Glycated Hemoglobin in Fractionated Erythrocytes

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Erythrocytes were fractionated, by centrifugation, on the basis of cell density, which is proportional to cell age. Glycated hemoglobin (Hb A₁c) in the fractionated cells was measured and compared with the Hb A₁c in whole blood and with the fructosamine concentrations in plasma of normal and diabetic subjects. In normal subjects, Hb A₁c increased according to the age of the erythrocytes, but hyperbolically rather than linearly, indicating the slow conversion of Hb A to Hb A₁c with saturation. The greatest correlation of Hb A₁c with plasma fructosamine was in younger middle-aged cells. A subgroup of subjects with impaired glucose tolerance had high Hb A₁c in older cells. Hb A₁c of the young cells reflects recent glycemic status, for monitoring the initial stage of the treatment of diabetics, whereas Hb A₁c of the older middle-aged cells more specifically reflects the longer term glycemic status, for screening and for the long-term monitoring index for treatment of diabetes.

Additional Keyphrases: diabetes mellitus · screening · monitoring therapy · cell age and glycation · fructosamine

Determination of glycated hemoglobin (Hb A₁c) in whole blood is well established and widely used for monitoring long-term (about two months) control of the concentration of blood glucose in known diabetic patients (1–8), and also as a tool for screening for diabetes mellitus (9–11).

Johnson et al. (12) developed a colorimetric assay for serum fructosamine, which originates mainly from the non-enzymatic glycation of serum protein. This assay is becoming widely used as an inexpensive, simple, short-term (two weeks) index to the concentration of glucose in blood (13). However, the assay is based on measuring the reducing activity of the glycated protein in an alkaline pH, so other reducing agents, which vary from serum to serum (14), interfere. In diabetic patients, various glycated proteins differ in their biological half-lives and reactivities to nitro blue tetrazolium (15), limiting the specificity of the fructosamine assay in this group.

Measurement of glycated albumin reflects blood glucose of even shorter term than fructosamine, because for the concentration of glycated albumin to enter the normal reference interval, whether as a result of strict diet or intensive therapy with insulin, takes four weeks (16). An index reflecting the concentration of blood glucose in the preceding week would be useful in the initial stage of treatment of type II diabetes as well as in evaluations of the unstable phase in patients with severe diabetes.

Young erythrocytes have a lower Hb A₁c content than do the older cells (17, 18). New erythrocytes, released into the bloodstream with little Hb A₁c, undergo glycation of hemoglobin throughout their life span as they are exposed to glucose in the plasma. The older the cell and the higher the concentration of plasma glucose, the greater the opportunity for this glycation to occur. We suggest that estimating Hb A₁c in erythrocytes of a specified age gives more-useful specific retrospective information about the glycemic status of a patient over a preceding time interval. How long a time interval is involved depends on the age of the cells in which Hb A₁c is measured.

In this study, we examined the relationship of cell aging to glycation, using an innovative method for erythrocyte fractionation before Hb A₁c assay. We examined the Hb A₁c concentration in fractionated erythrocytes from normal subjects and diabetic patients, and we compared these results with those for plasma fructosamine and whole-blood Hb A₁c in the same specimens.

Materials and Methods

Blood was sampled from two groups of patients: known diabetic patients being monitored at our outpatient clinic and admitted to our hospital after a 16-h fast, and “normal” fasting subjects who were being admitted to the hospital for health screening. This second group began a 75-g oral glucose-tolerance test at the time of preparandial blood collection. Blood for Hb A₁c and plasma fructosamine measurements was collected from the antecubital vein into a heparin-containing Vacutainer Tube (Becton Dickinson Co., Rutherford, NJ 07070; cat. no. 4903). Within 4 h, the blood specimens were manipulated for erythrocyte fractionation and separation from plasma (see below).

The plasma was stored at −80 °C until fructosamine was measured. Plasma glucose was measured in an automated glucose analyzer (Auto & Stat GA-1120; Kyoto Daichi Kagaku Co., Kyoto 601, Japan) with use of glucose oxidase (EC 1.1.3.4). All subjects were then classified into three groups according to the World Health Organization’s criteria (19): those with normal glucose tolerance, those with impaired glucose tolerance, and those with diabetes mellitus.

Reticulocytes were counted in thin smears after staining with new methylene blue solution according to the usual laboratory method (20). We measured pyruvate kinase (PK; EC 2.7.1.40) activity in the fractionated erythrocytes by Beutler’s method (21).

We measured Hb A₁c with an automated HPLC analyzer (Hi-Auto A₁c type 8121; Kyoto Daichi Kagaku Co., Kyoto 601, Japan) equipped with Auto-Sampler (type 9411) for loading 100 samples at a time. An improved model from one described earlier (22), this measurement in this analyzer takes only 4 min per sample.

Measurement of plasma fructosamine was automated by using a Cobas Mira centrifugal analyzer and a commercial kit from Nippon-Roche (Chiyoda-ku, Tokyo 100, Japan). Analyzer settings were as recommended by the kit manufacturers.

Erythrocyte Fractionation

By heating, we sealed one end of a polypropylene tube (1.75 mm inner diameter, 2.95 mm outer diameter, 7.0 cm, identical to the tube used to hold ink for a ballpoint pen)
and fitted a small funnel-like adaptor, also polypropylene, over the other end of the tube (Figure 1). After delivering 0.5 mL of heparinized blood into the adaptor, we centrifuged the tube, with the adaptor, at 836 × g for 5 min at 25 °C. The adaptor—now containing plasma, buffy coat, and an excess of erythrocytes—was connected to an aspirator and simultaneously removed from the tube. We then further centrifuged the tube at 14 170 × g for 15 min at 25 °C with a hematocrit centrifuge (Hematocrit KH-1200S; Kubota, Bunkyou-ku, Tokyo 113, Japan), which had been adapted to hold the polypropylene tube. After centrifugation, we cut through the tube at the border between plasma and the packed erythrocytes, leaving a 5- to 6-cm-long polypropylene tube of densely packed erythrocytes. The heavier, older cells were at the bottom; the lighter, newer cells were at the top. We aspirated a 1.5-μL sample of erythrocytes from the top of the packed erythrocytes through a 23-gauge venipuncture needle connected to an Autodiluter (Type 9211, Kyoto Daiiichi Kagaku). We bent the end of the needle, to limit its depth of insertion into the tube (Figure 1). We then cut a small segment with a length ¼ that of the packed-cell tube with a small guillotine by fitting the cut end of the tube onto the stopper. The procedure of aspirating the sample and cutting to discard the resulting small segments was repeated. The samples from the 16 segments so obtained were designated F1 (the lightest, youngest cells) to F16 (the heaviest, oldest cells). The packed cells are so viscous and the inner diameter of the tube is so small that the centrifuged packed cells are not intermixed during the sampling from the tube.

We then transferred each sample to a sample cup, where it was diluted with 0.45 mL of hemolyzing solution (8 mmol/L phosphate buffer, pH 7.4, containing 1 g of Triton X-100 per liter) by the autodiluter. We then placed the sample cups into the Auto-Sampler of the Hb A₁ analyzer.

By radiolabeling erythrocytes, Danon and Marikovsky (23) demonstrated that a mixture of phthalate esters facilitates the separation of erythrocytes by age. Therefore, to determine whether phthalate esters must be added for reliable separation of the erythrocytes by age, we repeated the whole fraction procedure, adding phthalate ester of known density (a mixture of dimethyl phthalate (relative density 1.189) and dibutyl phthalate (relative density 1.042)) to the tube before and after the second centrifugation, according to the method described previously (24).

**Results and Discussion**

Figure 3 shows reticulocyte counts and PK activities. Reticulocytes were most markedly numerous in F1, indi-
cating that the youngest cells were contained in F1. PK activity was greatest in F1 and declined linearly from F2 to F16. Microscopically, leukocytes were visible only in F1 but not in F2 to F16, most having been removed with buffy coat in the adaptor. Because PK activity is greater in leukocytes than in erythrocytes, the markedly increased PK in F1 may have been caused by contaminating leukocytes. Because the activity of PK in erythrocytes depends on their ages (25), its linear decrease of activity from F2 to F16 verifies that the cells in each fraction were in fact separated according to their age.

To determine Hb A1c in the erythrocytes from a normal subject and a diabetic patient, we fractionated each sample by the present method and by the method involving phthalate ester as separators (24). Values for Hb A1c in each fraction of the sample fractionated without separators were essentially identical to those of the sample fractionated with separators (Figure 4). However, in the latter samples, some fractions were unobtainable because of the presence of the separators: fractions 10 and 14 in the diabetic patient and fractions 6, 10, and 11 in the normal subject (Figure 4).

Intra-assay coefficients of variation (CVs) for Hb A1c were 0.88% for whole unfractonated erythrocytes (mean ± SD Hb A1c 5.67% ± 0.05% of total Hb), 2.43% in F1 (4.11% ± 0.10%), 0.88% in F6 (5.68% ± 0.05%), 1.22 in F11 (5.75% ± 0.07%), and 0.73% in F16 (5.45% ± 0.04%) (n = 20 each). This indicates that our technique fractionates erythrocytes reproducibly according to their age, without need for any separator or density liquid. This is possible because living erythrocytes are very deformable. Deformable erythrocytes suspended in density liquid act as separable cells, but by themselves they are able to act as a density liquid. They are therefore able to arrange themselves into a population having the same density during high-speed centrifugation. The method of Fitzgibbons et al. (17) requires 3 mL of blood, the addition of density liquid (dextran solution), a large centrifuge, 60 min of centrifugation, and wash-steps to remove density liquid from the erythrocytes for two erythrocyte fractionations in each sample. Obviously, erythrocyte fractionation was greatly simplified in the present study.

Figure 5 shows mean values for Hb A1c fractionated erythrocytes and whole blood, and the mean concentration of fructosamine in plasma of normal subjects and uncontrolled diabetic patients. Hb A1c values for fractionated erythrocytes and whole blood, and plasma fructosamine in newly diagnosed diabetes mellitus without treatment, are also shown in Figure 5. In normal subjects, Hb A1c increased steadily with cell aging, indicating the slow conversion of Hb A to Hb A1c, with saturaivity (26, 27), and a probable slight reversibility (28). In the uncontrolled diabetic patients (whom we defined as having Hb A1c >6.5% or fructosamine >3.0 mmol/L), the mean Hb A1c of fractionated erythrocytes was higher, but showed a similar pattern. Newly diagnosed patients who had not started treatment showed relatively higher Hb A1c in F1–F6, reflecting the recent high blood glucose concentration. On treatment, their values rapidly became normal.

Correlations between the concentration of Hb A1c of each fraction and plasma fructosamine, and Hb A1c of whole blood, are shown in Figure 6. The best correlation is seen for the younger middle-aged cells (F4–8), the poorest for the youngest and the oldest cells. This indicates that plasma fructosamine best reflects the retrospective pattern of plasma glucose concentration for about the preceding 20 days rather than more recently (the past seven days) or longer (100 days). The fact that there is about an r = 0.8 correlation of plasma fructosamine with Hb A1c in F9–F12 is to be expected, because fructosamine also reflects the glycated protein of longer life span such as glycated globulins.
ulin as well as the shorter-lived glycated albumin (16). Hb $A_{1c}$ of fractionated erythrocytes correlated better with that in whole blood in the older cells than it did with fructosamine; this correlation was poorest in the youngest cells. Our results suggest that Hb $A_{1c}$ of young erythrocytes is a much more specific and clinically useful index of recently changed blood glucose concentration than is fructosamine.

The Hb $A_{1c}$ content of young cells should first be restored to normal if one is to obtain a "normal" curve across cell fractions of the erythrocyte population, i.e., to obtain a "normal" value for an Hb $A_{1c}$ in whole blood, which is the ultimate goal of diabetic control. Such a patient with normal Hb $A_{1c}$ in whole blood would indicate that the subject's hemoglobin had not been exposed to highly increased concentrations of blood glucose. This goal should be attainable in patients with non-insulin-dependent diabetes mellitus, given proper treatment. In most patients with insulin-dependent diabetes mellitus, Hb $A_{1c}$ in whole blood is considered a reliable index of glycemic control status (6). However, in the severe diabetics treated with insulin, we found that Hb $A_{1c}$ of F1–2 was nearly normal, whereas that of F10–13 was markedly higher (see, e.g., the case shown in Figure 4). In severe cases of insulin-dependent diabetes it is impossible to avoid episodes of high plasma glucose, so erythrocytes inevitably are exposed occasionally to excess glucose, even when "diabetic control" is good (as assessed by monitoring the daily profile of blood glucose). As Figure 4 clearly shows, measurement of Hb $A_{1c}$ in middle-aged or older cells (and therefore of whole-blood Hb $A_{1c}$), to determine insulin dose, would result in dangerous hypoglycemia, as reported previously (4, 8). In such cases, measuring the Hb $A_{1c}$ in young erythrocytes may be more useful for rapid monitoring at the first step of insulin therapy. Goals for treatment in these cases must be developed for each individual and could be re-assessed frequently by separately measuring Hb $A_{1c}$ in the young and old cells.

In our subjects with impaired glucose tolerance (i.e., their plasma glucose was 1.4–2.0 g/L 120 min after ingesting 75 g of glucose), a subgroup had nearly normal values for Hb $A_{1c}$ in their whole blood and normal fructosamine concentrations in plasma, but higher proportions of Hb $A_{1c}$ in F10–16 (Figure 7). By avoiding the use of younger cells, Hb $A_{1c}$ of F10–16 might become more specific for glycemic status in retrospective evaluation. Although these subjects are not diagnosed as having diabetes mellitus, their cases should be carefully followed as they are treated by diet adjustment.

Fructosamine concentration depends on too many variables (the method of estimation measures nonspecific reducing substances as well as glycated albumin and globulin) to be of specific value in monitoring glycemic control (14, 15). Hb $A_{1c}$ of whole blood reflects only a mean value for glycated hemoglobin across the whole erythrocyte population, whereas the Hb $A_{1c}$ of new erythrocytes reflects glucose concentrations during the preceding week, and Hb $A_{1c}$ of the older middle-aged erythrocyte population correlates well with the longer-term blood glucose concentration. In our experience, fractionation of erythrocytes by age can be done reliably with small samples of whole blood when the described adaptation of currently available apparatus is used. We anticipate further simplification of this method by modifying the Auto-Sampler to take F1 and F10 directly from the centrifuge tube after one centrifugation.

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References