Review Article

GLYCATED HEMOGLOBIN-THE CLINICAL AND BIOCHEMICAL DIVIDE: A REVIEW

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ABSTRACT

Diabetes mellitus is a chronic metabolic disorder characterized by rise in blood glucose level called "hyperglycaemia". The main long term vascular complications are coronary artery disease, stroke, renal failure etc. The measurement of glycosylated hemoglobin (GHb) is one of the well established means of monitoring glycemic control in patients with diabetes mellitus. Hemoglobin (Hb) is composed of four globin chains. Adult hemoglobin (HbA) is the most abundant form in most adults and consists of two α and two β chains. Fetal hemoglobin (HbF), which is predominantly present at birth, consists of two α and two γ chains. Glycosylation is a non-enzymatic reaction between free aldehyde group of glucose and free amino groups of proteins. The biosynthesis of glycosylated hemoglobins (HbA_{1a}, HbA_{1b}, and HbA_{1c}) occurs slowly, continuously and almost irreversibly throughout the four month life span of erythrocytes and the process is non-enzymatic. Recent reports have shown that the concentration of total glycosylated hemoglobin measured by commonly used methods may change significantly over a period of hours. This reflects the short term fluctuations in glucose concentration. It is now realized that these rapid changes depend on the synthesis or dissociation of the labile fraction of HbA_{1c}, which is not separable from the stable form of HbA_{1c}, by most routine methods. Physicians should be aware of the expected variation in HbA_{1c} measurements performed in associated diseases.

Keywords: Glycated hemoglobin, Diabetes mellitus, Hyperglycemia, Coronary artery disease.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by rise in blood glucose level called "hyperglycaemia"[1]. The main long term vascular complications are coronary artery disease, stroke, renal failure etc. The measurement of glycosylated hemoglobin (GHb) is one of the well established means of monitoring glycemic control in patients with diabetes mellitus[2]. In 1968 Bookchin and Gallop subsequently reported that the largest of these minor fractions, designated HbA_{1c}, had a hexose moiety linked to the N-terminus of the β-globin chain[3]. The functions of many proteins depend upon post translational modification, hemoglobin is such a protein[4]. Hemoglobin (Hb) is composed of four globin chains. Adult hemoglobin (HbA) is the most abundant form in most adults and consists of two α and two β chains. Fetal hemoglobin (HbF), which is predominantly present at birth, consists of two α and two γ chains. HbF is a minor form in normal adults. HbA_2 is minor Hb after birth and consists of two α and two δ chains. The most common Hb variants worldwide in descending order of prevalence are HbS, HbE, HbC and HbD. All of these hemoglobins have single amino acid substitutions in the β chain. Normal adult hemoglobin consists primarily of hemoglobins A (90-95%), A2 (2-3%), F (0.5%), A_{Ia} (1.6%), A_{Ib} (0.8%), and A_{1c} (3-6%). Glycosylated hemoglobins (GHb) are the minor hemoglobin molecules separable by chromatographic techniques into three major components: A_{Ia}, A_{Ib}, and A_{1c}. Hemoglobin A_I refers to a combination of these three components[5].

Important perspective studies on chronic complications of Diabetes mellitus allowed us to establish with absolute certainty the role of glycosylated hemoglobin (HbA_{1c}) as a marker of evaluation of long term glycemic control in diabetic patients and the strict relationship between the risk for chronic complications and HbA_{1c} levels. Diabetes Control and Complication Trial (DCCT), a great extent study, has demonstrated that the 10% stable reduction in HbA_{1c} determines a 35% risk reduction for retinopathy, a 25- 44% risk reduction for nephropathy and a 30% risk reduction for neuropathy[6].

Glycosylation process

Glycosylation is a non-enzymatic reaction between free aldehyde group of glucose and free amino groups of proteins. A labile aldiminic adduct (Schiff base) forms at first, then, through a molecular rearrangement, a stable ketoaminic product slowly accumulates.

In the hemoglobin, the preferential glycosylation site is the amino-terminal valine of the β chain of the globin (about 60% of glycosylated globin). Other sites are: lysin 66 and 17 of the β chain, valine 1 of the α chain. The term HbA_{1c} refers to the hemoglobin fraction of the glucose bound stably (ketoamine) to beta terminal valines.

Other proteins which undergo glycosylation

Albumin, α_2 macroglobulin, antithrombin III, fibrinogen, ferritin, HDL and LDL, transferrin; all of them are short half-life proteins. The glycosylation process of short half-

life proteins stops at the formation of the stable ketoamine adduct.

Advanced Glycosylation End products (AGE)

The long half life proteins such as actin, collagen, fibronectin, myelin, nucleoproteins, spectrin, tubulin can also be glycosylated. These long half-life proteins (myelin and collagen) undergo a complex and irreversible rearrangement process, with the formation of Advanced Glycosylation End products (AGE). AGE form a family with many compounds, only partially identified; they accumulate in the structural proteins modifying the function of them. They bind to specific macrophage receptors inducing a release of hydrolytic enzymes, cytokines and growth factors able to promote the synthesis of fundamental substance and, acting at intracellular level, to determine a damage of the nucleic acids[7,8].

The biosynthesis of glycosylated hemoglobins (HbA_{1a}, HbA_{1b}, and HbA_{1c}) occurs slowly, continuously and almost irreversibly throughout the four month life span of erythrocytes and the process is non-enzymatic, as demonstrated by human studies using Fe-bound transferrin and measurement of specific radioactivity of the major and minor hemoglobin components during the entire life span of erythrocytes. Sugar phosphates, such as glucose-6- phosphate (G-6-P) present in red cells can react with hemoglobin 20 times faster than glucose, in fact, with greater specificity than glucose. Fructose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate, ribulose 5-phosphate, and glucuronic acid but not glucose 1-phosphate or glucose 1,6-diphosphate react with hemoglobin, with the rapid formation of the adduct, thus requiring an aldelyde or ketone group separated from a negatively charged COO– or PO_4 – group. It is very unlikely that G-6- PO_4 hemoglobin is a precursor of HbA_{1c}.

Concentration of G-6- PO_4 in red cells is 1/200th that of glucose, but G-6- PO_4 reacts with hemoglobin ten times more rapidly than glucose. Structure–function relationship can be studied with considerable significance on both natural and synthetic derivatives, on account of the specificities in relation to sites of glycation. Studies on the role of potentially catalytic residues on the polypeptide (protein) which may be crucially involved in the Schiff base formation and Amadori rearrangement by bringing into spatial juxtaposition of carefully designed helical peptides, is a noteworthy step in the echanistic understanding of protein glycation, with particular reference to the catalysis of Amadori rearrangement involved in the process[9,10].

Methods of estimation of glycated hemoglobin

In the last 20 years improved techniques in laboratory and new electrophoretical, chromatographic and immunological methods available, gave us a greater reliability on our results. However the use of different methods, the lack of a common calibration concerning the same method and the variability of instrumentation do not make reproducible results yet in different laboratories. For this reason studies and procedures of standardization are going on[11]. Methods of GHb assays have primarily evolved around three basic methodologies:

(1) Based on difference in ionic charge.

(2) Based on structural characteristics.

(3) Based on chemical reactivity.

The main methods are,

- Cation exchange chromatography
- Affinity chromatography
- High performance liquid chromatography
- Isoelectric focusing
- Radioimmunoassay
- Spectrophotometric assay
- Electrophoresis/Electroendosmosis
- Electrospary mass spectrometry

DISCUSSION

Handling of specimens before the assay is important as short period of hyperglycaemia before blood is taken, leads to an acute increase in the formation of aldimine which may increase the concentration of glycosylated haemoglobin by 10-20%--for example, from 9% to 11% of total haemoglobin-thus reducing the reliability of the test as a measure of long term diabetic control. Blood samples should therefore be treated to remove the aldimine before assay[12]. In measurement of HbA_{1c} the prevalence of the most common haemoglobin variants (HbS, HbC, and HbD) depends on the genetic background of the population being analysed. There are many Hb variants that result in false low HbA_{1c} level in diabetes. More than 700 Hb variants are known and about half of these variants are clinically silent, their presence may falsely interfere with measurement of HbA_{1c} by HPLC. Hence the identification of Hb variants is important to avoid inaccurate HbA_{1c} results[13].

Recent reports have shown that the concentration of total glycosylated hemoglobin measured by commonly used methods may change significantly over a period of hours. This reflects the short term fluctuations in glucose concentration. It is now realized that these rapid changes depend on the synthesis or dissociation of the labile fraction of HbA_{1c}, which is not separable from the stable form of HbA_{1c}, by most routine methods. In most cases, the labile fraction constitutes approximately 10% of the total glycosylated hemoglobin. This may increase to 25% when plasma glucose concentrations are high, as in unstable diabetics.

These day to day variations in glycosylated hemoglobin concentration secondary to changes in serum glucose are negligible in stable diabetics, but are very wide in unstable diabetics and are almost entirely dependent on the prevailing plasma glucose concentration. Thus, in

unstable diabetics with large swings in plasma glucose, a single HbA_{1c} measurement may be misleading as an index of long term control. It would therefore make sense to measure the stable fraction of glycosylated hemoglobin. However, this is not routinely available because most laboratories measure total HbA_1 or HbA_{1c}, which includes both labile and stable components. Therefore, in unstable diabetics, HbA_l measurements should be interpreted in relation to the simultaneous glucose concentration. To minimize the contribution of the labile fraction, glycosylated hemoglobin should be measured when the plasma glucose concentration is within or near the normal range.

Physicians should be aware of the expected variation in HbA_{1c} in associated conditions such as[14],

- False increases in HbA_{1c} levels may occur in the presence of HbF (Ex: Hereditary persistance of fetal Hb) and other negatively charged hemoglobins
- HbA_{1c} levels may also be increased in patients with renal insufficiency, caused by hemoglobin carbamylation resulting from condensation of urea with the same site to which glucose attaches.
- Increased HbA_{1c} occurs with advanced malignancy and iron deficiency anemia.
- Increased levels can be seen in people with a longer red blood cell lifespan, such as with Vitamin B_{12} or folate deficiency.
- Splenectomy can result in elevated levels of glycosylated hemoglobin
- False decreases may result when HbS (Ex: Sickle cell disease) or other positively charged variants are present
- Hemolytic anemia and chronic blood loss result in decreased red cell life span and therefore lower glycosylated hemoglobin levels.

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