure that measured values fell within the linear range of the ELISA standard curve. AGE units are calculated relative to an AGE-albumin standard synthesized and analyzed for AGE content as described (*11*).

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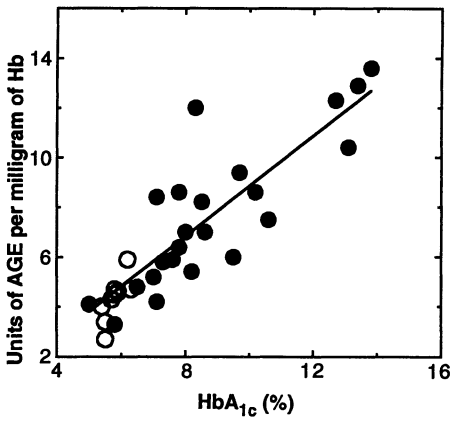


**Fig. 2.** Correlation between amounts of Hb-AGE and $HbA_{1c}$ for 9 normoglycemic (○) and 23 diabetic (●) individuals. $HbA_{1c}$ was measured by high-performance liquid chromatography (*18*) [($y = x - 1.6$, linear correlation coefficient ($r$) = 0.9; $P < 0.001$].

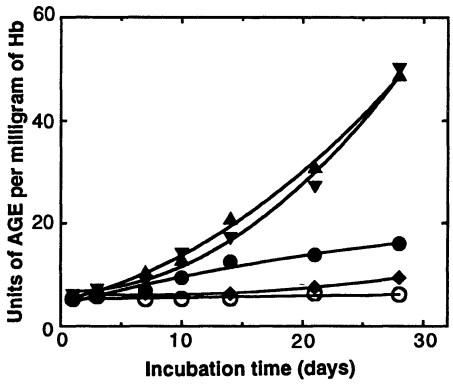


**Fig. 3.** Formation of Hb-AGE in vitro. Human hemoglobin (50 mg/ml) (Sigma) was incubated at 37°C in 0.4 M $NaPO_4$ buffer (pH 7.4) containing 0 mM glucose (○), 5 mM glucose (●), 20 mM glucose (▲), or 20 mM glucose and 50 mM aminoguanidine (◆). Aliquots that contained hemoglobin and 20 mM glucose were reduced with a 200-fold molar excess of sodium borohydride (*8*) before ELISA (▼). Aminoguanidine (50 mM) also inhibited (>95%) Hb-AGE formation in the 5 mM glucose condition (*19*). Aminoguanidine (50 mM) did not inhibit the detection of Hb-AGE when added to the glucose/hemoglobin incubations 1 hour before ELISA analysis (*19*). All incubations were performed after sterile filtration. One-milliliter samples were removed at the indicated time points and dialyzed against phosphate-buffered saline before ELISA analysis.

ly because the hemolysate preparations were neutralized with NaOH, which, in contrast to $NH_4OH$, does not catalyze FFI formation (*14*). Furthermore, anti-AGEs demonstrated no detectable cross-reactivity with synthetic FFI (*11*).

A correlation between the amounts of red cell $HbA_{1c}$ and Hb-AGE was observed (Fig. 2). This finding is consistent with previous work that demonstrates that protein-bound AGEs arise from Amadori product precursors (*1*). The formation of Hb-AGE from hemoglobin and glucose was confirmed in vitro. Purified human hemoglobin was incubated at 37°C with glucose concentrations that mimicked normoglycemia (5 mM) and hyperglycemia (20 mM). Hemoglobin-AGE formed in a time- and concentration-dependent manner (Fig. 3). That early Amadori glycation products are unreactive with anti-AGEs (*11*) was confirmed in this experimental system by the observation that sodium borohydride reduction, which alters the Amadori product epitope (*1, 2*), did not affect the detection of Hb-AGE products once formed. The addition of aminoguanidine, an inhibitor of advanced glycosylation, prevented the formation of Hb-AGEs. These data, together with prior studies of anti-AGE specificity (*11*), confirm that the Hb-AGE moiety is a stable, glucose-derived, post-Amadori product.

Amadori products are slowly reversible and attain equilibrium over a 3- to 4-week period. Advanced glycosylation end products, in contrast, remain irreversibly attached to proteins and continue to accumulate over the life-span of the protein (*1*). It was of interest, therefore, to examine the utility of Hb-AGE measurement as an in vivo marker of advanced glycosylation. Hemoglobin-AGE measurements were performed in blood specimens obtained from patients undergoing treatment with the advanced glycosylation inhibitor aminoguanidine. This patient group consisted of 18 individuals with long-standing diabetes mellitus (*15*). Blood samples were obtained before and after 28 days of aminoguanidine treatment, and the amounts of Hb-AGE were determined by ELISA. The mean Hb-AGE value decreased as a result of aminoguanidine therapy [13.8 ± 0.8 units AGE/mg Hb before therapy versus 10.0 ± 0.9 units AGE/mg Hb after therapy (mean ± SE), $P < 0.001$ by Student's paired $t$ test]. $HbA_{1c}$ values were not affected by aminoguanidine treatment [10.1% ± 0.8% before therapy versus 9.2% ± 0.8% after therapy (mean ± SE), $P$ = not significant]. No significant changes in the amounts of either Hb-AGE or $HbA_{1c}$ were observed in blood samples obtained from a group of six patients who received a placebo control.

The existence of an AGE-modified hemoglobin is noteworthy in several respects. First, AGEs generally have been considered to require a time course of months to years to form, even under hyperglycemic conditions (*1*). The present findings indicate that within the life-span of circulating red cells (120 days), significant amounts of AGE-modified hemoglobin form. If Hb-AGE units [expressed relative to a synthetic AGE-albumin standard (*11*)] are recalculated as a fraction of total red cell hemoglobin, Hb-AGE appears to account for 0.42% ± 0.07% of circulating hemoglobin. This level increases to a mean of 0.75% ± 0.08% in the diabetic group that was studied. These values contrast with corresponding $HbA_{1c}$ fractions of 5.8 and 8.9% for the normoglycemic and diabetic groups, respectively.

The reason for the apparently large amount of AGE accumulation on hemoglobin compared to connective tissue or basement membrane collagen is unknown (*11*). It may reflect the receptor-mediated turnover of connective tissue AGEs during normal remodeling (*5*) or indicate an inherently enhanced rate of AGE formation on hemoglobin as a protein substrate (*16*). Alternatively, circulating red cell hemoglobin may be susceptible to modification by reactive plasma AGEs that occur in increased amounts in patients with diabetes or renal insufficiency (*17*). Irrespective of mechanism, the application of quantitative ELISA methods to AGE measurements in vivo (*11*) indicates that AGE formation occurs more rapidly with hemoglobin than with connective tissue collagen.

The second implication of these findings is that Hb-AGE may serve as a useful biochemical index of advanced glycosylation in vivo. Kinetic considerations indicate that the formation of Hb-AGE reflects a time integral of blood glucose concentration that is significantly longer than that established for $HbA_{1c}$, a reversible glucose-derived adduct that reflects a blood glucose integral of only 30 days (*1, 3*). We did not establish the precise temporal relation between the formation of Hb-AGE and average glycemia; however, 4-week pharmacological intervention with aminoguanidine was sufficient to lower (by 28%) Hb-AGE levels in a treated diabetic population. Extrapolation of this rate leads to the prediction that an approximately 50% decrease in Hb-AGE would occur after 60 days, the time when one-half of the red cell mass has turned over. Thus, Hb-AGE may serve as an ideal integrator of blood glucose, reflecting the full 60-day half-life of the red cell. Hb-AGE measurements also should facilitate a variety of investigations into the pathophysiology of both diabetes- and age-related complications. These would include clinical studies aimed at elucidating the benefit of strict glucose control in preventing diabetic complications, as well as experimental investigations of the role of advanced glycosylation in the pathogenesis of such diabetes and age-related conditions as atherosclerosis, hypertension, and renal disease.

The tissue and end-organ damage caused by advanced glycosylation accumulates over

a period of months to years (*1*). Diabetic complications similarly progress over a period of several years. Hemoglobin-AGE measurements may prove useful in the evaluation of the pharmacological efficacy of newly emerging drugs aimed at inhibiting the advanced glycosylation pathway. The demonstration of decreased Hb-AGE levels as a result of aminoguanidine therapy provides the first direct evidence of the efficacy of this drug in preventing advanced glycosylation in human subjects.

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12. Five milliliters of whole blood was collected into heparinized tubes. We prepared hemoglobin for immunoassay following an established procedure [Z. Makita *et al.*, *Diabetologia* **34**, 40 (1991)]. We hemolyzed and delipidated washed red cell pellets by resuspending cells in 3 ml of distilled $H_2O$ and 2 ml of toluene. After vortexing, the insoluble material was pelleted by centrifugation at 3000*g* for 30 min. Hemolysate (100 μl) was then added to 3 ml of $H_2O$. This was followed by the addition of 1 ml of ice-cold 24% trichloroacetic acid. After mixing and incubation on ice for 15 min, precipitated hemoglobin was collected by centrifugation at 3000*g* for 45 min. The supernatant was discarded, and the pellet was redissolved in 0.15 ml of 1 N NaOH. Three-hundred microliters of 0.3 M $KPO_4$ buffer (pH 7.4) then was added, and tenfold dilutions (range 1:2 to 1:2000) were subjected to competitive ELISA as described (*11*). Competitive immunoreactivity was expressed relative to a synthetic AGE-albumin standard [1 AGE unit = 1 μg of AGE-albumin (*11*)]. Hemoglobin concentration was determined with the Lowry reagent with the use of purified bovine serum albumin as the standard [O. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)].
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# Negative Selection of Precursor Thymocytes Before Their Differentiation into $CD4^+CD8^+$ Cells


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**Thymic selection of the developing T cell repertoire is thought to occur at the $CD4^+CD8^+$ stage of differentiation and to be determined by the specificity of the T cell receptors (TCRs) that $CD4^+CD8^+$ thymocytes express. However, TCR signals can inhibit the differentiation of precursor thymocytes into $CD4^+CD8^+$ cells, which suggests that selection might occur earlier than thought. Indeed, in a negatively selecting male thymus, $CD4^-CD8^{lo}$ precursor thymocytes that express a transgenic TCR to male antigen are developmentally arrested as a consequence of antigen encounter and fail to become $CD4^+CD8^+$. Thus, negative selection can occur before the $CD4^+CD8^+$ stage of differentiation.**


T cell differentiation in the thymus consists of an ordered sequence of developmental steps that is influenced by the specificity of the TCR that individual thymocytes express (*1–4*). TCR-specific selection events are thought to act on thymocytes at the $CD4^+CD8^+$ stage of differentiation, selecting individual thymocytes for either deletion or further differentiation (*1–8*). $CD4^+CD8^+$ thymocytes appear to be the focus of thymic selection because their numbers are depleted in TCR-transgenic mice from negatively selecting mouse strains (*3, 4, 6, 7*) presumably because $CD4^+CD8^+$ transgenic thymocytes encounter antigen and are deleted. However, it is also possible that $CD4^+CD8^+$ transgenic thymocytes are low in number in negatively selecting strains because they fail to develop in these mice. To assess this latter possibility, we have examined the effect of TCR-mediated signals on the generation of $CD4^+CD8^+$ thymocytes from their immediate precursor cells in both normal mice and mice that express transgenic TCRαβ molecules (Tg-TCRαβ) specific for the antigenic complex created by the male antigen H-Y and H-$2D^b$ ($D^b$).

The immediate precursors of $CD4^+CD8^+$ thymocytes are $CD4^-CD8^{lo}$ thymocytes that constitute all the $CD4^-CD8^+$ cells present in the fetal thymus because mature $CD4^-CD8^+$ thymocytes do not appear until after birth (*1, 9*). To further enrich fetal thymocyte populations from both normal and Tg-TCRαβ mice for precursor cells, we eliminated thymocytes that had already become $CD4^+CD8^+$ in vivo by treatment with a monoclonal antibody (MAb) to CD4 and complement (C) (Fig. 1). Such $CD4^-$ fetal thymocyte populations consisted of two immature subsets that were distinguishable by their expression of CD8, being either $CD4^-CD8^-$ or $CD4^-CD8^+$ (Fig. 1). Placement of such $CD4^-$ fetal thymocytes into suspension culture resulted in the generation of $CD4^+CD8^+$ cells (Fig. 1), all of which were derived from $CD4^-CD8^+$ precursor cells. The derivation of these cells was determined by experiments in which precursor thymocytes were physically separated by electronic cell sorting into $CD4^-CD8^-$ and $CD4^-CD8^+$ cells (Fig. 1); $CD4^-CD8^-$ thymocytes did not undergo any detectable phenotypic change in culture (Fig. 1). Thus, precursors for $CD4^+CD8^+$ thymocytes could be uniquely identified among $CD4^-$ fetal thymocytes as $CD8^+$ cells and could be induced to further differentiate into $CD4^+CD8^+$ thymocytes by placement in suspension culture.

Studies with adult rat thymocytes have suggested that differentiation of cells expressing no CD4 and relatively little CD8


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