The Discovery of Glycated Hemoglobin

A Major Event in the Study of Nonenzymatic Chemistry in Biological Systems

SAMUEL RAHBAR

City of Hope National Medical Center and Beckman Research Institute, Duarte, California 91010, USA

ABSTRACT: Glycated hemoglobins are minor components of human hemoglobin (Hb). These are formed nonenzymatically by condensation of glucose or other reducing sugars with α- and β-chains of hemoglobin A. The subfraction HbA_{1c}, a nonenzymatic glycation at the amino-terminal valines of the β-chain, was identified by the author in the 1960s as a minor "abnormal fast-moving hemoglobin band" in diabetic patients during routine screening for hemoglobin variants. This finding later turned out to be an important biomolecular marker with clinical and pathological applications. Measurement of HbA_{1c} in diabetic patients is an established procedure for evaluating long-term control of diabetes, and the introduction of this measurement represents an outstanding contribution to the quality of care of diabetic patients in this century. More importantly, HbA_{1c} is the first example of *in vivo* nonenzymatic glycation of proteins, and its discovery opened new and still-growing avenues of research on Maillard reactions in biological systems, including the concept of advanced glycation/lipoxidation end products (AGEs/ALEs) and the development of diabetic complications and various diseases associated with aging. Although interest in the Maillard reaction is growing rapidly, much remains to be done in this field, including detection and characterization of all *in vivo* AGEs/ALEs, development and clinical applications of AGE inhibitors and breakers, as well as investigations into the possible roles of the Maillard reaction in regulatory biology and carcinogenesis.

KEYWORDS: HbA_{1c}; glycation; AGE/ALE; RAGE

EPIGRAPHS

What was once thought to be artifacts of hemoglobin electrophoresis turned out to be the most reliable index of disease progression, and an indicator of long-term glycemic control in patients with diabetes, superior to oral glucose tolerance test. In fact, the HbA_{1c} test has been a revolutionary change in diabetes management. Furthermore, HbA_{1c} concentrations predict mortality continuously across the whole population distribution in people without diabetes.

Address for correspondence: Samuel Rahbar, M.D., Ph.D., City of Hope National Medical Center and Beckman Research Institute, 1500 E. Duarte Road, Duarte, CA 91010. Voice: 626-256-4673 x 63131; fax: 626-301-8136.
srahbar@coh.org

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Most important is the fact that HbA_{1c} is the first example of *in vivo* nonenzymatic glycation of proteins, which has opened a new avenue of research and the concept of advanced glycation end products (AGEs) as one of the major causes of diabetes complications, macro-, and microangiopathies in the process of normal aging.

Haemoglobin has had good days and not such good days. The discovery of glycosylated haemoglobin in diabetes was one of its best days.

SIR HERMAN LEHMANN
(FROM A PERSONAL LETTER)

❦

The opening up and development of this area has been of fundamental importance for both the care of diabetic patients and to help unravel the cause of diabetic complications.

MAYER DAVIDSON
FORMER PRESIDENT OF ADA

❦

HbA_{1c} becomes nowadays one of the most important biochemical values to be measured in diabetic patients. The introduction of this measurement represents an outstanding contribution to the quality of care of diabetic patients in this century.

THE LATE MICHAEL BERGER
FORMER PRESIDENT OF EASD

HISTORICAL INTRODUCTION

As a postdoctoral fellow in immunology during 1962 to 1965, while teaching immunochemistry to medical students at the University of Tehran Medical School, I was involved in a research project on myeloma proteins and immunoglobulin light chains. At that time, I was also trying to learn more about protein structure in general, particularly about hemoglobin (Hb) structure, which was becoming in those years the shining-star molecule, a model protein, and one of the ABCs of biochemistry, molecular biology, pathology, genetics, and evolution. Hemoglobin and its cousin myoglobin were the very first proteins for which three-dimensional structures were worked out by X-ray crystallographic methods by Max Perutz and John Kendrew and also the first in amino acid sequence determination. Most important, perhaps, is the fact that with this one family of macromolecules—hemoglobin and myoglobin—one can illustrate nearly every important feature of protein structure, function, and evolution—for example, principles of amino acid sequence and protein folding, subunit motion and allosteric control in regulating activity, gene structure and control, and the effects of point mutations on molecular behavior. Finally, the widespread occurrence of globins and the vast body of amino acid sequence information that has been built up from many species and from human hemoglobin variants makes the globins the system of choice in any beginning study of molecular evolution and pathology.

Fascinated by all of the ongoing excitement in hemoglobin research, its newly discovered variants, characterization of sickle cell hemoglobin (Hb S) by Linus Pauling as the first molecular disease, X-ray crystallography of myoglobin and hemoglobin molecules by John Kendrew and Max Perutz, and the worldwide attempts to search for abnormal hemoglobin variants in different populations of the world

started by Sir Herman Lehmann, my mentor at Cambridge, I was motivated to switch my research from immunoglobulins to hemoglobins. My further incentive was the realization that Iran, with its highly diverse ethnic backgrounds, may be unique in the world and thus would be ideal for searching for souvenirs of the globin gene mutations. Compelling evidence for this idea was the high incidence of thalassemias in the north and west of the country and the surprisingly high incidence of sickle cell in the south and north. For these reasons, I began screening blood samples for hemoglobin abnormalities using paper and gel electrophoresis. Paper electrophoresis was a tedious, time-consuming technique; hemoglobin bands were diffused and sometimes overlapped. Gel electrophoresis had better resolution but was not ideal for screening a large number of blood samples. In 1963 Graham and Grunbaum introduced a new system for hemoglobin electrophoresis using cellulose acetate membrane as a supporting medium with high resolution; this system was able to separate different hemoglobin bands in 20 minutes, as 8 hemolysate samples could be applied on a single 5-cm-wide membrane. I found the system ideal. The same day I read the article I fashioned an electrophoretic cell using Plexiglas sheets and within 24 hours started the screening procedure. This method was of great help to me. It made me able to screen about 60 samples a day using a single electrophoresis cell. The sources of our specimens were discarded samples from the university hospitals and the national blood transfusion centers in Tehran.

In the following months, with the help of my two technicians, using several electrophoretic cells, we were able to screen approximately 300 blood samples each weekday (a total of 220,000 samples in 15 years). From the first months of our study, five or six blood samples containing abnormal hemoglobin were detected each week. By that time, we had devised a program for presumptive identification of the abnormal variant, such as Hb S, Hb C, and Hb D. In 1966, our Abnormal Hemoglobin Research Unit was able to perform comprehensive studies for the characterization of variants such as peptide mapping (fingerprinting) and amino acid analysis of the aberrant peptides. A large number of known and hitherto unknown hemoglobin variants were discovered and reported.[1]

A STRANGE, FAST-MOVING HEMOGLOBIN COMPONENT

An electrophoresis pattern of normal human hemolysates on cellulose acetate and starch gels at pH 8.6 usually shows a major HbAo with concentration of 98% of total hemoglobin and a second slow-moving HbA2 of 2%. Once every two to three weeks, we observed a fast-moving hemoglobin band in cellulose acetate electrophoresis from blood samples received mostly from university hospitals. On electrophoresis in alkaline buffers, the unusual hemoglobin component was not separated as a sharp band from normal HbA, but appeared as a trailed diffused area in front of HbA and appeared to be different from the known fast-moving hemoglobin variants such as HbJ, HbI, or HbN. The unusual component was sharply resolved on agar gels in citrate buffer at pH 6.0, moving faster than HbA in the position of HbF, and constituted 10 to 15% of normal HbA. Because this unusual hemoglobin was stable as revealed by the heat denaturation test at 50ºC and by the isopropanol test, it was not considered to fit with or relate to other hemoglobin variants we observed everyday in our study since β-chain variants such as HbS constitute 40–45% of total hemoglobins,

and α-chain variants constitute 25% of total hemoglobin. Thus, the unusual fast moving hemoglobin remained a mystery to us until 1967.

The first patient who presented the unusual fast-moving hemoglobin was identified for further study. A 67-year-old female named Zobaydeh Khatoon was traced to Vaziri Hospital. Upon review of her hospital chart labeled "DIABETIC," we found out that she was suffering from a severe uncontrolled diabetes mellitus. At first this was considered to be a coincidence. A person with hereditary abnormal hemoglobin could also suffer from diabetes. But when the same observation was made in several other blood samples in the following weeks, and all the patients were found to be diabetic, the possibility of a real relationship could no longer be ignored. Our study then focused on diabetes mellitus, and during the three months that ensued the abnormal band was detected in the blood of all 47 diabetic patients studied. The finding of the "diabetic hemoglobin components" was thus reported in 1968.[2] Huisman and Dozy had reported an increase in the fast-moving HbA1 in four diabetic patients who had been treated with tolbutamide. This increase was attributed to the reaction of tolbutamide to Hb. Attempts to reproduce this phenomenon by incubating red cells and hemolysates *in vitro* with tolbutamide were, however, unsuccessful.[3]

In 1968, my studies on abnormal Hb in diabetes were continued at Albert Einstein College of Medicine, in collaboration with Professor Helen Ranney and Drs. Paul Gallop, Olga Blumenfeld, and Robert Bookchin. That medical school was then an institution of extraordinary creativity and great dynamism. Dr. Elsa Paulsen, who was in charge of the diabetic clinic at Jacobi Hospital in the Bronx, provided blood samples from 92 diabetic patients, and the existence of a diabetic component was reconfirmed. The purification techniques described by Schneck and Schroeder, and Allen, Schroeder, and Balog were employed for structural studies using column chromatography on a Bio-Rex 70 cation exchanger. Soon it was demonstrated that the diabetic component has a chromatographic characteristic similar to that of HbA_{1c}, a minor Hb component previously described by Schneck and Schroeder and known to exist in hemolysates of normal adults in a proportion of one to four percent. Structural studies later established that the diabetic Hb was indeed identical with HbA_{1c}, which was elevated two- to threefold in diabetic patients.[4,5]

HEMOGLOBIN A_{1C}: A GLYCATED HEMOGLOBIN

In enzymatic glycosylation, the linkage of sugars to certain residues such as serine, asparagine, and hydroxylysine is under strict enzymatic (genetic) control. Enzymatic glycosylation serves a wide variety of functions, such as diversifying antibodies, facilitating secretion of a protein from its cell of origin, enhancing a protein's survival in the circulation, providing a protective coat or barrier, or forming specific receptors for hormones and other humoral substances. In contrast, proteins can also condense nonenzymatically with sugars to form a variety of adducts. This phenomenon, sometimes called the *browning (Maillard) reactions*, has been well known in the food industry for 90 years.[6] It was generally assumed that nonenzymatic glycation depended on the presence of a high concentration of free sugar and required high temperatures for the reaction to take place. (The term *glycation* was coined to differentiate nonenzymatic glycosylation from enzymatic glycosylation).

Glycation reactions involve condensation of aldehydes, ketones, and reducing sugars with amino groups on proteins, peptides, and amino acids. The carbonyl group on sugars forms a Schiff base with the amino group, followed by an Amadori rearrangement.[7,8] Following the discovery of HbA_{1c} and other glycated hemoglobins in the 1960s as the first examples of *in vivo* glycated proteins, it has become increasingly apparent that nonenzymatic glycation can occur under physiological conditions.[9–17]

HEMOGLOBIN A_{1C} IN HEALTH AND DISEASE

The global prevalence of diabetes is predicted to rise from 135 million in 1995 to 300 million by 2025. In the US alone, 70 million diabetic patients with their associated complications cost $3 billion a year. Large prospective studies such as the DCCT, UKPDS, EDIC, and EPIC-NORFOLK multicenter clinical studies were designed to investigate the long-term risks of complications of diabetes and their correlation with Hb A1C levels. Nearly 10,000 publications on HbA_{1c} have appeared in the literature in the past 30 years.

What we learned from these studies is:

- Diabetes mellitus increases the risk of cardiovascular.
- HbA_{1c} concentrations predict cardiovascular risk in people with diabetes.
- HbA_{1c} concentrations predict mortality continuously across the whole population distribution in people without diabetes and at concentrations below those used to diagnose diabetes.
- People with high HbA_{1c} concentrations may benefit from control of blood pressure and cholesterol concentration.
- HbA_{1c} may provide a practical screening tool for diabetes or impaired glucose tolerance.[18]

The EURODIAB Prospective Complication Study reported an association of HbA_{1c} with inflammatory markers of endothelial function in diabetes:[19]

- HbA_{1c} is strongly and consistently associated with all inflammatory markers tested including C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor (TNF).
- Measures of inflammation were associated with diabetes, duration glycemic control, the advanced glycation end products pentosidine, body mass index (BMI), triglycerides, HDL (inversely), and systolic/blood pressure. These factors were directly associated with HbA_{1c}.
- Measures of inflammation were strongly associated with markers of endothelial dysfunction including soluble vascular cell adhesion molecule-1 and soluble E-selectin.
- A close link between poor glycemic control, inflammation, and vascular endothelial dysfunction has also been demonstrated in type 2 diabetes.
- HbA_{1c} may reflect the biological activities of hyperglycemia, Amadori products, and AGEs, all of which induce inflammation.

- AGE pentosidine was strongly associated with the general score of inflammatory markers independent of HbA_{1c}.

ADVANCED GLYCATION AND ADVANCED LIPOXIDATION END PRODUCTS (AGEs/ALEs)

Modification of proteins by sugar continues beyond the Amadori product (such as fructosylysine). The Amadori product is a precursor to AGEs, which are more permanent, irreversible modifications of proteins. However, the Schiff base product also plays a major role in protein modification through the Namiki pathway.[20] Glycation is a spontaneous nonenzymatic amino carbonyl reaction between reducing sugars and long- lived proteins and lipids; it is a major form of chemical modifications of biomolecules that compromise their function. These chemical damages are detectable in the form of advanced glycation and lipoxidation end products (AGEs, ALEs), amino acids modified by reactive oxygen species (ROS), reactive nitrogen species (RNS) chlorine, and racemized amino acids.[21] Glycation is a major source of ROS, RNS, and reactive α-dicarbonyl intermediates that are generated by both oxidative (glycoxidative) and nonoxidative pathways of glycation.

The toxic effects of AGEs (both endogenous and exogenous) result from structural and functional alterations in plasma and extracellular matrix (ECM) proteins—in particular, from the crosslinking of proteins and the interaction of AGEs with their receptors and/or binding proteins. This leads to enhanced formation of ROS with subsequent activation and release of proinflammatory cytokines, growth factors, and adhesion molecules.[22] AGE accumulation in collagen, a long-lived structural protein in the extracellular matrix region of the kidney, is thought to effect changes in elasticity, ionic charge, thickness, and turnover of basement membrane components. Immunohistochemical studies using anti-AGE antibodies have revealed the presence of AGE-modified proteins in several tissues under pathological conditions, including the kidneys of patients with diabetic nephropathy chronic renal failure, atherosclerotic lesions of arterial walls, and amyloid fibroids in hemodialysis-related amyloidosis, suggesting the potential involvement of AGE modification in the pathogenesis of age-related disorders. Most recently, the change of emphasis to in vivo AGE formation of intercellular regulatory proteins through modification of cysteine residues has provided a possible link between nonenzymatic and enzymatic chemistry in biological systems.[21]

CONCLUSIONS AND PERSPECTIVES

Several lines of evidence indicate the accumulation of AGEs to high levels in tissues in age-related chronic diseases, such as diabetes, atherosclerosis, arthritis, amyloidosis, and Alzheimer's disease. The challenge is to find out whether AGE accumulation and other protein modifications, including amino acid modifications of proteins by products of lipid peroxidation, are causative or correlative with respect to chronic diseases. Here is some evidence in favor of the causative role of AGEs in pathology:

- Levels of AGEs/ALEs are correlated with the severity of diabetic complications.
- Aggregation, crosslinking, and insolubilization of lens proteins and collagen by AGE-modification are seen in cataracts and arthritis, respectively.
- Glycation inactivates several enzymes and hormones such as insulin, interferon-γ, and sarcoendoplasmic reticulum Ca2+ ATPase, an intracellular sulfide containing enzymes that is modified on cysteine.[23]
- Some AGEs or their precursors are the source of oxidative stress by the generation of superoxide, hydroxyl group, and hydrogen peroxide, which accelerate the damage to proteins.[24,25]
- AGE-modified proteins promote lipid peroxidation reaction and vice versa.
- Reactive carbonyl and dicarbonyl (RCS) compounds derived from sugars and/or lipids such as glyoxal (GO), methylglyoxal (MGO), malonedialdehyde (MDA), and hydroxynonenal (HNE) rapidly react with proteins to generate AGE-protein crosslinks and tissue damage.[20,24]
- MGO, an intermediate of AGE-formation, has been found to generate ROS.[26]
- A strong correlation between blood levels of MGO and the severity of diabetic complications has been reported.[27]
- CML-rich proteins are redox active (glycochelates) and can catalyze oxidative damage.[28]
- AGE-modified proteins accumulated in tissues can generate an inflammatory response through recruitment of macrophages and monocytes. Proinflammatory processes are a known phenomenon in atherosclerosis and Alzheimer's disease.[29]
- Injection of AGE-modified proteins prepared *in vitro* to healthy animals induces damage to kidney and vascular walls similar to what is seen in diabetes. Treatment of affected animals with anti-AGE antibody ameliorates the nephropathy.[30]
- Tissue accumulation of AGEs is not uniform in different organs. AGE tissue specificity has been demonstrated in kidney, retina, and brain arteries using a specific antibody against each AGE component.[31]
- Type 2 diabetes and atherosclerosis are etiologically related. Modification of proteins by glycation and glycoxidation in diabetes, or lipid peroxidation in atherosclerosis, is thought to be central to the pathogenic process.[20]
- Silent cumulative random damage to the genome resulting from Maillard reactions and oxidative stress, and DNA's imperfect repair system has been proposed as the primary factor in aging.[33]
- DNA damage by Maillard products consists of transpositions, deletions, insertions, and substitutions resulting from errors in repair processes.[33]
- Nucleotide glycation was found to be associated with mutagenesis and apoptosis.[32]
- Caloric restriction in animal models leads to life span extension associated with a decrease in the rate of AGE formation and crosslinks in skin collagen of rodents.[34]

- Inhibition of AGE formation in diabetes results in limiting oxidative and inflammatory damage in tissues, retarding the progression of pathology.
- Recent discoveries of natural defense mechanisms (both cellular and enzymatic), against glycation and AGE formation provide further evidence that nature has recognized the Maillard and oxidative reactants to be deleterious to biological systems and has developed elaborate mechanisms to protect the biomolecules.[35] Other enzyme systems involved in the enzymatic regulation of AGE formation include enzymes that metabolize α-dicarbonyl (α-oxaloaldehydes) such as endogenous enzymes capable of reducing 3-DG.[35] Two of these NADPH-dependent enzymes are oxoaldehyde reductase and aldose reductase, which are able to detoxify reactive dicarbonyl intermediates.[35] Enzymatic antiglycation include glyoxylase systems I and II, fructosamine-3-kinase, and fructosyamine oxidize (amadoriase), which are able to deglycate at different stages of the Maillard reaction.[35]
- Cellular defense mechanisms, including several AGE receptors characterized on the surface of monocytes, macrophages, and endothelial, mesangial, and hepatic cells, such as macrophage scavenger receptor types I and II, AGE-R1, R-2, and R-3. These receptors are involved in AGE-protein turnover and mediate inflammation.[35]
- RAGE, a member of the immunoglobulin superfamily, has been found to have wide distribution in tissues. RAGE is a multiliganded membrane receptor that mediates cell signaling.[36] AGE-RAGE binding on macrophage and macroglia lead to generation of ROS and activation of nuclear transcription factor NF-κB and other cytokines. Blockade of RAGE using soluble RAGE, the extracellular ligand binding domain of RAGE, suppresses the levels of some inflammatory cytokines. Treatment of diabetic rats with the soluble RAGE completely suppressed diabetic atherosclerosis.[37,38]
- Glycation, inflammation, and RAGE have been proposed as a scaffold for the macrovascular complications of diabetes and beyond.[39]
- Experience with the use of a growing number of AGE inhibitors such as aminoguanidine, pyridoxamine, and LR-90 in diabetic humans and animals and their effects on preventing or ameliorating diabetic complications are evidence for the causative effects of AGE pathology.[40–42]
- Most recently, a firm link of protein glycation to endothelial cell dysfunction has been established.[43]

Although interest in the field of Maillard reaction is growing rapidly (the number of publications has gone up from 200 in the year 2000 to 504 in 2003), much remains to be done to detect and characterize all *in vivo* AGE products, their origin, and aspects of such a complex phenomenon.

- Recent studies suggesting lipids as a major source of protein modification have established a strong correlation between obesity and diabetes. Further studies are needed to investigate the relationship between obesity and AGE accumulation in the tissues of obese people.

- Structural studies on protein modification on sites other than lysine and arginine, such as cysteine, histidine, tyrosine, and tryptophane, need further research.
- Studies are needed to investigate the biological impact of dietary AGEs, tobacco AGEs, and other environmental factors.
- We are just beginning to understand specific DNA modification by the Maillard reaction *in vivo*. These studies may help to find relationships between somatic mutations, aging, and carcinogenesis.
- Increasing uses of mass spectrometry, gas chromatography, and immunological methods will be of great importance to identify and quantify specific AGE products in tissues, including amino acid modification and their oxidized products.
- Studies are needed to focus on the role of AGE receptors, particularly RAGE and its involvement in inflammation and carcinogenesis, and the possible therapeutic use of soluble RAGE.
- The most intensive efforts should be dedicated to developing new inhibitors of glycation and AGE formation side-by-side with the development of AGE breakers.

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