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Glycosylated Hemoglobin and Plasma Protein in Newborns of Normal and Diabetic Women

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Summary

The glycosylated hemoglobin (glyco Hb) and the glycoprotein of the plasma were determined using affinity chromatographic techniques in cord blood and maternal blood of normal (Group I), White's Class A diabetic (Group II), and insulin-dependent diabetic (Group III) mothers. The results revealed significant increase of glyco Hb ($P < 0.005$) and glycoprotein ($P < 0.005$) in the newborns of Group III mothers in comparison to those of Group I. The glyco Hb of maternal blood in this group also showed significant increase ($P < 0.005$) while the increase in Hb A₁ and plasma glycoprotein were nonsignificant. All values for cord blood and maternal blood of White's Class A diabetics revealed no significant change from the normal group. We conclude that the fetuses of insulin-dependent diabetic mothers are hyperglycemic *in utero*.

Abbreviations

glyco Hb, glycosylated hemoglobin
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IDDM, insulin-dependent diabetes mellitus

The heterogeneity of human Hb has been known for some time (7, 17). Several chromatographically minor Hbs can be separated from adult red cell lysates using cation exchange chromatographic techniques which were designated A_{1a1}, A_{1a2}, A_{1b}, and A_{1c} based on their order of elution with A_{1c} being the major subfraction (19, 23). Hb A_{1c} is formed by the reaction of the β chain amino terminus of Hb A₀ (major hemoglobin) with glucose whereas Hb A_{1a1} and Hb A_{1a2} are formed by reaction of fructose-1,6-diphosphate and glucose-6-phosphate, respectively (9). Hb A_{1b} is a glyco Hb whose identity is not known. However, other glycohemoglobins that are formed by glycosylation of the α chain amino terminus and ϵ -amino groups of certain lysyl residues on both chains are not normally separated by cation exchange chromatographic technique (25). Glycosylation of Hb occurs by a nonenzymatic reaction that is continuous throughout the lifespan of the red blood cell (8). It follows that glyco Hb levels will reflect the time-averaged blood glucose level over a period of 8–12 weeks (16, 20). This has led to increasing interest of the clinicians in the study of the glyco Hb levels in relation to diabetes mellitus (14, 15). Levels of Hb A₁ (A_{1a+b+c}) are increased in diabetes mellitus depending upon the degree of metabolic control

(3, 9). This elevation of Hb A₁ was also reported in pregnant women with diabetes mellitus whether diet controlled or insulin-dependent diabetics (11, 24). Several minor Hbs, namely F_{1a1}, F_{1a2}, F_{1b}, and F_{1c}, were separated from cord blood red cell lysates using similar cation exchange chromatographic techniques (1, 2). Hb F_{1c} contains γ chains mostly acetylated, but it contains minor glycosylated Hb as well (1, 2). In a previous study, it has been shown that the level of Hb F_{1c} is increased in newborns of diabetic mothers; however, it was not shown whether this increase was due to an increase in acetylated or glycosylated form of Hb F_{1c} (12).

The specific interaction of boronic acid ligands with 1,2-*cis*-diol compounds resulted in affinity chromatographic techniques for the separation of some glycoproteins (27). Affinity chromatographic methods utilizing *m*-aminophenylboronic acid immobilized on agarose have been recently introduced to quantitate total glyco Hb even in the presence of Hb F and various abnormal hemoglobins (4, 5). This technique is particularly suited for the quantitation of glyco Hb in cord blood. The purpose of this study was to use the new technique to find out if there are any significant differences in the levels of the glycosylated products (Hb and plasma proteins) between newborns of normal and diabetic mothers. This would give a more accurate indication of the time-averaged blood glucose levels *in utero* in these pregnancies.

MATERIALS AND METHODS

Subjects. Forty-eight pregnant women participated in this study. Informed consent was obtained from all patients. They were classified into three groups as follows: Group I, 20 normal women with a normal glucose screening test, *i.e.*, a blood glucose value <130 mg/dl 1 h after a 50-g glucose load (10); Group II, 20 women with an abnormal oral glucose tolerance test, *i.e.*, White's Class A diabetics who did not require insulin therapy. Group III, eight women with IDDM of whom seven were Class B, and one was Class C. The management of the diabetic patients was according to the routine protocol in our institution (10).

Blood samples. Samples of blood (5–7 ml) were collected from mothers at the time of their admission in labor and from the cord (3–4 ml) at delivery. Blood was collected into tubes containing EDTA and stored at 4°C. After 24 h, the red cells were separated from the plasma by centrifugation and washed three times with isotonic saline. Both the cells and the plasma were used immediately for glyco Hb and glycoprotein determinations.

Affinity chromatographic quantitation of glyco components. Microcolumns (Glyc-Affin system from Isolab, Inc., Akron, OH) prepacked with the affinity gel (*m*-aminophenylboronic acid immobilized on agarose), and supplied reagents of the same company, were used. The following procedures which were recommended by the manufacturer were followed precisely. For glyco Hb determination, a 50- μ l aliquot of the cells suspended in saline was lysed with 400 μ l of the sample preparation reagent and 100 μ l of the lysate were applied to the column and developed at 25°C. The nonglyco Hb fraction was eluted first with the first fraction elution agent (Solution II) or 0.05 M HEPES buffer, adjusted to pH 8.5 with HCl and containing 10 mM MgCl₂ and 0.02% sodium azide. The glyco Hb fraction (bound to the gel) was eluted with the second fraction elution buffer or 100 mM sorbitol. So every sample was fractionated into two fractions: glyco and nonglyco Hb. The absorbance of each fraction was read at 415 nm in a Beckman spectrophotometer. The percentage of glyco Hb was calculated from the readings. For glycoprotein determination, 500 μ l of plasma were mixed with 500 μ l of first fraction elution agent. A 400- μ l aliquot of the diluted sample was applied to the column. The glyco and nonglyco protein fraction was eluted in the same way as mentioned under glyco Hb. One hundred μ l of each fraction were mixed separately with 4 ml of diluted protein reagent (Isolab, Inc.). Colors developed were read at 595 nm. The percentage of glycoprotein was calculated from these readings.

Ion exchange chromatographic quantitation of Hb A₁ in maternal blood. Microcolumns (Fast Hemoglobin Test System from Isolab, Inc.) prepacked with Bio-Rex 70 (IRC-50) cation exchange resin and reagents of the same company were used. The procedure supplied by the company was followed precisely. Fifty μ l of blood were lysed with 200 μ l of the sample preparation reagent and 50 μ l of the lysate were applied to the column. Fast hemoglobin (Hb A₁) fraction was eluted first with Solution 1 while the other hemoglobin fraction was eluted with Solution 2. The absorbance of each fraction was read at 415 nm in a Beckman spectrophotometer. The percentage of Hb A₁ was calculated from these readings.

RESULTS

The results were analyzed by a standard computer program. The statistical tests performed are one-way analysis of variance (Bartlett's test), and paired analysis between the mothers and their newborns. Table 1 shows that the percentages of glyco Hb ($6.2 \pm 0.7\%$) of cord blood samples in eight newborns of IDDM mothers were significantly higher than in the normal group ($4.7 \pm 0.7\%$) ($P < 0.005$). The glycoprotein levels of the plasma also showed significant increase ($P < 0.005$). Cord blood samples from White's Class A diabetic women, however, showed no significant difference from the normal group. The percentage of glyco Hb of IDDM mothers (maternal blood) was significantly higher than in the normal group ($P < 0.005$), while the increases in the level of fast hemoglobin (Hb A₁) and glycoprotein were nonsignificant. The results of these parameters in the maternal blood samples of White's Class A diabetic women were not different from the normal group (Table 2). Paired analysis between the mothers and their own newborns revealed no correlation for glyco Hb and glycoprotein in the normal group ($\gamma = 0.08$), whereas marginal correlations were observed in IDDM and White's Class A diabetic groups ($\gamma = 0.37$ – 0.42).

DISCUSSION

This work is a logical extension of a previously reported study, where an elevated Hb F_{1c} (determined by ion exchange chromatography) was observed in the newborns of mothers with IDDM (12). At that time, it was not clear if this increase was due to an increase in the glycosylated or acetylated portion of Hb F_{1c}. In addition, the total glyco Hb including Hb glycosylated at sites other than the γ chain amino terminus could not be determined by the ion exchange chromatographic technique. Utilizing glycoaffinity technique, this work confirms that glyco Hb and glycoproteins of the plasma are significantly elevated in the newborns of insulin-dependent diabetic mothers. Since glycoprotein and glyco Hb levels reflect the time-averaged blood glucose concentration in the preceding 2 to 3 weeks or months, these results may indicate that the fetuses of IDDM mothers had mean blood sugar values higher than fetuses of normal mothers. On the basis of the glyco Hb (6.4%) and Hb A₁ (7.1%) levels, White's Class A diabetics must have been well controlled which explains

Table 1. Comparison of the percentages of glycohemoglobin and glycoprotein in cord blood of different groups of patients

Samples*	Red blood cell glycohemoglobin† (%)	Plasma glycoprotein† (%)
Normal group (20)	4.7 $\pm$ 0.7 [‡] (3.3–5.8)	8.8 $\pm$ 1.6 (6.4–12.8)
Newborns of White's Class A diabetic mothers (20)	4.4 $\pm$ 1.2 (3–7.6)	9.0 $\pm$ 1.9 (6.1–11.7)
Newborns of insulin-dependent diabetic mothers (8)	6.2 $\pm$ 0.7 [‡] (4.9–6.8)	13.4 $\pm$ 3.7 [‡] (10.0–19.1)

* Number of samples analyzed is in parentheses.

† Mean $\pm$ SD, with range in parentheses.

‡ Bartlett's test; $P < 0.005$.

Table 2. Percentages of glycohemoglobin, fast hemoglobin (Hb A_{1c}), and glycoprotein in maternal blood of different groups of patients studied

Samples*	Red blood cell glycohemoglobin† (%)	Hb A _{1c} † (%)	Plasma glycoprotein† (%)
Normal group (20)	7 ± 0.6 (5.9–8)	7.4 ± 0.9 (6.1–8.9)	12.8 ± 1.4 (10.0–15.4)
White's Class A diabetic mothers (20)	6.4 ± 1.2 (4.8–8.4)	7.10 ± 0.7 (5.9–8.3)	10.8 ± 2.5 (4.6–15.9)
Insulin-dependent diabetic mothers (8)	8.9 ± 0.9‡ (7.6–9.7)	8.1 ± 1.6 (5.5–9.6)	14.3 ± 3.9 (8.6–19.4)

* Number of samples analyzed is in parentheses.

† Mean ± SD, with range in parentheses.

‡ Bartlett's test; $p < 0.005$.

why the glyco Hb and glycoproteins of their newborns were not different from the newborns of normal mothers. In this report, we were able to compare glyco Hb values in the newborns with the values of their own mothers. They were lower in the newborns (4.7 ± 0.7 versus $7.0 \pm 0.6\%$) confirming previous findings (4). The decreased glyco Hb levels in the newborns may be due to a decrease for both red cells survival (21) and average blood sugar level in fetuses compared to adults. Another possibility is that Hb F is glycosylated less readily than Hb A. However, the results of previous *in vitro* glycosylation studies (6) and normal levels of glyco Hb reported earlier in a homozygote for hereditary persistence of fetal hemoglobin condition do not confirm this (4). It is also noteworthy that even the plasma glycoprotein levels were significantly lower in the normal newborns than in their mothers. However, such a difference was not seen in the newborns of mothers with IDDM.

Current techniques for measurement of the relative concentration of glyco Hb fall under various categories such as ion exchange chromatography, electrophoresis, isoelectric focusing, and immunological and chemical analyses. Considering first the last category, the fluorometric technique has been considered an efficient means of determining the true number of total glyco groups (13). Second, the thiobarbituric acid assay is believed to provide a reasonable estimation of the protein-bound ketoamine (26). However, these procedures are very time-consuming. Ion exchange chromatography is subjected to interferences by temperature variations and the presence of Hb F, abnormal Hbs, and the labile aldimine (Schiff base). However, affinity chromatography is not influenced by such interferences (4). Moreover, comparison of the glyco Hb results obtained by affinity chromatographic technique with those of chemical methods resulted in a good correlation (5). In addition, the inter- and intra-assay coefficients of variation for glyco Hb as determined in earlier studies (4, 5) and for glycoprotein determined during this study was very satisfactory (<5%).

Fetal macrosomia in diabetic pregnancies has been attributed to fetal hyperglycemia that is secondary to maternal hyperglycemia and consequent fetal hyperinsulinemia (Pederson theory). Direct evidence for this has been lacking since it is not possible to determine fetal blood glucose during pregnancy. Glyco Hb levels in cord blood may reflect the average levels of fetal blood glucose during the third trimester of pregnancy. Plasma glycoprotein level on the other hand may reflect the mean blood glucose level during the last few weeks of pregnancy. This is due to relatively high turnover of albumin which has a half-life in plasma of about 17 days (18). The results of this study indicate

the presence of higher than average fetal blood glucose levels in insulin-dependent diabetic pregnancies. This presents further support to the Pederson theory (22).

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