Evaluation of Autofluorescent Property of Hemoglobin–Advanced Glycation End Product as a Long-Term Glycemic Index of Diabetes

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Current methods for measuring long-term glycemia in patients with diabetes are HbA_{1c} and advanced glycation end products (AGEs), which are estimated by phenyl boronate affinity chromatography and competitive enzyme-linked immunosorbent assay, respectively. In this study, we hypothesize that the intrinsic fluorescence property of hemoglobin-AGE (Hb-AGE) may be a simple, accurate, and therefore better index for long-term glycemic status due to its highly specific nature and longer half-life. To establish this contention, in vitro and in vivo experiments were carried out. The former was performed by incubating commercially available hemoglobin with 5 and 20 mmol/l glucose and the latter through experimentally induced (streptozotocin) diabetes in an animal model (male Wistar rats) to identify the new fluorophore formed due to the nonenzymatic glycosylation of hemoglobin. An adduct exhibiting fluorescence at 308/345 nm of excitation/emission wavelengths has been identified and its time-dependent formation established. Under in vitro conditions, the first appearance of the new fluorophore was noticed only after a period of 2 months, whereas under in vivo conditions, it increased significantly after 2 months of hyperglycemia. Consistent with the observations, studies on patients with type 2 diabetes demonstrated an elevated level of this new fluorescent adduct in patients with persisting high levels of plasma glucose for >2 months. Based on the results obtained, Hb-AGE appears to be an efficient fluorescence-based biosensing molecule for the long-term monitoring of glycemic control in diabetes. *Diabetes* 52:1041–1046, 2003

Conventional diagnosis of diabetes and determination of immediate glycemic status in such patients is based on fasting plasma glucose levels or oral glucose tolerance test (1). Glycation of total serum proteins provides another indicator of the glycemic status over the previous 7–14 days and is currently assessed by fructosamine assay. Although the method is cheap and rapid, there are variations in the levels of serum fructosamine when serum protein concentrations are altered. HbA_{1c} reflects glycemic status over the previous 4–6 weeks and is currently commonly used as a clinical and research tool. Levels of HbA_{1c} show a fair correlation with levels of plasma glucose (2–4). Methods used for the isolation of glycohemoglobin can be subdivided into four groups: chemical, electrophoretic, chromatographic, and enzyme-immunoassay (4–7).

Wolffenbuttel et al. (8) have suggested that long-term modification of hemoglobin by advanced glycosylation end products (Hb-AGEs) would be a better index for long-term glycemia in patients having diabetes. The observation that AGEs are formed on hemoglobin suggests that HbA_{1c} is a precursor for Hb-AGE, which is stable to dialysis, acid precipitation, and proteolysis. A good correlation between HbA_{1c} and Hb-AGE in hyperglycemic patients, especially those with poor glycemic control, was also reported (9).

The only available method for quantification of AGEs is an immunochemical assay (10). In this test, high titer-specific antiserum was prepared for glycated ribonuclease that recognized the cross-reactive epitopes formed during the reaction of different sugars with diverse carrier proteins. Another study (11) reported the presence of a common structure in AGE preparations, regardless of whether AGE products are generated from proteins, amino acids, or monoaminocarboxylic acids. Mitsuhashi et al. (12) have shown that the immunological recognition of AGEs by competitive enzyme-linked immunosorbent assay (ELISA) is both an AGE-carrier protein and anti-AGE antibody dependent. They further suggested that in vitro AGE-modified proteins might not be appropriate standards for AGEs that occur naturally in vivo.

As of now, the competitive solid-phase ELISA is used for AGE determination. However, neither AGE antibodies nor AGE carrier proteins have been standardized, which entails considerable methodological problems. Some of the literature results are based on the use of polyclonal and others on monoclonal AGE antibodies. The lack of availability of stable certified glycohemoglobin standards hamper standardization of the various methods discussed. Additionally, all of the existing methods are time consuming, requiring between an hour to several hours to obtain results. In view of the limitations in the AGE assay, the authors explored the intrinsic fluorescence characteristics of Hb-AGE as a technique for its qualitative and quantitative identification. Increase in brown pigment formation

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AGE, advanced glycation end product; ELISA, enzyme-linked immunosorbent assay; Hb-AGE, hemoglobin-AGE.

and fluorescence was observed with the advanced glycation of long-lived tissue proteins such as lens crystallin, collagen, and myelin (13,14). Collagen AGE-specific fluorescence was characterized by measuring emission at 440 nm upon excitation at 370 nm and the intensity increased with an increase in glycemia (13). Pentosidine is another intermediate adduct that accumulates in the tissue proteins with aging and diabetes and displays emission appearing at 385 nm when excited at 335 nm (15). Advanced products of the Maillard reaction, including compounds as carboxymethyllysine and pyrraline (16), which are dominant compounds formed as a result of glycoxidation of proteins, are not fluorescent. To the best of our knowledge, the intrinsic molecular fluorescence of Hb-AGEs has not yet been well established. This study, therefore, was carried out to standardize the excitation and emission wavelengths of Hb-AGE fluorescence. Time-dependent formation of Hb-AGE was also analyzed under an in vivo condition using the animal model. Further, formation of Hb-AGE was measured in blood samples obtained from normoglycemic and hyperglycemic patients.

RESEARCH DESIGN AND METHODS

Glycohemoglobin was obtained from three different sources.

In vitro study. Bovine hemoglobin (Sigma, St. Louis, MO), D (+) glucose, and 0.4 mol/l sodium phosphate buffer (pH 7.4) were used for the in vitro experiment. All solutions were made in deionized water. Two different concentrations of glucose (5 and 20 mmol/l) were used for incubation of hemoglobin to mimic the normoglycemic and hyperglycemic conditions, respectively, while the control solution lacked glucose. Hemoglobin at a concentration of 50 mg/ml (10,12) was dissolved in 0.4 mol/l sodium phosphate buffer (pH 7.4) and filter-sterilized by using a 0.22-μm Millipore filter. The samples were incubated in a rotary-shaking water bath at 37°C for 120 days under sterile and dark conditions. Intermittent sampling was done to check any microbial contamination. The samples were withdrawn at weekly intervals and diluted to obtain the final concentration of 1.0 mg/ml with 0.4 mol/l sodium phosphate buffer (pH 7.4). Fluorescence measurements were carried out in a spectrofluorimeter.

In vivo study. Approval was obtained from the Ethics Committee and Expert Council of the All India Institute of Medical Sciences, New Delhi, for the animal experiments. Six-month-old healthy male Wistar rats weighing 200–250 g were fed ad libitum with pelletite (Hindustan Lever, Bangalore, India) and water. All approved animal care protocols were followed.

Diabetes was induced in 14 rats with a single intraperitoneal injection of streptozotocin (60 mg/kg body wt) dissolved in 1.0 mol/l citrate buffer (pH 4.5). Six control rats received an equivalent volume of citrate buffer in the same manner. The blood glucose concentration of control rats remained <150 mg/dl throughout the experimental time (13 weeks), whereas the glucose level of diabetes-induced rats increased to 350–550 mg/dl. The parameters monitored were plasma glucose level, HbA_{1c} (Sigma kit), total hemoglobin (Cyanmethamoglobin method), and fluorescence of Hb-AGE. The blood samples (0.5 ml) were collected from orbital veins of diabetic/normal rats at an interval of 7 days, centrifuged at 3,000 rpm to remove the plasma and buffy coat, and hemolysed and diluted to get the optimum protein concentration of 1.0 mg/ml in 0.4 mol/l sodium phosphate buffer (pH 7.4). Fluorescence measurements were carried out as mentioned in the previous section.

Clinical study. A total of 23 patients >30 years of age with type 2 diabetes were recruited from the Internal Medicine ambulatory care department and diabetes research clinic. The patients were on various combinations of antihyperglycemic treatment and free from severe end-organ damage and acute systemic illnesses. Seven subjects were recruited as normal control subjects after excluding diabetes during an oral glucose tolerance test. Informed consent was obtained from all of the subjects.

One milliliter of venous blood was obtained from human subjects after an overnight fast, and the sample was processed in a similar manner as in the rats. The hemolysate was diluted to the final protein concentration of 1 mg/ml for the fluorescence measurement. The parameters analyzed include plasma glucose, total hemoglobin concentration, HbA_{1c}, and Hb-AGE fluorescence using previously described procedures.

Fluorescence measurement. The LS 50B Luminescence spectrometer (Perkin-Elmer, Cambridge, U.K.) was used for measuring the fluorescent signal. Fluorescence intensity standards (Perkin-Elmer) were used to calibrate and monitor the performance of the instrument. To determine the optimum excitation and emission wavelengths for Hb-AGE, an excitation/emission prescan was performed using excitation monochromator limits ranging between 200 and 800 nm and the emission monochromator limits ranging between 250 and 800 nm. The excitation/emission slit width combination of 2.5 and 5.0 nm was used to obtain maximum intensity values. Based on the prescan value, the excitation wavelength with maximum intensity was fixed at 308 nm and the corresponding maximum emission wavelength determined at 345 nm. Further scattering effects were also removed. Since Rayleigh scattering is largely a random effect, the scatter peaks in the two spectra may not be identical. Hence, the spectra were normalized before performing the background subtraction. After normalization, normalized blank spectrum was subtracted from the normalized sample spectrum to get the difference spectrum. The resultant spectra, after removing the Rayleigh and Raman scattering, were used for peak analysis. Major peaks were identified using threshold intensity values and analyzed for the emission wavelength.

Statistical analysis. Data are presented as the mean ± SD, and the correlation between HbA_{1c} and Hb-AGE fluorescence intensity was analyzed using Spearman's correlation coefficient. The analysis was performed using the Vassarstats package.

RESULTS

In vitro study. Based on the prescan values of in vitro glycated hemoglobin, the excitation wavelength for the emission scan was set at 308 nm. Our analysis shows that upon excitation in the UV range of the spectrum at 308 nm, in vitro glycated hemoglobin gives the maximum intensity of emitted light at 345 nm (Fig. 1). In both cases (5 and 20 mmol/l glucose), the autofluorescent emission peaks were clustered around 345 nm. The emitted fluorescence intensity values of hemoglobin incubated with 5 and 20 mmol/l glucose are plotted as a function of time (Fig. 2). Initially, the fluorescence of the reaction mixture is close to zero up to the 4th week of incubation in both cases. Thereafter, intensity was noted to increase from the 5th week and thereafter. The maximum intensity of 9.52 (for 5 mmol/l) and 52.02 (for 20 mmol/l) was obtained during the 12th week of incubation and the intensity stabilized subsequently. These observations indicate that a fluorophore adduct first appears after 5 weeks of incubation during the glycation process, which has excitation and emission wavelengths of 308 and 345 nm, respectively. Further, this new fluorophore starts to accumulate from the 5th week and thereafter and tends to increase until the 12th week—after which a stabilized intensity was noticed. Moreover, the intensity in 20-mmol/l samples was five times higher than that of 5-mmol/l samples. Hence, this new fluorophore provides consistent results under the given experimental conditions and can thus be used as a marker of glycation.

In vivo study. An attempt was made to standardize the samples using their erythrocyte count. However, due to variation of hemoglobin content within a cell, standardization was then carried out based on the hemoglobin protein concentration. The concentration of hemoglobin plays an important role in fluorescence measurement, since the relationship between intensity and concentration of analyte is not linear at higher concentrations of the analyte. This nonlinearity arises as a result of the inner filter effect, which causes quenching of emitted light. Our experiments confirm that protein at 1.0 mg/ml concentration is the optimum concentration of hemoglobin for fluorescence measurement (10). The Hb-AGE fluorescence intensity, HbA_{1c}, and plasma glucose levels in hyperglycemic rats is depicted in Figure 3. There were considerable variations in

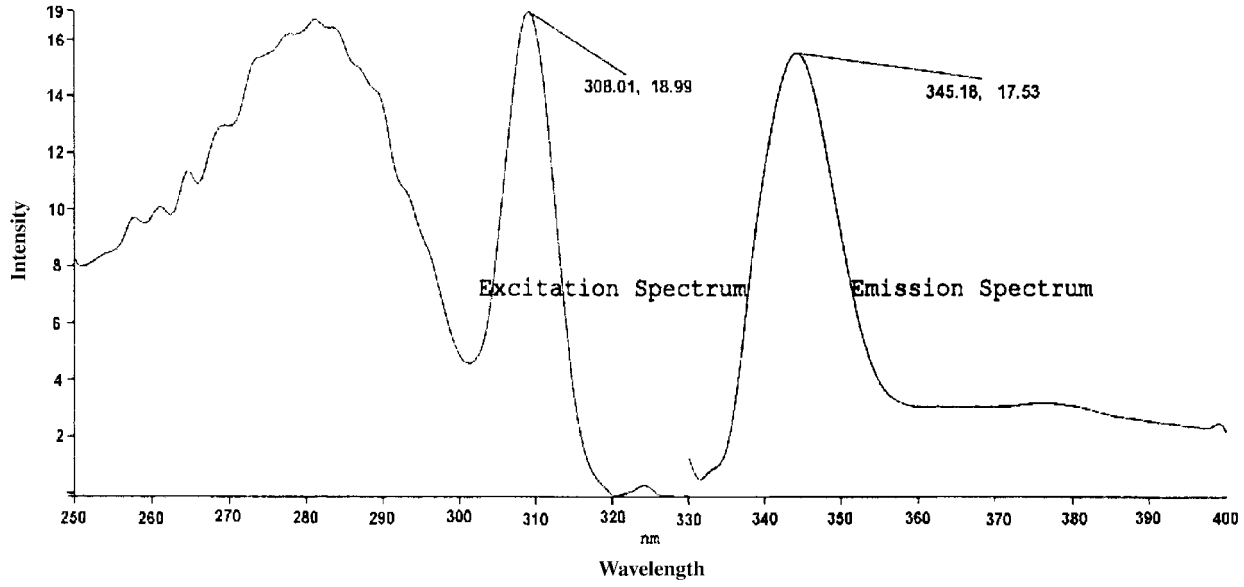

FIG. 1. Characteristic fluorescence spectra of Hb-AGE.

the levels of plasma glucose immediately after the induction of hyperglycemia. After 4–6 weeks, a significant rise in the level of HbA_{1c} was observed. The fluorescence intensity at 308/345 nm was initially low and became substantial after ~10 weeks followed by a linear increase thereafter. The values of plasma glucose level, HbA_{1c}, and Hb-AGE in control rats were 109.5 ± 11.5 mg/100 ml, 4.25 ± 0.26%, and 4.77 ± 0.22 (arbitrary units), respectively. These are means of measurements made at random intervals during the 13-week period.

Clinical study. A total of 23 patients (11 women and 12 men) with type 2 diabetes (category I) and 7 nondiabetic age-matched control subjects (category II) were recruited for the study. The mean age was 47.6 ± 8.1 and 46.6 ± 7.4 years for category I and II subjects, respectively. The mean glucose level (mg/dl), HbA_{1c} (%), and Hb-AGE fluorescence intensity (arbitrary units) in category I subjects were 170.6 ± 40.0, 8.2 ± 0.61, and 10.2 ± 5.2, respectively, while the corresponding values in the control subjects were 85.6 ± 10.66, 5.9 ± 0.66, and 1.7 ± 0.2, respectively. Whereas, a positive correlation was found between levels of plasma glucose, HbA_{1c}, and Hb-AGE fluorescence intensity in category I subjects (Fig. 4), there was a lack of correlation in category II subjects. Prescan measurements showed that the maximum excitation of human Hb-AGE was also obtained at 308 nm, which was then used to determine the emission wavelength. The latter were again observed to be ~345 nm. Thus, these findings corroborate with the in vitro study observations.

DISCUSSION

In this study, autofluorescence of Hb-AGE as a feasible long-term index of glycemia was examined. AGEs of

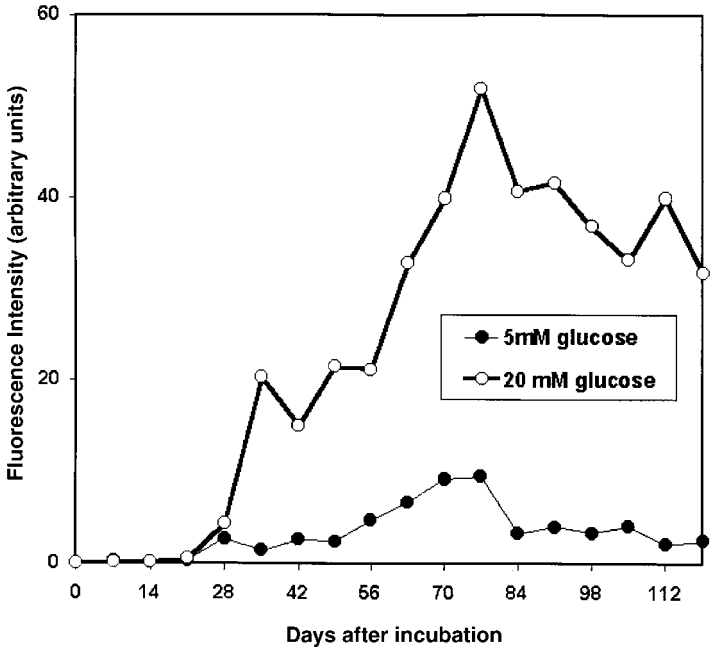

FIG. 2. Time course of formation of Hb-AGE during in vitro glycation; ● = 30 mmol/l glucose, ○ = 20 mmol/l glucose. Hb-AGE fluorescence intensity is indicated in arbitrary units.

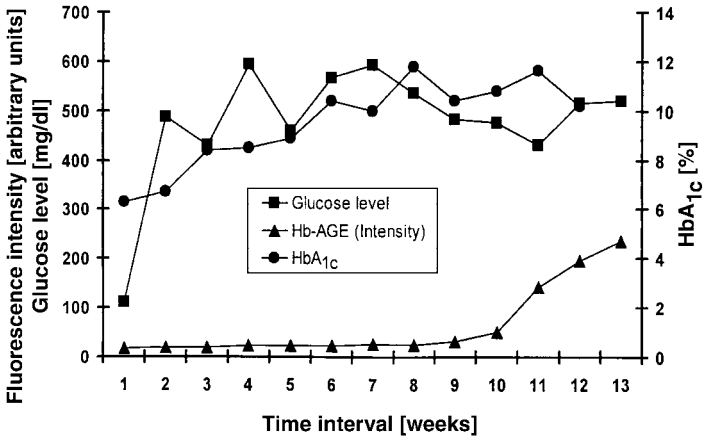

FIG. 3. Time course of glycation of hemoglobin in the streptozotocin-induced diabetic rats studied up to 13 weeks after onset of diabetes. Plasma glucose levels are also shown during this period.

proteins in general comprise a heterogeneous population, and the precise nature of most AGEs remains to be elucidated (17). Amadori products are slowly reversible and attain equilibrium over a 3- to 4-week period. AGEs, in contrast, remain irreversibly attached to proteins and continue to accumulate over the lifespan of the proteins. The rate of turnover of hemoglobin is 104 days in hyperglycemic individuals and 120 days in normoglycemic subjects. Since this molecule has a short half-life compared with other proteins that also undergo glycation in the body, the autofluorescence of Hb-AGE can be attributed to hyperglycemia, not to the process of aging. The formation of glycated hemoglobin has been observed both under in vitro and in vivo conditions (18). A correlation between the amounts of red-cell HbA_{1c} and Hb-AGE quantified by ELISA has been reported previously (19). Our results on these aspects are consistent with earlier reports.

Spectroscopy-based fluorescence determination has a much higher sensitivity than the absorbance spectroscopy (20). Measurements down to 10^{-5} μg/ml are feasible by fluorescence measurement technique. AGEs of various proteins are known to have yellow-brown pigmentation (having a characteristic fluorescence spectrum) and the ability to form protein-protein cross-links. Protein glycation is highly dependent substrate concentration and, given the relatively slow kinetics of the overall process, it is best observed for hemoglobin. Hence, the intrinsic molecular fluorescence nature of Hb-AGE can prove to be useful for long-term monitoring of diabetes. In vitro studies show that a new fluorophore adduct is developed after a prolonged period of incubation of hemoglobin with glucose. This fluorophore adduct having the excitation/emission wavelengths of 308/345 nm seems to be characteristic of Hb-AGE and is specific for the fluorescent AGEs formed during the nonenzymatic glycosylation of hemoglobin. The absence of any significant peak at these wavelengths for pure hemoglobin confirmed the formation of a new fluorophore adduct in glycated hemoglobin. As expected, the wavelength of maximum excitation coincides with the absorbance maxima of this adduct (Fig. 1). We feel that the possibility of nonspecific fluorescence is minimal, since all other proteins present in the plasma are removed by centrifugation during the isolation of the erythrocytes and the subsequent washing step. Fluorescence for the known fluorescent adducts like pentosidine (335/385 nm) and collagen-related fluorescence (370/440 nm) was found to be absent. The role of glyco-oxidation cannot be ascertained from these experiments, since both in vitro and in vivo experimental design did not and could not preclude the possibility of glyco-oxidation. However, even if the dominant glyco-oxidation product carboxy-

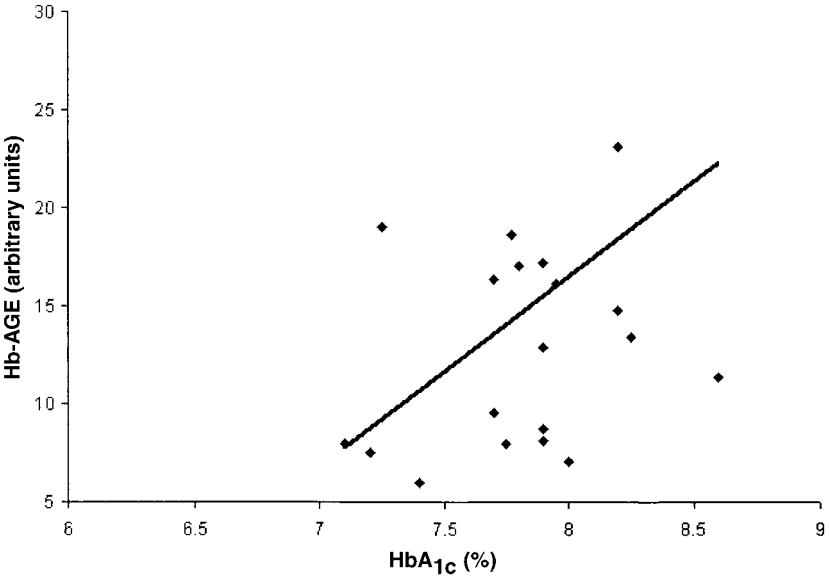

FIG. 4. Correlation between HbA_{1c} and amounts of Hb-AGE in group I subjects (R_s = 0.427, P < 0.04). HbA_{1c} was measured by phenylboronate affinity chromatography and Hb-AGE by fluorescence intensity (excitation/emission wavelength = 308/345 nm).

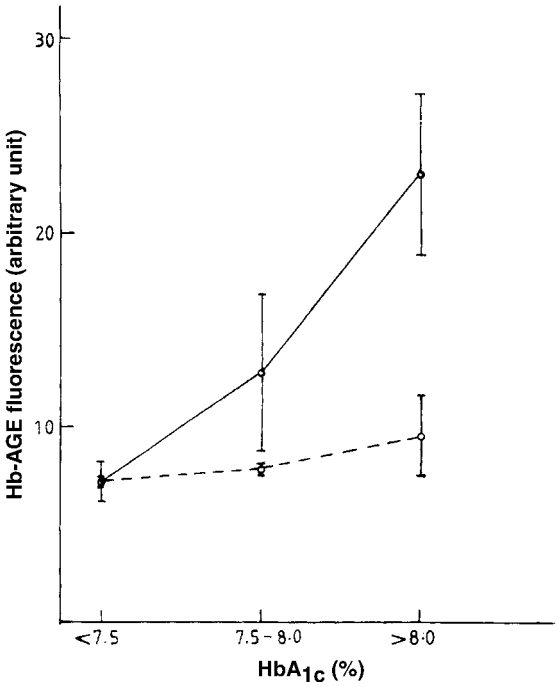

FIG. 5. The values (mean ± SE) of HbA_{1c} and Hb-AGE as categorized on the basis of glycemic control: group I HbA_{1c} <7.5%; group II HbA_{1c} = 7.5- 8.0%; group III HbA_{1c}>8.0%.

methyllysine is formed, it will not affect our measurements because it is nonfluorescent (16).

The same fluorophore, formed under in vitro condition was observed in experimentally induced hyperglycemic condition. In both cases, a prominent emission peak at 345 nm after 4–6 weeks occurs that correlated significantly with the increase in HbA_{1c} formation. Despite heavy fluctuation in the glucose level of the diabetic rat during the study period of 13 weeks, the Hb-AGE fluorescence intensity level showed a steadily increasing value after 2 months. Further, the HbA_{1c} formed during the first 6 weeks of diabetic condition does not show any further increase with disease status, possibly due to its modification to a new adduct. This adduct may not be detected by the phenylboronate affinity chromatographic column. Similar AGE formation under both in vitro and in vivo conditions was reported earlier by incubating BSA with glucose (21).

Clinical analysis on human subjects indicated the presence of the new fluorophore at very low concentrations in normal subjects, while it was significantly higher in the hyperglycemic patients. In this study, the diabetic patients had HbA_{1c} values in the range of 7.1- 8.6%, of which the majority were <8% (Fig. 5). Levels <8% are considered to be in the range of good glycemic control, and we observe that HbA_{1c} and Hb-AGE fluorescence are correlated at $R_s = 0.427$ ($P < 0.04$). The Hb-AGE did not correlate with HbA_{1c} in normal subjects ($R_s = -0.445$). In a previous study (9) in which Hb-AGE was evaluated by ELISA, the authors observed a less tight correlation ($R_s = 0.37$, $P < 0.001$) in well-controlled patients (HbA_{1c} 5.4–7.5%) and better correlation ($R_s = 0.51$, $P < 0.001$) in poorly-controlled patients (HbA_{1c} 8.9–13.3%). This indicates that the Hb-AGE fluorescence values compare reasonably well with Hb-AGE measured by ELISA.

These experiments also shed some light on the temporal relation between the conversion of the reversible adduct HbA_{1c} to the irreversible moiety Hb-AGE. From the in vivo data it appears that there may be a threshold for Hb-AGE formation. In rats, the first appearance of Hb-AGE fluorescence occurs after 9 weeks of induction of diabetes, when the HbA_{1c} values have stabilized ~10%. The correlation between HbA_{1c} and Hb-AGE (Fig. 4) in clinical samples also gives an indication of a threshold. When Hb-AGE value is zero, the correlation fit intersects the X-axis of HbA_{1c} at a value of ~6.8%. This would imply that the onset of Hb-AGE formation occurs when circulating HbA_{1c} levels rise to ~6.8%. In normoglycemic individuals who have mean HbA_{1c} concentrations of 5.9%, the Hb-AGE values show a negative correlation with the former. At this stage one can only speculate that the rate of formation of fluorescent AGEs from the Amadori products is different under conditions of hyperglycemia. Further, the increase in the mean Hb-AGE values (Fig. 5) in groups II and III diabetic subjects (HbA_{1c} >7.5%) is nonlinear as compared with the increase in HbA_{1c} values. This indicates that Hb-AGE has a longer persistence than HbA_{1c} in the circulating erythrocytes. This observation reinforces its utility for monitoring glycemic control. The significant variation in the formation of Hb-AGE due to hyperglycemia should be useful for follow-up and monitoring of diabetic patients. For example, low levels of Hb-AGE in diabetic patients may imply good metabolic control over the previous 2–3 months. As opposed to HbA_{1c}, the measurement technique for Hb-AGE is relatively simple and precludes the possibility of inter-laboratory variations (22).

A growing body of epidemiological evidence indicates an "epidemic" of type 2 diabetes globally. The complications of the rapidly escalating number of patients can be prevented, or progress can be slowed, if glycemic control is tight and follow-up monitoring is performed reliably and correctly. This is an apt situation where Hb-AGE has a potentially important role. A reliable long-term monitor of glycemia can reduce management errors and reduce hospital visits. Furthermore, it creates important opportunities for research.

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