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A novel advanced glycation index and its association with diabetes and microangiopathy

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Abstract

Formation of advanced glycation end products (AGEs) is an important mechanism by which chronic exposure to high glucose levels leads to vascular complications. Measurement of AGEs is hence of great importance for clinicians and researchers concerned with the management and prevention of diabetic vascular disease. The aim of this study was to evaluate a simple methodology to detect AGEs in the serum and to correlate their levels with diabetes and microangiopathy, specifically retinopathy and nephropathy. We studied 157 subjects, which included nondiabetic control subjects (n = 38), type 2 diabetic patients without microangiopathy (n = 65), and type 2 diabetic subjects with retinopathy (n = 29) or both retinopathy and nephropathy (n = 25). All subjects were assessed for their glycemic and lipid status. Serum AGEs were monitored by recording the Maillard-specific fluorescence that resulted from sequential addition of serum into the buffer. The resultant linear regression was modeled to yield the slope values that were termed *advanced glycation index* (AGI) in arbitrary units. The serum levels of AGI (mean ± SD) were higher in diabetic subjects without complications (6.0 ± 1.6 units) compared with nondiabetic subjects (4.6 ± 1.0 units), still higher among diabetic subjects with retinopathy (7.6 ± 1.2 units) and highest in diabetic subjects with both retinopathy and nephropathy (8.3 ± 2.0 units). Among diabetic subjects, AGI had a significant positive correlation with duration of diabetes (r = 0.25, P = .006), glycated hemoglobin (r = 0.27, P = .004), cholesterol (r = 0.24, P = .009), triglycerides (r = 0.23, P = .014), and serum creatinine (r = 0.30, P = .001), and a significant negative correlation with creatinine clearance (r = −0.27, P = .003). Logistic regression analysis using diabetic microangiopathy as the dependent variable showed an association with AGI even after including age, duration of diabetes, and glycated hemoglobin (P < .001) into the model. Advanced glycation index is a simple method to detect AGEs, and it correlates well with diabetes, particularly with microangiopathy.

1. Introduction

The etiology of vascular complications of diabetes mellitus is poorly understood, and several metabolic abnormalities have been postulated as possible triggers for micro- and macroangiopathies [1,2]. The glycation or Maillard hypothesis proposes that complications of diabetes are a consequence of accelerated, cumulative, nonenzymatic modification of proteins and other biomolecules by glucose or its metabolic intermediates during hyperglycemia. This nonenzymatic reaction between the reducing sugars and the free amino groups of proteins leads to the formation of advanced glycation end products (AGEs) through a complicated network of pathways [3,4]. Although the early Amadori modifications are reversible, the later nonenzymatic glycation reactions ultimately culminate in the formation of irreversible AGEs (carboxymethyl lysine [CML], pentosidine, argpyrimidine, pyrroline, etc), which are significantly correlated to diabetic complications [5]. However, the measurement of AGEs is difficult due to their low concentration in tissue proteins. Specific and accurate measurement of AGEs requires gradient high-pressure liquid chromatography analysis or gas or liquid chromatography-mass spectrometry that is not available in most clinical settings [6]. The available

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enzyme-linked immunosorbent assays detect only individual AGEs, and other methods require preanalytical steps that may alter or convert the Amadori adducts to AGEs and thus affect the results. We report here on a simple assay to assess advanced glycation in a clinical setting.

2. Materials and methods

2.1. Subjects

The study group comprised 157 subjects, which included nondiabetic control subjects (n = 38), type 2 diabetic patients without microangiopathy (n = 65), and type 2 diabetic subjects with diabetic retinopathy (n = 29) or both retinopathy and nephropathy (n = 25). The diabetic subjects were selected from outpatients attending Dr Mohan's MV Diabetes Specialties Centre, a tertiary referral center for diabetes care at Chennai (formerly Madras) in Southern India. Nondiabetic control subjects were recruited from the ongoing Chennai Urban Rural Epidemiological Study (CURES), a population-based study in Chennai, the details of which have been published elsewhere [7,8]. Details such as age, sex, and duration of diabetes were recorded, and a complete clinical examination was done. Of the 119 type 2 diabetic subjects, 65 (55%) were on oral hypoglycemic agents and the rest were on oral hypoglycemic agent plus insulin. Blood pressure was recorded in sitting position in the right arm to the nearest 2 mm Hg with a mercury sphygmomanometer (Diamond Deluxe Model, Pune, India).

Biochemical analyses were done on Hitachi-912 Autoanalyser (Hitachi, Germany) using kits supplied by Boehringer Mannheim, Mannheim, Germany. Fasting plasma glucose (glucose oxidase-peroxidase method), serum cholesterol (cholesterol oxidase-peroxidase-amidopyrine method), serum triglycerides (glycerol phosphate oxidase-peroxidase-amidopyrine method), high-density lipoprotein cholesterol (direct method), and serum creatinine (modified kinetic method of Jaffe) were measured. Low-density lipoprotein cholesterol was calculated using the Friedewald formula [9]. Glycated hemoglobin (HbA_{1c}) was estimated by high-pressure liquid chromatography using the Variant machine (Bio-Rad, Hercules, Calif). Urine samples were collected in the early morning after an overnight fast. Urine creatinine was measured using Jaffe's method. Because creatinine clearance is one of the well-accepted methods of estimating the glomerular filtration rate and is used as a relative index of renal function, we calculated creatinine clearance using the Cockcroft and Gault equation [10,11]. Urinary protein was measured on spot urine by sulfosalicylic acid technique and expected protein excretion was calculated. Urine microalbumin concentration was measured using commercially available immunoturbidometric assay kits from Randox (Randox, UK) on Hitachi 902 Autoanalyser (Roche Diagnostics GmbH, Mannheim, Germany) as reported elsewhere [12].

2.2. Definitions

2.2.1. Nephropathy

Nephropathy was diagnosed if the patients had either persistent proteinuria (≥150 mg/d) or microalbuminuria (if albuminuria estimated by albumin creatinine ratio exceeded 30 μg/mg of creatinine) [12].

2.2.2. Retinopathy

Fundus photography was done to evaluate retinopathy in the diabetic study subjects. The pupils were dilated using one drop each of phenylephrine 10% and tropicamide 1% into both eyes, and the drops were repeated until the best possible mydriasis was obtained. A trained photographer carried out 4-field color retinal photography with a Zeiss FF 450 plus camera (Carl Zeiss Medica, Zena, Germany) using 35-mm color transparencies. The photographs were graded against standard photographs of the Early Treatment Diabetic Retinopathy Study grading system for severity of retinopathy as described earlier [8]. Informed consent was obtained from all study subjects and the institutional ethics committee approved the study.

2.2.3. Estimation of advanced glycation index

Serum was diluted 50 times in phosphate-buffered saline, and the intrinsic AGE-specific fluorescence was monitored spectrofluorimetrically (Fluoromax-3, Jobin Yvon Horiba, NJ) by exciting the samples at 370 nm and collecting the emission readouts at 440 nm. To ascertain the excitation and emission characteristics of AGEs measured in serum, AGE adduct (bovine serum albumin + glucose in phosphate buffer for 40 days) was made in vitro and tested for fluorescence spectra. Fluorescence intensities of AGE adduct measured at excitation and emission wavelengths at 370 and 440 nm, respectively, were abolished with aminoguanidine (cotreatment during the AGE adduct formation), thereby indicating a relative and reliable measurement of AGEs in serum. Fluorescence estimations

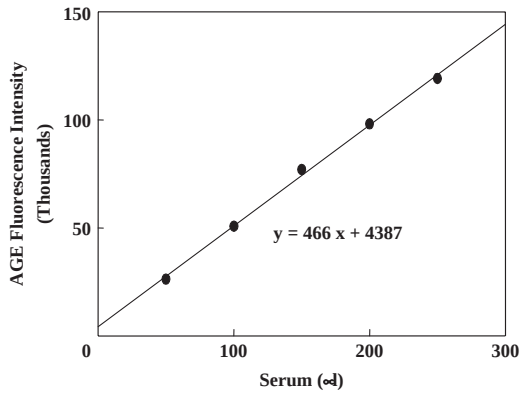

Fig. 1. Increases in AGE fluorescence on sequential addition of serum were curve fitted to a linear regression, $y = mx + b$ (details in methodology). The slope of the regression line was termed as *AGI*. Slope values (AGI) were scaled as 100 = 1 arbitrary unit in the subsequent figures.

Table 1
Clinical characteristic of study subjects

Parameters	Nondiabetic subjects (n = 38)	Diabetic subjects without complications (n = 65)	Diabetic subjects with retinopathy alone (n = 29)	Diabetic subjects with retinopathy and nephropathy (n = 25)	ANOVA, *P*
Age (y)	54.6 ± 4.8	56.0 ± 10.5	58.0 ± 6.5	56.6 ± 8.0	.427
Systolic blood pressure (mm Hg)	114 ± 10	135 ± 16[a]	132 ± 12[a]	149 ± 16[a,b,c]	<.001
Diastolic blood pressure (mm Hg)	72 ± 8	81 ± 7[a]	82 ± 6[a]	87 ± 10[a,b]	<.001
Fasting plasma glucose (mg/dL)	88 ± 8	146 ± 57[a]	160 ± 60[a]	170 ± 71[a]	<.001
HbA_{1c} (%)	5.8 ± 0.5	8.0 ± 2.0[a]	8.9 ± 2.0[a]	9.5 ± 2.5[a,b]	<.001
Serum cholesterol (mg/dL)	187 ± 35	185 ± 37	201 ± 44[a,b]	209 ± 56	.054
Serum triglycerides (mg/dL)	128 ± 77	164 ± 88	172 ± 96[a,b]	217 ± 163[a]	.011
Serum creatinine (mg/dL)	0.93 ± 0.09	0.94 ± 0.09	0.96 ± 0.2[a,b]	1.4 ± 0.6[a,b,c]	<.001
Creatinine clearance (mL/min)	82.1 ± 18.7	81.9 ± 21.4	76.5 ± 21.6	66.0 ± 28.4[a,b]	.015

Values are mean ± SD.
ANOVA indicates analysis of variance.
[a] $P < .05$ compared to control group.
[b] $P < .05$ compared to diabetic subjects without complications.
[c] $P < .05$ compared to diabetic subjects with retinopathy alone.

at these wavelengths represent measurement of Maillard-specific AGE peptides in circulation [13-16]. The concentrations of the AGE products were directly proportional to the fluorescence intensity, and the increase in fluorescence intensity at each addition of serum sample was curve fitted to a linear regression line (Fig. 1). The slope of the regression line was termed *advanced glycation index* (AGI) and expressed in arbitrary units (100 units = 1 unit of AGI). The AGI value (which represents the serum AGE levels) is a 5-point linear regression estimate, rather than the single-point derivation of AGE fluorescence levels reported in earlier studies. Experiments were done in batches and each time the instrument was checked for excitation and emission readings with appropriate water Raman and lamp spectra.

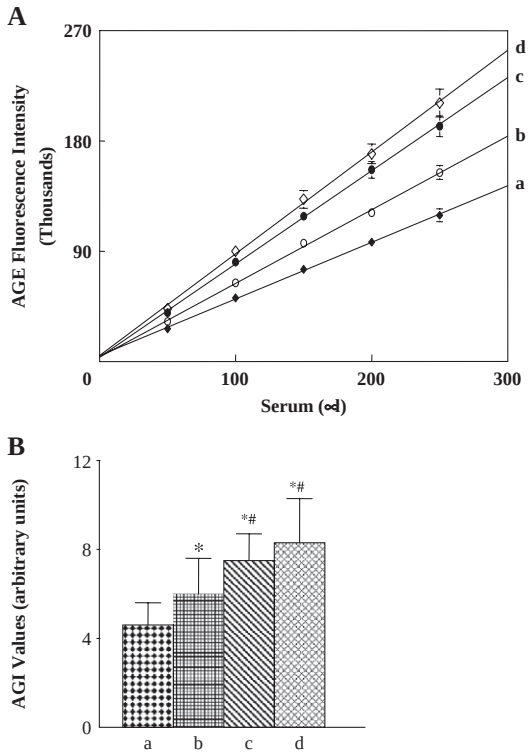

Fig. 2. A, Cumulative AGE fluorescence data fitted as linear regression model for the study subjects: (*a*) nondiabetic subjects, n = 38; (*b*) type 2 diabetic subjects without microangiopathy, *n* = 65; (*c*) type 2 diabetic subjects with retinopathy alone, *n* = 29; and (*d*) with both retinopathy and nephropathy, n = 25. B, Bar diagram showing the AGI values (mean ± SD) computed from the slopes from A. Asterisk indicates $P < .001$ compared to nondiabetic subjects; number symbol, $P < .001$ compared to diabetic subjects without microangiopathy.

2.2.4. Precision of AGI

To determine the precision of the assay, 5 samples each in the range of 2 to 6 and 8 to 12 units were repeated 5 times. The intraassay and interassay coefficients of variation for the range of 2 to 6 units were 4.9% and 5.9%, respectively, whereas those for the range between 8 and 12 units were 4.5% and 5.4%, respectively. The lower limit of detection was 2 units.

Table 2
Pearson correlation analysis of AGI with other risk variables in nondiabetic and diabetic subjects

Parameters	Nondiabetic subjects (n = 38)		Diabetic subjects (n = 119)	
	r	*P*	*r*	*P*
Duration	–	–	0.25	.006
Fasting plasma glucose	0.40	.015	0.225	.074
HbA_{1c}	0.29	.079	0.27	.004
Total cholesterol	0.014	.932	0.24	.009
Serum triglycerides	0.175	.293	0.23	.014
Serum creatinine	0.34	.036	0.30	.001
Creatinine clearance	−0.29	.080	−0.27	.003

Table 3
Logistic regression analysis using diabetic microangiopathy as dependent variable

Description	Odds ratio	95% Confidence interval	P
AGI	2.008	1.470-2.742	<.001
Age	1.002	0.953-1.053	.953
Duration of diabetes	1.068	0.999-1.142	.053
HbA_{1c}	1.217	0.997-1.486	.054

2.3. Statistical analysis

Statistical analysis was carried out on Windows-based SPSS package (version 4.0.1, SPSS, Chicago, Ill). Numbers are expressed as mean ± SD. Student *t* test or 1-way analysis of variance as appropriate was used to compare continuous variables, and χ^2 tests were used to compare proportions among groups. Pearson correlation analysis was done to determine the relation of AGI with other risk variables. Logistic regression analysis was done in diabetic subjects using diabetic microangiopathy as the dependent variable to determine the association of AGI with diabetic microangiopathy.

3. Results

Table 1 shows the clinical characteristics of the study groups. There was no significant difference in the age or sex distribution between the study groups. Diabetic subjects with retinopathy and nephropathy had higher systolic blood pressure and creatinine levels compared to all other groups ($P < .05$). This subgroup also had higher diastolic blood pressure, HbA_{1c}, and lower creatinine clearance values compared to nondiabetic subjects and diabetic subjects without complications ($P < .001$).

Fig. 2 illustrates the mean slopes (Fig. 2A) and their respective mean AGI values (Fig. 2B) in the study groups. The mean AGI values in diabetic subjects without complications (6.0 ± 1.6 units) were higher than in the nondiabetic subjects (4.6 ± 1.0 units). The values were still higher among diabetic subjects with retinopathy alone (7.6 ± 1.2 units) and highest in diabetic subjects with both retinopathy and nephropathy (8.3 ± 2.0 units). The differences reached statistical significance when values were compared between diabetic subjects without complications and nondiabetic subjects ($P < .001$). Similarly, diabetic subjects with microangiopathy had significantly higher values compared with diabetic subjects without complications ($P < .001$).

In nondiabetic subjects, AGI showed a significant correlation with serum creatinine ($P = .036$) and fasting plasma glucose ($P = .015$). In diabetic subjects, AGI had a significant positive correlation with duration of diabetes ($r = 0.25$; $P = .006$), HbA_{1c} ($r = 0.27$; $P = .004$), cholesterol ($r = 0.24$; $P = .009$), triglycerides ($r = 0.23$; $P = .014$), and serum creatinine ($r = 0.30$; $P = .00$), and an inverse correlation with creatinine clearance levels ($r = -0.27$; $P = .003$) (Table 2). Patients with reduced creatinine clearance (≤70 mL/min) also showed significantly higher fluorescent AGE levels compared with those with creatinine clearance greater than 70 mL/min (mean AGI, 7.4 vs 6.5; $P \leq .05$).

Logistic regression analysis was done using diabetic microangiopathy as dependent variable and AGI as independent variable. Advanced glycation index showed a significant association ($P < .001$) with microangiopathy even after adding age, duration of diabetes, and HbA_{1c} into the model (Table 3).

4. Discussion

The AGI used in this study represents a Maillard-specific index of circulating AGE compounds as demonstrated in earlier studies [13-16]. Involvement of AGEs in the pathogenesis of diabetic complications may comprise a series of related chemical structures of dissimilar immunogenic characteristics [17]. Measuring individual AGEs such as CML may not reveal completely the AGE-associated severity of diabetic complications. Moreover, the AGEs that can be measured today by enzyme-linked immunosorbent assay and other techniques represent only a fraction of the AGEs that are formed in vivo [14]. It has also been demonstrated that serum levels of non-CML AGEs are significantly associated with the severity of diabetic nephropathy and retinopathy, suggesting a role of non-CML AGE in the progression of microvascular complications [18]. Recent studies indicate that non-CML AGEs may play a role in accelerating micro- and macrovascular complications of diabetes [5,19,20]. Therefore, we used a very simple method to measure total AGEs in serum samples and analyzed the association of the same with diabetes and microangiopathy.

A number of studies have confirmed that circulating AGE levels are higher in diabetic subjects [21-24]. However, a clear correlation between AGE levels and HbA_{1c} has not been demonstrated in some studies [22,24]. In our study, we observed that AGI had a significant correlation with HbA_{1c} in diabetic subjects. Moreover, there is a significant association of AGI with diabetes and its complications, which is independent of HbA_{1c}. As suggested by Miyata et al [25], glycemic control may influence early Maillard products, whereas other factors, for example, complication-related oxidative or α-dicarbonyl stress, are of greater importance for the late stage of the reaction where irreversible cross-linking of proteins occurs. The predominant source of AGEs is endogenous, and a recent study established that genetic factors might account for 74% of normal population variance in CML levels [26]. Because genes that are in common with those determining fasting glucose or HbA_{1c} could not explain CML heritability, Leslie et al [26] have suggested that the genetic factors contributing to CML levels must be distinct from those contributing to fasting glucose and HbA_{1c} levels. Moreover, because increased serum AGI in diabetes subjects is independent of age and metabolic parameters, it is possible that AGI could be used as an adjuvant measure for

predicting microvascular complications in diabetes. However, a causal role cannot be established in a cross-sectional study such as this but would require longitudinal studies.

The present study also demonstrates a strong correlation between triglycerides and serum AGEs in diabetes subjects. A similar association was recently demonstrated in a study in children and adolescents with type 1 diabetes [19]. These observations suggest a link between triglycerides and formation of AGEs. Some AGE structures could also be formed from oxidized lipid precursors termed *advanced lipoxidation end products*, which together may precipitate endothelial dysfunction, atherosclerosis, and diabetic complications [27]. Further studies are needed to see whether higher levels of oxidized lipids lead to increased fluorescent AGEs.

The association of AGE levels with diabetic retinopathy observed in our study is consistent with earlier studies [22,28-30]. Advanced glycation end product–induced interactions could lead to morphological and functional changes in retinal capillaries, including loss of pericytes, basement membrane thickening, and increased permeability [31]. It has also been shown that subjects with proliferative diabetic retinopathy have higher levels of plasma pentosidine [32], glycated albumin, and corneal AGEs [33].

In our study, the relationship of increased AGEs with diabetic nephropathy is shown by higher level of AGI in subjects with nephropathy and retinopathy compared with those with retinopathy alone. In addition, AGI were positively correlated with creatinine levels and inversely related to creatinine clearance values. We also noted that the levels of AGEs were higher in patients with creatinine clearance of less than 70 mL/min compared with those with values greater than 70 mL/min. Higher concentrations of creatinine and pentosidine levels in serum were also shown in patients with reduced creatinine clearance [34-36]. Miyata et al [37] and Miyazaki et al [38] have recently characterized a pentosidine-like compound in plasma and demonstrated that endogenous creatinine plays a direct role as a protein modifier in the formation of pentosidine. As suggested by Li et al [39], the AGE peptides produced by the normal catabolism of AGE-containing proteins are released into the circulation to be cleared by the kidney, showing the link between AGE accumulation in tissues and renal dysfunction. Thus, it is possible that either high levels of AGEs could play a causal role in the development and progression of nephropathy or they could be an epiphenomenon caused by the decrease in urinary excretion. Recent studies [40-42] support the former view, but this would need to be established by longitudinal studies.

Our assay of advanced glycation assessment in serum with computation of AGI has several advantages. It is simple and cost-effective and requires no preanalytical modification of testing samples. Secondly, increased AGE-specific values in our study correlate well with diabetes and its microangiopathic complications. Because the accumulation of AGEs may be prevented by intensive therapy of hyperglycemia, antioxidant therapy, and/or anti-AGE strategies, AGI measurement may serve as a simple surrogate marker for monitoring therapeutic benefits in clinical trials. Support for this comes from a recent study [43], which showed that abnormalities in skin collagen due to glycation (measured as fluorescence changes) predict the worsening of diabetic retinopathy over 4 years, independent of HbA_{1c} and diabetes duration. In addition, in a 10-year population-based study [36], improvement in diabetes control was accompanied by a decrease in the concentration of AGE products. In conclusion, our results suggest that AGI measurement in the serum may be useful as a biomarker of potential vascular complications in clinical settings and for epidemiological studies. In an ongoing prospective study, we intend to see whether increased AGI levels can predict the occurrence or progression of diabetic complications, independent of HbA_{1c} levels.

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