Increased Collagen-Linked Fluorescence in Skin of Young Patients With Type I Diabetes Mellitus

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Our objective was to determine whether the fluorescence of skin collagen, which may reflect the accumulation of advanced glycosylation end products, is increased in young patients with type I (insulin-dependent) diabetes. Our study design was a cross-sectional case-control study in a referral-based diabetic clinic in an academic hospital. Study subjects comprised a convenience sample of 18 type I diabetic patients aged 17–30 yr and 8 age-matched healthy control subjects. The fluorescence of collagen was measured in skin biopsy material. Collagen-linked fluorescence (CLF) was increased in diabetic patients (mean 10.5 [range 5.8–15.8] U/mg) compared with control subjects (7.6 [5.6–10.1] U/mg, $P < 0.02$). In diabetic patients, CLF was related to age ($r = 0.581$) and duration of diabetes ($r = 0.697$) but not concentration of glycosylated hemoglobin ($r = 0.082$). Partial correlation analysis demonstrated that duration of diabetes is the main factor determining the fluorescence of collagen in these patients. There was a relationship between CLF and presence of diabetic retinopathy after the data were adjusted for patient age and duration of diabetes ($P = 0.023$). Increased fluorescence of skin collagen can be detected in young type I diabetic patients and is primarily related to duration of diabetes. ***Diabetes Care*** **13:468–72, 1990**

Nonenzymatic glycosylation of proteins in human tissues proceeds beyond the stage of ketoamines (Amadori products), particularly on proteins with a long half-life, e.g., myelin or collagen. Ketoamines are transformed through a complex sequence of rearrangements, dehydrations, and condensations into a group of compounds of unestablished structure that are collectively known as advanced glycosylation end products (AGEs) (1–3). AGEs are brown and fluorescent.

The measurement of collagen-linked fluorescence (CLF) reflects the AGE content of proteins (1). The accumulation of AGE leads to an increase in the degree of protein cross-linking (4), and AGE–cross-linked collagen and myelin can trap nonglycosylated proteins (5,6). AGEs are eliminated by macrophages in a receptor-dependent manner (7,8), and the binding of AGE-modified proteins to their receptors promotes increased secretion of cytokines (9).

The accumulation of AGE on proteins is relevant to both the mechanisms of aging and the development of diabetic complications (1,3). CLF increases with age (10,11) and, to a greater extent, in patients with type I (insulin-dependent) diabetes (11), in whom its increase was associated with the presence of diabetic complications (12). No information about the detection and measurement of CLF in young diabetic patients is available. In this study, CLF was measured in skin biopsy material from patients with type I diabetes, all of whom were <30 yr old.

RESEARCH DESIGN AND METHODS

Eight nondiabetic individuals (6 men, 2 women) with mean age 23 yr (range 19–29 yr) comprised the control group, and 18 patients aged 24 yr (17–30 yr) with type I diabetes (11 men, 7 women) were studied.

All patients were screened for the presence of diabetic retinopathy by direct ophthalmoscopy followed by fluorescein angiography and nephropathy by the measure-

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ment of urinary albumin excretion with the Albuscreen latex agglutination slide test (Cambridge Life Sciences, Cambridge, UK; 13). The results of ophthalmic examinations were missing in one case, and this patient was excluded from the analysis of the relationship between CLF and retinopathy.

In the diabetic group, two patients had pretibial necrobiosis lipoidica diabeticorum, one had coexisting diabetic nephropathy manifested by proteinuria (Albustix positive) and treated proliferative retinopathy, and five had evidence of background retinopathy manifested by microaneurysms, retinal hemorrhages, and/or hard exudates.

Incisional skin biopsies were obtained under local lidocaine anesthesia from clinically normal skin of the buttocks. This site was chosen because it is protected from actinic damage. Friction sites and areas of the buttocks used for injection of insulin were avoided for biopsy. The biopsy specimens were frozen and stored at −20°C until analysis.

Histological and electron-microscopy studies on this group of patients were reported previously (14,15). Collagen concentration was determined as hydroxyproline, assuming 14% hydroxyproline content (16). Hydroxyproline was measured colorimetrically with an HCl digestion technique (17). Glycosylated hemoglobin (HbA_1) was measured on venous blood samples by electroendosmosis (Ciba-Corning, Halstead, UK; 18). All other reagents were obtained from Sigma (Poole, UK) and were of the highest grade available.

On the day of analysis, the skin was thawed and scraped free of subcutaneous fat with a surgical blade, and the residual tissue was washed in 0.15 M saline, dried with filter paper, and weighed.

Skin samples weighing between 10 and 79 mg were used. Homogenization, washing, and lipid extraction procedures were performed according to Monnier et al. (12). A Sorvall RT 6000 centrifuge (Du Pont, Stevenage, UK) was used for the initial centrifugation of skin homogenate (2200 g, 4°C, 15 min). Collagenase digestion was carried out for 24 h at 37°C with shaking by use of 250 U of type VII collagenase (Sigma) per 10 mg of collagen.

This resulted in >95% of the hydroxyproline content of the sample being present in the soluble fraction. After digestion, the samples were centrifuged (2200 g, 15 min), and the supernatant was diluted with 3 ml of water. Fluorescence was measured on supernatant at an emission wavelength of 440 nm and excitation wavelength of 370 nm against a collagenase blank on the LS3B fluorescence spectrometer (Perkin-Elmer, Beaconsfield, UK). Results are arbitrary fluorescence units per milligram of skin collagen.

A blank containing collagenase in buffer was included in each experiment. The operator was unaware of the identity of samples at any time during analysis.

The within-batch imprecision of fluorescence measurement was 10.1 and 11.0% (coefficient of variation) at a fluorescence level of 16.8 and 22.7 U/mg, respectively. The between-batch imprecision at fluorescence levels 15.4 and 12.8 U/mg was 7.8 and 10.2%, respectively.

TABLE 1
Characteristics of control subjects and diabetic patients

	Control	Diabetic
n	8	18
Age (yr)	23 (19–29)	24 (17–30)
HbA_1 (%)		9.8 (6.4–14.5)
Duration of diabetes (yr)		9.9 (0.75–21.00)
Collagen-linked fluorescence (U/mg)	7.6 (5.6–10.1)	10.5 (5.8–15.8)*

Values are means with ranges in parentheses.
*$P < 0.02$.

Statistical analysis was performed with nonparametric methods (19). Means and ranges are given throughout. The relationship between investigated variables was assessed by linear regression analysis (least-squares method), partial correlation analysis, and multivariate analysis of variance (ANOVA).

The study protocol was approved by the Medical Ethics Committee of the Western Infirmary/Gartnavel General Hospital, Glasgow, and written informed consent was obtained from all participants.

RESULTS

The data on patient age, HbA_1 concentrations, and results of the CLF measurement are presented in Table 1. CLF was higher in diabetic patients than in the control group. CLF correlated both with age of diabetic subjects ($r = 0.581$, $P < 0.01$; Fig. 1*A*) and duration of diabetes ($r = 0.697$, $P = 0.0012$; Fig. 1*B*). There was, however, no correlation between CLF and HbA_1 (Fig. 1C). HbA_1 did not correlate with the duration of diabetes ($r = 0.185$, $P = 0.46$). Partial correlation analysis demonstrated that the relationship with duration of diabetes was stronger (partial coefficient of correlation $r = 0.436$) than the correlation with age ($r_p = 0.188$) and confirmed the lack of relationship between CLF and HbA_1 ($r_p = 0.041$). The presence of diabetic retinopathy was related to age of the patients ($P = 0.012$) and duration of diabetes ($P = 0.006$) but not to the concentration of HbA_1 ($P = 0.850$).

The association between the presence of diabetic retinopathy and CLF persisted after adjustment for age and duration of diabetes was performed with multivariate ANOVA using age and diabetes duration as covariates ($P = 0.023$).

DISCUSSION

The AGEs accumulate on proteins with long half-lives by a slow transformation of ketoamine-linked glucose molecules that are formed during the early stages of the glycosylation reaction. AGEs are formed by condensation of at least two mol-

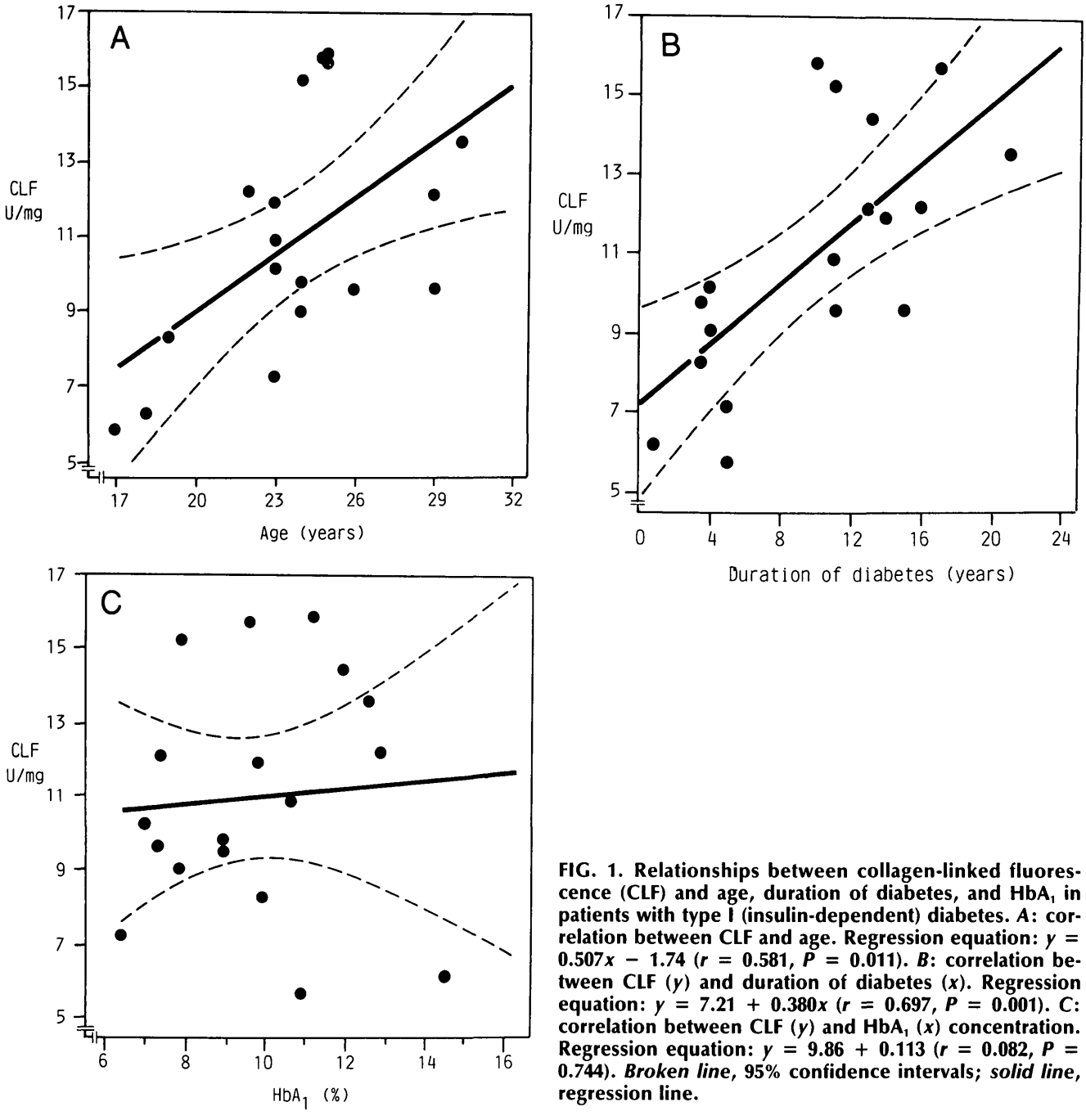

FIG. 1. Relationships between collagen-linked fluorescence (CLF) and age, duration of diabetes, and HbA_1 in patients with type I (insulin-dependent) diabetes. *A*: correlation between CLF and age. Regression equation: $y = 0.507x - 1.74$ ($r = 0.581$, $P = 0.011$). *B*: correlation between CLF (y) and duration of diabetes (x). Regression equation: $y = 7.21 + 0.380x$ ($r = 0.697$, $P = 0.001$). *C*: correlation between CLF (y) and HbA_1 (x) concentration. Regression equation: $y = 9.86 + 0.113$ ($r = 0.082$, $P = 0.744$). *Broken line*, 95% confidence intervals; *solid line*, regression line.

ecules of glycosylated lysine (3), and their accumulation leads to increased cross-linking of proteins (4,20).

The properties of human collagen change during aging and in association with the metabolic abnormalities of diabetes. These changes can be attributed in part to the accumulation of AGE and a consequent increase in the cross-linking of collagen. The solubility of human collagen declines with age (21), and this decline is of greater magnitude in diabetic patients (particularly those with type I diabetes) than in age-matched nondiabetic individuals (21,22). The aging of postmature collagen results in increased resistance to degradation by acetic acid, pepsin (21), and collagenase (10,16,22). The molecular weight of fragments released by pepsin increases with age, which is consistent with a greater degree of collagen cross-linking (21). The reduction in solubility of collagen parallels the accumulation of ketoamine-linked glucose in tendons (21) and in dura mater (11). Kohn et al. (10) have demonstrated an age-related increase in the tendon-breaking time of the rat that was accompanied by the accumulation of ketoamine-linked glucose and fluorescent chromophores with a spectrum consistent with the products of the browning reaction. These fluorescent chromophores also accumulate dur-

ing in vitro glycosylation of collagen (20). In elderly and diabetic humans, collagen adducts have fluorescence spectra identical to that of in vitro glycosylated (browned) collagen (11). Sugar-derived cross-links have been characterized by Tanaka et al. (20). Although determination of the structure of AGE is of considerable research interest (3,23,24), the measurement of protein-linked fluorescence remains the only method of their assessment measurement that has been applied to clinical studies.

The observation that AGE can accumulate on human proteins in vivo has led to a hypothesis by Monnier et al. (1) and Cerami et al. (3) regarding the possible role of this process in the mechanisms of aging and the development of late complications of diabetes. The elegant study by Monnier et al. (12), who investigated patients with long-standing type I diabetes (20–40 yr duration), demonstrated an increase in CLF and a correlation of CLF with severity of diabetic retinopathy, arterial stiffness, and joint stiffness. In a different study, the thickness of the skin related to the collagen content was increased in type I diabetic patients, and a significant correlation was demonstrated with both the duration of diabetes and glycemic control but not diabetic complications (25).

This study confirms that CLF is increased in diabetic skin and extends this observation to a younger age-group of diabetic patients than those investigated by Monnier et al. (12), who were aged 29–52 yr, whereas in our study, the patients were 17–30 yr old. We confirmed that in this group of patients, the correlation between age and CLF persists, similar to that observed in older age-groups (12). Our results also suggest that, in these patients, duration of diabetes rather than age is a main determinant of the increase in CLF. In our patients, CLF was associated with the presence of diabetic retinopathy after adjustment for patient age and duration of diabetes. These findings agree with previously published data by Monnier et al. (12).

Previously reported histological and electron-microscopic changes in the skin observed in this group of patients showed no relationship to either glycemic control or the presence of diabetic complications (14,15). However, prominent collagen deposition was observed in the skin of diabetic patients (15). In our study, no correlation was observed between HbA_1 and CLF. Other investigators have reported a correlation between Amadori products on collagen and HbA_1 but not with either patient age or duration of diabetes (26,27). The amount of Amadori products on collagen was not related to the severity of diabetic complications (27). This is not surprising because the formation of Amadori products is reversible and reaches equilibrium in ~28 days, whereas the formation of AGE is irreversible and proceeds over a prolonged period (3). The formation of AGE may reflect the average degree of glycemia over months to years by contrast to HbA_1, which reflects short-term glycemia; however, Eble et al. (28) demonstrated that AGE formation, once initiated, can proceed in the absence of glucose. The recent discovery of a specific receptor for AGE-modified proteins on macrophages (7,8) may indicate that the quantity of AGE-modified proteins in tissues may also be dependent on the rate of their removal (3).

Our findings show that an increase in CLF can be detected in young patients with type I diabetes. The relationship between the presence of diabetic retinopathy and CLF warrants further studies to determine whether the measurement of CLF in the early stages of the disease may be of value in predicting the development of diabetic complications.

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