Glycation Index of Hair for Non-invasive Estimation of Diabetic Control

Kunio Kobayashi* and Hirotune Igimi

Diagnostic Science Department, Shionogi & Co., Ltd., 2-5-1, Mishima, Settsu 566, Japan.
Received November 20, 1995; accepted January 5, 1996

We propose a new indicator for diabetic control that shows the extent of glycation of hair protein (keratin), the glycation index \( A_{390} / A_{412} \) which is based on the ratio of glycated protein- to cystine-induced coloration, where \( A_{390} \) and \( A_{412} \) represent each absorbance in the color reactions of glycated protein and cystine in the hair protein. Samples can be quickly and non-invasively collected and easily stored. This index for the back and scalp hairs from hypercholesterolemic mice with hyperglycemia, diabetic rats and diabetic patients gave significantly higher values (2.0—6.0-fold) than those of normal subjects \( (p < 0.01) \). The glycation indices \( (\text{mean} \pm S.D.) \) of hairs from diabetic and non-diabetic subjects were \( 3.00 \pm 0.96 \) \((n = 21)\) and \( 1.51 \pm 0.45 \) \((n = 30)\), respectively. These indices \( (\eta) \) correlated well with the levels of glycoxidoglobin (HbA\(_{1c}\), \( x \)) in diabetic and non-diabetic subjects: \( y = 0.69x - 2.03 \) \((r = 0.82, n = 31, p < 0.01) \). Within-run precision (reproducibility, CV) for the assay of the glycation indices of hairs from the three groups was 6.7—9.4% \((n = 10 \text{ each})\).

The proposed glycation index of hair gave reasonable results for animals and humans with normo- and hyperglycemia, suggesting that it is reliable and can be diagnostically useful.

Key words glycation index; scalp hair; non-invasive clinical examination; diabetic control; diabetes mellitus; hyperglycemia

Glycated proteins (glycoxidoglobin, fructosamine and glycated albumin) and glucose in blood have been widely accepted as reliable indicators of metabolic control in diabetes mellitus, and their assay methods have been established. However, if a non-invasive method with facile sample collection was available, it would greatly facilitate clinical diabetic control. The presence of free amino groups in hair keratin which may be glycated\(^1\) offers the possibility of such a method. If the glycated hair protein content can be correlated with the level of glycated protein in blood,\(^2\) this would offer a stable measure of long-term diabetic control.\(^1\) Hair samples can be quickly and non-invasively obtained and easily stored. Conventionally, the extent of glycation of hair proteins has been evaluated by the measure of furosine produced from the glycated proteins.\(^3\) However, the level of furosine did not correlate strongly with the level of glycoxidoglobin (HbA\(_1c\) or HbA\(_{1c}\)) in blood \((r = 0.43—0.59, n = 5)\).\(^3\)

The ratio of cystine-induced coloration to the weight of hair is variable because the content of cystine in the hair protein (keratin) is fixed in each animal species.\(^5\) Therefore, the extent of glycation of hair protein may be expressed by a glycation index \( A_{390} / A_{412} \), where \( A_{390} \) and \( A_{412} \) represent each absorbance in the color reactions of glycated proteins and cystine in the hair protein, respectively, without precise weighing of the hair sample.

In the present study, we evaluated the proposed glycation index of hair and found that it may be useful for following the extent of long-term average glycemia in humans and animals with normo- and hyperglycemia.

MATERIALS AND METHODS

Subjects Scalp hairs and/or blood were sampled from normal volunteers (ages 25—53 years, \( n = 30 \)) and diabetic patients (ages 8—83 years, \( n = 21 \)). The blood samples were subjected to the measurement of glycoxidoglobin (HbA\(_{1c}\)). The spontaneously diabetic rats used in this study (male, BB-DP line) had shown symptoms of diabetes mellitus for 2 and 4 months and had been kept under insulin treatment (2—5 units/d). Non-diabetic rats (male, BB-DR line) were aged 3—5 months. Hypercholesterolemic (male, FLS-ob/ob line) and normal (male, DS and C\(_5\)LS lines) mice were also used for this study. The back hairs and blood were sampled to measure glycated protein and blood glucose, respectively.

Chemicals Dithiothreitol (DTT), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), ethyl alcohol (ethanol), hydrazine monohydrate, phenylhydrazine hydrochloride and other reagents of analytical reagent grade were from Wako Pure Chemical Industries, Ltd., Osaka, Japan. N-p-Tolyl-o-isogulosamine was prepared by the method of Inoue et al.\(^8\).

Apparatus Absorbance was measured using a spectrophotometer (Hitachi Spectrophotometer, Model U-3210, Hitachi, Ltd., Tokyo, Japan).

Preparation of Reagents 50% ethanol: 50% aqueous solution of ethanol. 50 mM DTT solution: DTT (771 mg) was dissolved in 100 mL of distilled water. 10 mM DTNB solution: DTNB (396 mg) was dissolved in 100 mL of 67 mM phosphate buffer (pH 8.5) containing 10% ethanol. Hydrazine reagent: aqueous solution of hydrazine monohydrate (4 M) adjusted pH 9.4 with acetic acid. Phenylhydrazine solution: phenylhydrazine hydrochloride (150 mg) was dissolved in 100 mL of 40% aqueous solution of acetic acid. Aqueous solution of N-p-tolyl-o-isogulosamine (250 \( \mu \)M) was used as a standard solution for the assay of glycated protein.

Coloration of Cystine in Hair Protein\(^9\) A hair sample (5—10 mg) less than 1 mm in length was rinsed with 1 mL of ethanol at 37°C for 0.5 h. After removing ethanol using a capillary pipet, the sample was incubated in a mixture of 0.5 mL ethanol and 50 mM DTT at 37°C for 15 min. After removing the solvent, the sample was washed 3 times with 1 mL of 50% ethanol, then incubated in a mixture of 0.5 mL of 50% ethanol and 1 mL of 10 mM DTNB solution at room temperature (25—27°C) for 5 min. The absorbance \( A_{412} \) of the supernatant of the
reaction mixture was measured at 412 nm.

**Coloration of Glycated Hair Protein**

The same hair sample used for the coloration of cystine was washed 2 times with 1 ml of 50% ethanol. It was heated in a mixture of 0.1 ml each of distilled water and hydrazine reagent at 100 °C for 30 min, then 0.6 ml of phenylhydrazine solution was added to the reaction mixture, followed by incubation at 60 °C for 1 h. The absorbance (A_{390}) of the supernatant obtained after centrifuging (1400 × g, 10 min) the incubation mixture was measured at 390 nm.

**Glycation Index and Level of Glycated Protein in Hair**

Absorbance of the reagent blank was the same as that of the sample blank in both the colorations of cystine and glycated protein in the hair. The glycation index (A_{390}/A_{412}) was calculated using both net absorbances (A_{390}, A_{412}) corrected by each reagent blank. The level of glycated protein (nmol/mg hair) was obtained by reference to 250 μM aqueous solution of N-p-tolyl-d-isoglucomamine (standard solution).

**Measurements of Blood Glucose and Glycohemoglobin**

The levels of blood glucose and glycohemoglobin (HbA_1c) were measured by enzymatic method (an automated clinical analyzer, H-7150, Hitachi, Ltd.) and by HPLC method (Glycohemoglobin Analyzer, HLC-723 GHbIII, Tosoh, Ltd., Tokyo, Japan), respectively.

**Statistical Analysis**

Statistical significance was assessed by Student’s “t” test for unpaired data.

**RESULTS AND DISCUSSION**

As shown in Fig. 1, the cystine-induced coloration (A_{412}) of hair correlated well with the weight of the sample with coefficients of correlation (r) of 0.98, 0.79 and 0.90, respectively, in humans, rats and mice (non-diabetic and diabetic subjects), indicating that the extent of hair glycation can be represented by a cystine equivalent without precise weighing of the hair sample. The slopes for the regression lines of hairs from mice and rats were higher (9.7- and 4.7-fold) than that from humans, suggesting different contents of cystine in hairs from these groups. The glycation index (A_{390}/A_{412}) of scalp hair from humans and back hair from experimental animals (rats and mice)
correlated well with the level of glycated protein in those hairs. These results show that the glycation index of hair reflects the level of glycated protein in hair (Fig. 2).

The mean glycation index (1.50) of hair from diabetic rats was higher (6.0-fold) than that (0.25) of non-diabetic rats ($p<0.01$) (Fig. 3A). This result was similar to the relation (3.1-fold) in the level of glycated protein in hair. Hypercholesterolemic mice with hyperglycemia also showed a higher glycation index of hair (0.45, 2.3-fold) than that (0.20) of normal mice, with the results showing good parallels with those of rats (Fig. 3B). As shown in Fig. 3C, the mean glycation index (3.00) of hairs from diabetic patients was significantly higher than that (1.51) in the non-diabetic population ($p<0.01$). The glycation index of hairs in hyperglycemic or diabetic subjects showed larger standard deviation than that in normal or non-diabetic subjects. This result was estimated to be due to various levels of glycemic control in diabetic subjects, in particular, 7 diabetic patients whose levels had been well controlled by drug treatment or diet, had the same low glycation index as that of the non-diabetic population. Thus, the glycation index may be a useful indicator of therapeutic effect in diabetes mellitus.

The glycation index ($y$) of hair correlated well with the level of glycated protein (HbA$_1c$, $x$) in human: $y = 0.69x - 2.03$ ($r = 0.82, n = 31, p < 0.01$) (Fig. 4). This result shows that the index reliably reflects the level of glycated proteins in the blood and may be available to help with diabetic control. The blood glucose level did not show a high correlation with the glycation index ($r = 0.79$ and 0.73, rats and mice, respectively), probably due to the time difference between the life-span of glycated protein in hair and the glucose metabolism. Fructosamine and glycated hemoglobin could not be measured because the amounts of blood samples from animals were too small.

When 10—30 mm, 5—10 mm and less than 1 mm of hair was used for the assay, no statistical difference was found among the glycation index values ($p > 0.50$), indicating that hair of less than 30 mm can be used for the assay. Within-run reproducibility (coefficient of variation, C.V.) for the assay of glycation indices of hair from the three groups was 6.7—9.4% ($n = 10$ each) (Table 1). This level of within-run precision is allowable for routine clinical examinations.

The scalp hairs which had been treated with an oxidizing and a reducing agent for a permanent wave, or hair bleach for decoloration gave large false-negative results, while those stained with a hair dye gave large false-positive results. These false-negative or positive results were believed to be caused by the increase of cystine-induced coloration ($A_{412}$) or of glycated protein-induced coloration ($A_{390}$), respectively. Hair-dressing materials such as hair cream, hair oil or hair conditioner which adhere to the hair surface, did not interfere with the assay, because hairs were washed with 50% ethanol during the assay.

The level of glycated protein in hair has been suggested to provide a stable measure of long-term diabetic control and an estimation of the time of onset of diabetes mellitus. As hair grows at a rate of about 10 mm per month.

![Fig. 4. Correlation between Glycation Index and Glycohemoglobin (HbA$_1c$) in Humans](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glycation index ($A_{390}/A_{412}$, mean ± S.D.)</th>
<th>C.V. (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.8 ± 0.17</td>
<td>9.4</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>1.6 ± 0.16</td>
<td>9.4</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>19.4 ± 1.31</td>
<td>6.7</td>
<td>10</td>
</tr>
</tbody>
</table>

Sample 1, scalp hair from a healthy volunteer; sample 2, scalp hair from a well-controlled diabetic patient; sample 3, hair sample (glycated protein, 16 mmol/mg hair) which had been glycated with 0.1 M glucose at 37°C for 24 h in vitro.
in humans, the glycation index of hair in the portion 20 mm from the scalp may reflect the average diabetes control for the previous 2 months with as much precision as the glycohemoglobin (HbA1c) level, which has heretofore been widely used as a reliable indicator. Conventionally, the glycated protein in scalp hair from humans has been examined by acid hydrolysis, followed by measurement of the fructose produced from the glycated protein (fructose-lysine). However, this method requires use of a strong acid (6 N HCl), long-time reaction (30 h) at a high temperature (95 °C), and complicated procedures such as analysis by HPLC.3–5

Our method for assay of the glycation index of hair reveals the extent of glycation of hair proteins with only a small amount (less than 10 mg) of hair sample and without the need for precise weighing. Our results suggest that the glycation index proposed here can be clinically helpful in diagnosing the pathological status of hyperglycemia in diabetic subjects.

REFERENCES