Advanced Glycation End Products and Endothelial Dysfunction in Type 2 Diabetes

KATHRYN C.B. TAN, MD[1]
WING-SUN CHOW, MBBS[1]
VICTOR H.G. AI, MBBS[2]
CHRISTINE METZ, PHD[3]
RICHARD BUCALA, MD, PHD[4]
KAREN S.L. LAM, MD[1]

OBJECTIVE — Data from experimental studies have suggested that the increased formation of advanced glycation end products (AGEs) is one of the causes of endothelial dysfunction in diabetes. This study was performed to investigate whether changes in endothelium-dependent vasodilation, a marker of endothelial function, were related to serum AGEs concentrations in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS — For this study, 170 patients with type 2 diabetes and 83 healthy nondiabetic control subjects of similar age were recruited. Serum AGEs were assayed by competitive enzyme-linked immunosorbent assay. Endothelium-dependent and -independent vasodilation of the brachial artery was measured by high-resolution vascular ultrasound.

RESULTS — Serum AGEs were increased in diabetic patients compared with control subjects (4.6 ± 0.7 vs. 3.1 ± 0.8 unit/ml; $P < 0.01$), and both endothelium-dependent (5.1 ± 2.5 vs. 9.1 ± 4.1%; $P < 0.01$) and endothelium-independent vasodilation (13.2 ± 4.6 vs. 16.4 ± 5.5%; $P < 0.01$) were impaired. On univariate analysis of all subjects, serum AGEs correlated with endothelium-dependent vasodilation ($r = -0.51$, $P < 0.01$); a weaker association was found with endothelium-independent vasodilation ($r = -0.24$, $P < 0.01$). On multiple regression analyses including age, sex, smoking status, and plasma lipids, only serum AGEs remained a significant independent determinant of endothelium-dependent vasodilation ($r^2 = 0.34$, $P < 0.01$).

CONCLUSIONS — Increased serum concentrations of AGEs in patients with type 2 diabetes is associated with endothelial dysfunction, independent of other cardiovascular risk factors. Further studies to determine whether treatment targeting AGEs will lead to an amelioration of endothelial dysfunction are warranted.

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Hyperglycemia may cause tissue damage by several mechanisms, one of which is nonenzymatic glycation of intra- and extracellular proteins. Glucose possesses a reactive aldehyde moiety that reacts nonenzymatically with the amino groups of proteins, forming slowly reversible Amadori products. Rearrangement reactions then occur to produce a chemically related group of moieties, termed advanced glycation end products (AGEs), which remain irreversibly bound to proteins (1). AGEs are a heterogeneous group of compounds that can cause a number of adverse cellular events, including reduction of enzymatic activity, damage to nucleic acids, cross-linking and impaired degradation of proteins, and induction of cytotoxic pathways. They have been implicated in the pathogenesis of many of the complications of diabetes (2–4).

Based on experimental studies, it has been suggested that the increased formation of AGEs is one of the causes of endothelial dysfunction in diabetes. Binding of AGEs to their cellular receptors has been shown to enhance oxidant stress and induce a state of endothelial cell activation (5). AGEs cause increased vascular permeability, stimulate cell adhesion molecule expression, and induce migration of macrophages and T-cells into the intima (6,7). AGEs also quench NO (nitric oxide) in vitro and may reduce NO-dependent vasodilation (8). Smulders et al. (9) recently demonstrated an association between urinary excretion of pentosidine, an AGE product, and markers of endothelial dysfunction, such as plasma concentrations of von Willebrand factor and soluble vascular cell adhesion molecule-1 in patients with type 1 diabetes. Because we have previously shown that endothelium-dependent vasodilation, another marker of endothelial function, was impaired in patients with type 2 diabetes (10), this study was performed to investigate whether the impairment of endothelium-dependent vasodilation was related to changes in serum concentration of AGEs in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

— We recruited 170 patients with type 2 diabetes, whose diagnosis was confirmed according to World Health Organization criteria, from the diabetes clinics at Queen Mary Hospital. Patients on insulin therapy were eligible if they had been previously managed their diabetes with diet and an oral agent at some point and had no known history of diabetic ketoacidosis. To rule out the confounding effect of renal impairment in

From the [1]Department of Medicine, University of Hong Kong, Queen Mary Hospital, Honk Kong; [2]Department of Diagnostic Radiology, Queen Mary Hospital, Hong Kong; and [3]Picower Institute for Medical Research, Manhasset, New York; and [4]Yale University School of Medicine, New Haven, Connecticut.

Address correspondence and reprint requests to Dr. Kathryn Tan, Department of Medicine, University of Hong Kong, Queen Mary Hospital, Pokfulam Rd., Hong Kong. E-mail: kcbtan@hkucc.hku.hk.

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Abbreviations: AGE, advanced glycation end product; CML, N^{ε}-[carboxymethyl]-lysine; ELISA, enzyme-linked immunosorbent assay; GTN, glyceryl trinitrate; HPLC, high-performance liquid chromatography; IMT, intima-media thickness; OD, optical density; UAE, urinary albumin excretion.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Clinical characteristics and serum AGEs of control subjects and diabetic patients*

	Control subjects	Diabetic subjects
n	83	170
Male/female (%)	51/49	51/49
Age (years)	52.8 ± 7.5	54.5 ± 8.8
BMI (kg/m^2)	25.0 ± 3.4	26.1 ± 3.7*
Smokers (%)	13	14
Duration of diabetes (years)	—	9.8 ± 6.8
Fasting glucose (mmol/l)	5.3 ± 1.2	8.3 ± 2.1†
HbA_{1c} (%)	5.8 ± 0.7	8.1 ± 1.2†
AGEs (unit/ml)	3.1 ± 0.8	4.6 ± 0.7†
Total cholesterol (mmol/l)	5.6 ± 0.9	5.8 ± 1.0
Triglycerides (mmol/l)	1.3 (0.8)	1.5 (0.9)*
LDL (mmol/l)	3.6 ± 0.8	3.9 ± 1.0†
HDL (mmol/l)	1.3 ± 0.4	1.2 ± 0.3*
Systolic blood pressure (mmHg)	126.4 ± 16.3	130.4 ± 12.1*
Diastolic blood pressure (mmHg)	78.4 ± 10.9	76.0 ± 8.7

Data are *n* or means ± SD except for triglyceride levels, which are given as median (interquartile range). *$P <$ 0.05, †$P < 0.01$ vs. control subjects.

causing an elevation of serum AGEs, diabetic patients with macroalbuminuria and/or impaired renal function were excluded from the study. Clinical data were obtained from medical records. Macrovascular disease was defined as evidence of ischemic heart disease (according to clinical history and Minnesota coding of electrocardiogram), stroke, transient ischemic attack, or peripheral vascular disease. The diagnosis of retinopathy was based on fundoscopic findings by ophthalmoscopy and peripheral neuropathy by assessment of vibration perception threshold. Two consecutive 12-h overnight urine collections were performed for the measurement of the urinary albumin excretion (UAE) rate. Persistent microalbuminuria was defined as a UAE rate of 20–200 μg/min in both overnight urine collections. The clinical characteristics of the diabetic patients are shown in Table 1. Specifically, 76% of the patients were on oral hypoglycemic agents, and the rest were on insulin therapy; 36% had microalbuminuria, 18% had nonproliferative retinopathy, 3% had had previous laser treatment, and 7% had neuropathy. We also recruited 83 healthy control subjects from the community by advertisement. In all subjects, fasting blood samples were taken for the measurement of glucose, lipids, HbA_{1c}, and serum AGEs. Carotid intima-media thickness (IMT) and endothelial function were assessed by high-resolution vascular ultrasound. The study was approved by the Ethics Committee of the University of Hong Kong, and informed consent was obtained from all subjects.

Serum AGEs were measured by competitive enzyme-linked immunosorbent assay (ELISA) using a well-characterized polyclonal rabbit antisera raised against AGE-RNase (11). Specifically, 96-well plates were coated with 50 μl/well of AGE-RNase (3.75 μg/ml) in coating buffer (0.1 mol/l sodium bicarbonate; pH 9.6) overnight at 4°C. Wells were washed three times with washing buffer, and blocked by SuperBlock blocking buffer in PBS for 2 h at room temperature. After wells were rinsed three times, 50 μl of serum (1:4 dilution) were added, followed by 50 μl of 1:500 diluted anti-AGE antibody in dilution buffer containing 2% goat serum. Plates were incubated at room temperature with gentle agitation. Wells were then rinsed three times. Alkaline phosphate-conjugated anti-rabbit IgG (1:2000) in dilution buffer was added to each well and incubated for 1 h at 37°C. After being washed, color was developed by the addition of 100 μl *p*-nitrophenyl phosphate substrate (Sigma). Optical density (OD) at 405 nm was determined by an ELISA reader. Results were calculated as 1 − (experimental OD − background OD/total OD − background OD). A 50% competition was defined as 1 unit of AGEs.

Plasma total cholesterol and triglyceride levels were determined enzymatically on a Hitachi 912 analyzer (Roche Diagnostics, Mannheim, Germany). HDL cholesterol was measured using a homogenous method with polyethylene glycol–modified enzymes and α-cyclodextrin. LDL cholesterol was calculated by the Friedewald equation. HbA_{1c} was measured in whole blood using ion-exchange high-performance liquid chromatography (HPLC) with the Bio-Rad Variant Hemoglobin Testing System (Bio-Rad Laboratories, Richmond, CA).

High resolution B-mode ultrasound (ATL HDI 3000 ultrasound system; Advanced Technology Laboratories, Bothell, WA) was used to measure the IMT of the carotid artery. The anterior, lateral, and posterolateral projections were used to longitudinally image the right and left common carotid arteries. At each projection, three determinations of IMT were made at 2 cm proximal to the bulb and at the site of greatest thickness. The values at each site were averaged, and the greatest value of the averaged IMT was used as the representative value for each individual. Carotid plaque was defined as the presence of a focal lesion measuring at least twice the thickness of the IMT (12). If a plaque was present in one of the projections, that value was excluded from the analysis and IMT was averaged on the remaining values.

Endothelium-dependent and -independent vasodilation of the brachial artery was assessed by high-resolution ultrasound, as previously described (10). In brief, diameter measurements of the right brachial artery were taken at rest and during reactive hyperemia after occlusion by inflation of pneumatic tourniquet, raised to a pressure of 300 mmHg for 4.5 min. The resting scan was repeated after a 20-min period for vessel recovery. Sublingual glyceryl trinitrate (GTN) spray (400 μg) was then administered, and measurements were taken after 5 min. Flow-mediated (endothelium-dependent) and GTN-induced (endothelium-independent) vasodilation was calculated as the percent change in diameter compared with baseline.

Results are expressed as means ± SD or as the median and interquartile range if the data were not normally distributed. Comparisons between two different groups were done using an independent sample *t* test, and skewed data were logarithmically transformed before analysis. Pearson's correlations were used to test the relationship among variables. Multi-

Table 2—*Carotid LMT and vasomotor function of control subjects and diabetic patients*

	Control subjects	Diabetic subjects
n	83	170
Carotid LMT (mm)	0.57 ± 0.13	0.61 ± 0.12*
Brachial artery diameter (mm)	3.8 ± 0.7	3.9 ± 0.7
Endothelium-dependent vasodilation (%)	9.1 ± 4.1	5.1 ± 2.5†
Endothelium-independent vasodilation (%)	16.4 ± 5.5	13.2 ± 4.6†

Data are means ± SD. *$P < 0.05$; †$P < 0.01$ vs controls.

ple stepwise linear regression analysis was used to simultaneously assess the relation between vasomotor function and various variables.

RESULTS — The diabetic patients had significantly higher fasting glucose and HbA_{1c} than the control subjects. Serum AGEs were increased in the diabetic patients compared with the control subjects ($P < 0.01$) (Table 1). No correlation was found between serum AGEs and the UAE rate. There was a significant correlation between AGEs and HbA_{1c} ($r = 0.43$, $P < 0.01$) when the diabetic patients and control subjects were analyzed together, but no correlation was found with age. Results of carotid IMT and endothelium-dependent and -independent vasodilation are shown in Table 2. Although only 3% of our diabetic patients had a clinical history of macrovascular disease, 43% of them had evidence of carotid plaques on an ultrasound test. Both endothelium-dependent and -independent vasodilation were impaired in diabetic patients. The degree of impairment was similar between patients without and with micro- and/or macrovascular complications (endothelium-dependent vasodilation: 5.4 ± 2.7 vs. 4.9 ± 2.4%, respectively; endothelium-independent vasodilation: 13.4 ± 4.7 vs. 13.1 ± 4.6%, respectively).

On univariate analyses of all subjects, serum AGEs correlated with endothelium-dependent vasodilation ($r = -0.51$, $P < 0.01$) (Fig. 1*A*) and with endothelium-independent vasodilation ($r = -0.24$, $P < 0.01$) (Fig. 1*B*). These associations remained significant ($P < 0.01$), even after adjusting for age, sex, and smoking status. Endothelium-dependent vasodilation also correlated with HDL levels ($r = 0.23$, $P < 0.01$) (Fig. 2), whereas endothelium-independent vasodilation correlated with HDL ($r = 0.20$, $P < 0.01$) and triglyceride levels ($r = -0.13$, $P < 0.05$). No significant correlations were found with blood pressure. Because endothelium-dependent and -independent vasodilation also correlated with plasma lipids, multiple regression was performed to determine whether the effect of serum AGEs on vasomotor function was independent of that of other risk factors. On multiple regression analysis including age, sex, smoking, and plasma lipids, only serum AGEs remained a significantly independent determinant of endothelium-dependent ($r^2 = 0.34$, $P < 0.01$) and endothelium-independent vasodilation ($r^2 = 0.16$, $P < 0.01$). Repeating the analyses in the diabetic cohort alone with the inclusion of the presence of diabetic complications as a confounding variable did not change our results. Serum AGEs were the only significant determinant of endothelium-dependent ($r^2 = 0.27$, $P < 0.01$) and endothelium-independent vasodilation ($r^2 = 0.12$, $P < 0.01$).

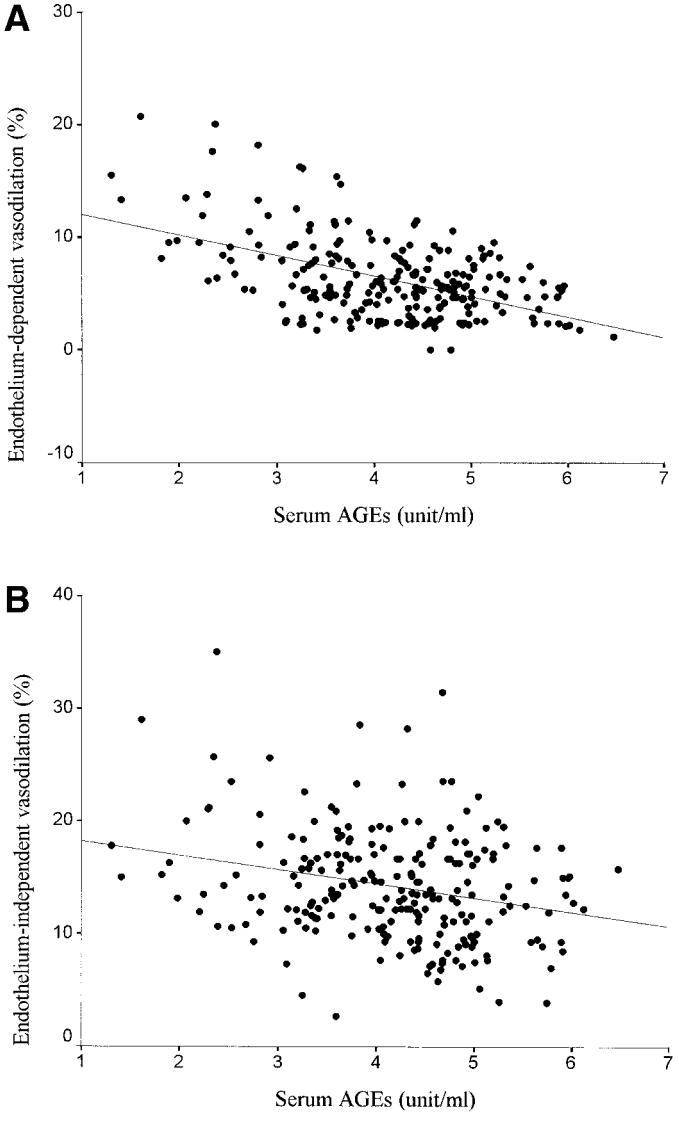

Figure 1—A: *Correlation between serum AGEs and endothelium-dependent vasodilation;* r = *−0.51,* P < *0.01.* B: *Correlation between serum AGEs and endothelium-independent vasodilation;* r = *−0.24,* P < *0.01.*

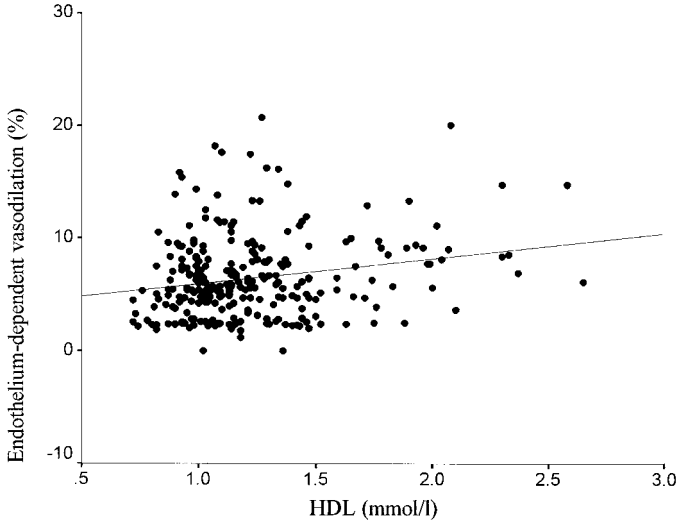

Figure 2—*Correlation between plasma HDL and endothelium-dependent vasodilation;* r = *0.23*, P < *0.01*.

CONCLUSIONS — AGEs are a complex group of compounds, with the structure of only a few AGEs having been identified. These include N^{ϵ}-[carboxymethyl]-lysine (CML), pentosidine, imidazolone, and pyrraline. Although the chemistry of AGEs is not fully understood, increased levels of circulating and tissue AGEs have been demonstrated in both animal and human studies of diabetes. In animal models of diabetes, concentrations of AGEs increase within a few weeks after the animal is rendered diabetic; furthermore, the increase is systemic, occurring in the kidneys, skin, and vascular tissue (13–15). Using the ELISA method, increased concentrations of serum AGEs have been demonstrated in children with type 1 diabetes (16). In adults with diabetes, the concentrations of AGEs, such as CML and pentosidine, measured by HPLC are approximately double (17). The increase in serum AGE concentration is most marked in diabetic subjects with end-stage renal disease (18–20). In addition, some studies have shown a correlation between serum AGEs and diabetic complications (17,20). In the current study, we demonstrated that the increase in serum AGEs correlated with the degree of impairment of endothelium-dependent and, to a lesser degree, endothelium-independent vasodilation in patients with type 2 diabetes.

Our study had several limitations. Because it was cross-sectional in nature, we could demonstrate only associations and not causal relations. In addition, serum levels of AGEs probably reflected only the local AGE burden within the blood vessel wall rather than the number, nature, and sites of AGE deposition within the endothelial and smooth muscle cells and extracellular matrix. Our study also had insufficient power to evaluate the relation of serum AGEs with diabetic complications, and we made no attempt to do so. Using the same anti-AGE antibody, Kilhovd et al. (21) showed that levels of AGEs were significantly increased in patients with type 2 diabetes. Those researchers further reported that levels of AGEs, but not of CML, were significantly higher in patients with type 2 diabetes and coronary heart disease than in patients without diabetes. The structural heterogeneity of AGEs and the lack of a standardized method for quantifying AGEs so far have made it difficult to determine which AGE(s) is relevant to the diabetic complications observed in vitro or in vivo. The polyclonal anti-AGE antibody we used primarily recognizes the nonfluorescent cross-link arginine-lysine imidazole, but may also recognize other epitopes (11,22). Specific imidazolone immunoreactivity has been detected in atherosclerotic lesions in the aorta as well as renal lesions in diabetic subjects (23); other AGEs present within the AGE-ribonuclease mix may also be pathogenic.

We have shown that the serum concentration of AGEs is an important determinant of the degree of impairment of endothelium-dependent vasodilation, independent of other risk factors. The underlying mechanism(s) by which AGEs cause endothelial dysfunction is not fully understood. Deposits of AGEs have been observed in fatty streaks and atherosclerotic lesions by immunohistochemistry (24). Because AGEs are known to quench NO in vitro, the accumulation of AGEs in the extracellular matrix of atheromatous arteries might result in NO depletion, enhanced subintimal protein and lipoprotein deposition (8,25), and increased smooth muscle cell proliferation (26). There also have been recent data suggesting that the vasoactive properties of AGEs may not be mainly attributable to the NO quenching effect of AGEs (27), but that additional mechanisms might be involved. It has been suggested that constitutive NO synthase expression in endothelial cells is suppressed by AGEs, with a reduction in NO synthesis. Chakravarthy et al. (28) reported that there was a reduction in protein and gene expression of endothelial NO synthase when endothelial cells were cultured in the presence of AGEs in vitro and in basal NO production. AGEs also induced the production of the vasoconstrictor endothelin-1 by endothelial cells through nuclear factor-κB activation (29). A much weaker relation was also found between serum AGE concentration and endothelium-independent vasodilation. The accumulation of AGEs has been demonstrated in vascular smooth muscle cells (24); furthermore, similar to endothelial cells, smooth muscle cells also express receptors for AGEs. AGEs have been shown to mediate the chemotactic migration of smooth muscle cells through the induction of transforming growth factor-β, one of the chemotactic factors for smooth muscle cells (30), and also to stimulate mitogen-activated protein kinase activation and smooth muscle cell proliferation (26,31). Whether AGEs can lead to a reduction in the sensitivity of vascular smooth muscle cells to NO merits further investigation.

In conclusion, the serum concentration of AGEs is increased in patients with type 2 diabetes and is associated with endothelial dysfunction independent of other cardiovascular risk factors. Because endothelial dysfunction contributes to the pathogenesis of both micro- and macroangiopathy in diabetes (32), it is important to determine whether treatment targeting AGEs will lead to an amelioration of endothelial dysfunction in patients with type 2 diabetes. Therapeutic agents for the treatment of AGE accumulation are currently being developed (4), and further intervention studies are warranted.

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