Glycation of Skin Collagen in Type I Diabetes Mellitus

Correlation With Long-Term Complications

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SUMMARY

Nonenzymatic glycosylation (glycation) of collagen was measured by boronate affinity chromatography in skin biopsies from 41 type I diabetics with mean duration of diabetes of 25 yr (range 20–40 yr) and from 25 age-matched controls. Mean level of Amadori products was significantly increased in diabetic [7.85 ± 1.78 (SD) nmol/mg collagen] versus control subjects [3.34 ± 1.06 (SD) nmol/mg collagen, $P < .001$] but did not correlate with age, diabetes duration, or severity of retinopathy, nephropathy, arterial stiffness, and joint stiffness. Similarly, mean collagen content per biopsies was 42% increased in diabetic versus control subjects ($P < .001$) but did not correlate with age, diabetes duration, or severity of complications. A weak but positive correlation between glycohemoglobin level and glycation of skin collagen was observed. These results indicate that Amadori products cannot explain by themselves the pathogenesis of diabetic complications unless individual tissue response to glycation is different in subjects with and without complication. They do not exclude a role for the late stages of the Maillard reaction, nonenzymatic browning, in the formation of some of these complications. DIABETES 1986; 35:916–21.

Several observations point to alterations of collagen structure and function in diabetes. Collagen from diabetic humans is more cross-linked, less soluble, and less digestible by bacterial collagenase and cyanogen bromide.[1-3] Collagen content is increased in capillary basement membranes and glomeruli of diabetic humans.[4,5] Increased stiffening of collagen-rich tissue, such as lungs,[6] arteries,[7] and skin and joints,[8] has been noted with type I diabetes. Such changes appear to be associated with severe microangiopathy. Rosenbloom et al. described a syndrome of short stature, limited joint mobility, and waxy skin with increased risk of developing microvascular disease in diabetic children. Scleroderma-like changes were found in 18% of children with insulin-dependent diabetes mellitus.[10] Some of these children had severe restrictive pulmonary disease, and their skin was thickened and its collagen poorly extractable. Increased stiffening of arteries, assessed by measurement of pulse wave velocity (PWV), was noted in diabetic subjects with microangiopathy, whereas those without microangiopathy had normal arterial elasticity.[11]

Several authors,[12-17] including ourselves, have hypothesized that such changes and diabetic complications could result from excessive nonenzymatic glycosylation or from its more stable end products formed during nonenzymatic browning of glycated collagen.[18] Increased glycation of collagen has been found in practically all diabetic tissues studied so far. Overall results indicate a two- to fourfold increase in diabetics versus controls. For practical or ethical reasons, few authors have attempted to correlate the degree of glycation of diseased tissue with the severity of the complication. Uitto et al.[18a] found increased glycation of glomerular basement membrane in four out of five kidneys from diabetic subjects, but no correlation with severity of nephropathy was noted. Using autopsy material, Vogt et al.[19] found a correlation between glycation of aortic tissue and an arbitrary index for the severity of late complications of diabetes. However, diabetes duration and age of the subjects were quite heterogenous. The study has not been confirmed yet. In 3 diabetic children with scleroderma-like changes, Buckingham et al.[10] noted a marked increase in glycation of skin collagen. Because of the small number of subjects studied and the lack of "no-complication" controls, however, no conclusions could be drawn as to the role of glycation of collagen in the limitation of joint mobility. A more systematic study of this question was published recently.[20] Ketoamine-linked glucose was measured in skin biopsies from 36 type I diabetics of which 12 had limited joint mobility. Although glycation was increased in most diabetic subjects, its extent was identical in subjects

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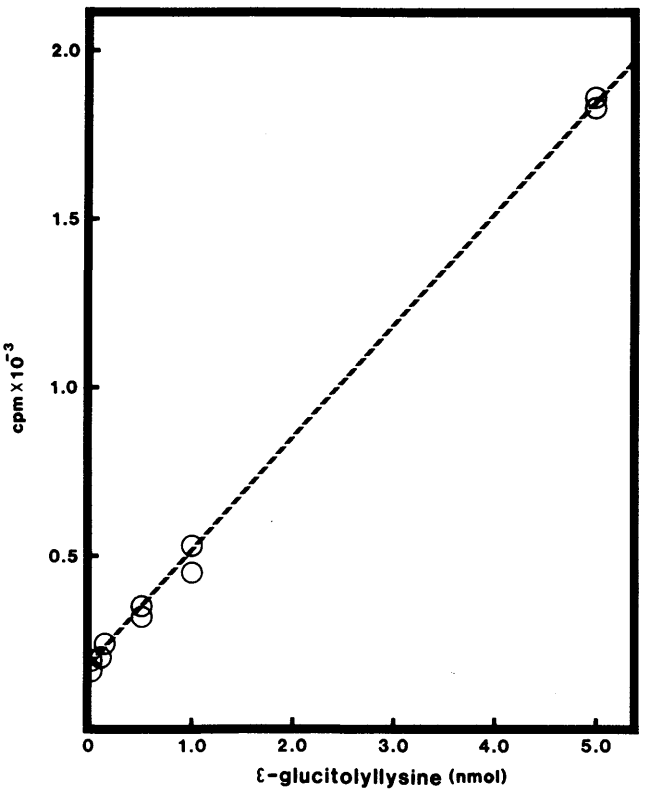

FIG. 1. Calibration line for assay for Amadori products with m-phenylamino boronic acid sepharose minicolumns. [^{3}H]borohydride-reduced and acid-hydrolyzed-α-formyl-ε-deoxyfructoselysine (ε-glucitolyllysine) were used as standard.

with and without limited joint mobility, thus raising doubts concerning its role in the pathogenesis of this complication.

In our study, we investigated the glycation of skin collagen from type I diabetic subjects and sought a correlation not only with joint mobility, but also with the severity of retinopathy, nephropathy, and arterial stiffness.

MATERIALS AND METHODS

Subjects. Twenty-two female and 19 male type I diabetic subjects were enrolled in the study through an advertisement in the major local newspaper. Age of the subjects was 29–52 yr with a mean age of 36.4 yr. Diabetes duration was from 20 to 40 yr with a mean of 25.1 yr. Mean age of onset was 11.2 yr with a range of 4–20 yr. Presence of type I diabetes was based on the criteria of the National Diabetes Data Group,[21] which included history, family history, information from private physicians, insulin requirement (mean 45 U/day, range 20–80 U/day), and absence of obesity. Twenty-five nondiabetic control subjects, 11 women and 14 men between the ages of 28 and 41 yr, were recruited from hospital and university staff.

Retinopathy was assessed by the ophthalmologist (K.F.) by use of funduscopy with dilated pupils and graded 0 (no fundus abnormality), 1 (background retinopathy), or 2 (proliferative retinopathy). The only patient with maculopathy was included in the grade 1 group. Nephropathy was graded 0 (<0.5 g urinary protein/24 h), 1 (<1.0 g/24 h), or 2 (>1.0 g/24 h, with or without decreased creatinine clearance). None of the patients, however, were uremic. Arterial stiffness was assessed by the aortic PWV index, measured by the method of Pillsbury et al.[7] Piezoelectric transducers (Electronics for Medicine PSA 23) were placed over the right carotid and femoral arteries with the subject in the supine position. Lead II of the ECG was recorded. The subject was allowed to rest for 10 min before recording. Ten cycles were recorded with a Gould strip chart recorder at a speed of 125 mm/s, and mean PWV was calculated by dividing the trunk length by the time difference between the carotid and femoral pulse. Grade 0 stiffness was present if PWV was within 2 SD of nondiabetic control subjects, grade 1 if PWV was between 2 and 4 SD of control subjects, and grade 2 if PWV was >4 SD.

Joint mobility was assessed essentially with the criteria of Rosenbloom et al.[9] Because, however, some control subjects in their 30s and 40s had already some degree of limited joint mobility of the fingers, we used a computerized method to assess finger joint mobility. With the subject in the sitting position, palmar surfaces of both hands were covered with ink and pressed onto a sheet of paper with a plastic bag filled with 1 kg of sand. The areas of the inked handprints were measured by computerized planimetry and divided by the total surface area of the hands determined from a photocopy copy of the hands. Age-adjusted joint mobility of the fingers was normal if covered ratio fell within 2 SD of control subjects (0.48–0.78) and decreased if the ratio was <0.48. Overall joint stiffness was graded 0 if joint mobility was similar to that of control subjects for all joints, 1 if joint mobility was limited only to fingers or one other large joint, and 2 if not only finger joints but several other joints had limited mobility.

Skin biopsy technique. Informed consent was obtained from all participants. Skin-punch biopsies of 4-mm diameter were obtained under local anesthesia (1% xylocaine with

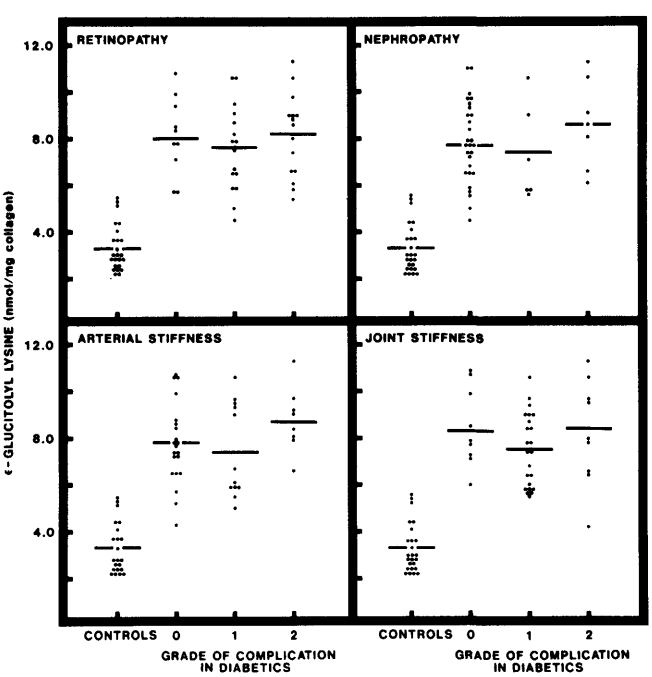

FIG. 2. Collagen-linked Amadori products as function of severity of diabetic complication. *P* values are given in Table 1.

TABLE 1
Level of collagen-linked Amadori products and collagen content per biopsy in correlation with type and severity of diabetic complication

Subjects	*N*	Mean age (yr)	Mean duration of diabetes (yr)	ε-Glucitolyllysine (nmol/mg collagen)	Collagen per biopsy (mg)
Controls	25	35.0 ± 3.8	0	3.34 ± 1.06	1.75 ± 0.43
Diabetics	41	36.4 ± 5.1	25.1 ± 5.1	7.85 ± 1.78*	2.50 ± 0.69†
Retinopathy					
0	11	35.9 ± 4.9	24.5 ± 6.3	7.95 ± 1.66	2.64 ± 1.02
1	16	36.4 ± 4.8	29.6 ± 3.6	7.52 ± 1.91	2.58 ± 0.60
2	14	36.8 ± 5.9	26.0 ± 5.2	8.17 ± 1.76	2.43 ± 0.48
Nephropathy					
0	29	36.3 ± 5.4	25.3 ± 5.3	7.78 ± 1.70	2.53 ± 0.73
1	5	35.0 ± 4.9	22.6 ± 1.7	7.36 ± 2.01	2.33 ± 0.69
2	7	38.0 ± 3.9	26.4 ± 3.7	8.50 ± 1.93	2.49 ± 0.60
Arterial stiffness					
0	21	34.8 ± 5.0	23.9 ± 4.7	7.70 ± 1.72	2.38 ± 0.81
1	12	36.7 ± 7.5	24.5 ± 3.5	7.43 ± 2.01	2.66 ± 0.60
2	8	40.2 ± 7.6	29.0 ± 5.6	8.75 ± 1.40	2.53 ± 0.45
Joint stiffness					
0	9	36.1 ± 5.0	24.1 ± 4.5	8.31 ± 1.89	2.46 ± 1.20
1	23	34.8 ± 5.3	24.2 ± 4.8	7.53 ± 1.53	2.55 ± 0.48
2	9	37.4 ± 6.2	28.2 ± 5.1	8.23 ± 2.26	2.38 ± 0.57

Values are given in mean ± SD.
*$P < .0001$; †$P < .001$, for comparison of Amadori products and collagen content between diabetic and control subjects, respectively. *P* values are not significant (>.05) for comparisons between various grades of severity of complication.

0.001% epinephrine) from the upper medial quadrant of the left buttock, about 15 cm below the iliac crest. Care was taken to avoid areas of insulin injection. The specimen was soaked at room temperature for 60 min in 0.9% saline and rinsed three times with 5 ml of saline. Biopsies were stored at −70°C under nitrogen until the end of the study. Throughout the study, specimens were analyzed with identical batches of buffers and chemicals.

Preparation of collagen digest. The skin sample was frozen in liquid nitrogen to harden the tissue and allow removal of epidermis and fat with a sharp razor blade. The rest was minced into pieces, suspended in 10 ml of deaerated cold phosphate-buffered saline (pH 7.4), and homogenized on ice for 60 s with a Polytron homogenizer (Brinkman, Zurich, Switzerland). The suspension was transferred into 20-cm × 10-mm borosilicate tubes with Teflon-lined caps. All subsequent steps were performed in these tubes. Centrifugation was done at 650 × *g* for 30 min at 4°C (IEC Model PR-2 centrifuge). The supernatant was removed and the collagen-rich pellet was washed three times with deaerated deionized water that had been further purified with a Millipore MF Cartridge system (Millipore, Bedford, MA).

Lipids were extracted from the pellet with 5.0 ml of $CHCl_3$:MeOH (2:1) by mild shaking overnight. Two milliliters of methanol and 0.5 ml of water were then added to rehydrate the insoluble collagen and allow it to settle at the bottom of the tube. The sample was centrifuged as described above and the solvent was removed by suction. The pellet was washed two times with 5 ml of methanol, three times with water, two times with 0.02 M Hepes buffer (pH 7.5) containing 0.1 M $CaCl_2$ (buffer H), and stored overnight at 4°C in buffer H. The buffer was then removed and the pellet resuspended in 1.0 ml of buffer H containing 280 U of type VII collagenase (Cat. no. 0773, Sigma, St. Louis, MO). Two microliters of chloroform and 2 ml of toluene were each added to prevent bacterial growth. Digestion was carried out for 24 h at 37°C with shaking. A blank containing collagenase in buffer H was included. The digest was transferred into 1.4-ml microtubes and centrifuged with a microcentrifuge (Beckman, Palo Alto, CA) for 3 min. The remaining pellet was found to contain, on the average, <2% of total collagen. The clear supernatant containing digested collagen was used for assays of Amadori products and hydroxyproline content as described below.

Assay of Amadori products. Amadori products were assayed with borohydride reduction and affinity chromatography, essentially according to Brownlee et al.,[14] with the Glycogel Test Kit from Pierce (Rockford, IL). The buffers were those of the manufacturer. An amount equivalent to 200 μg of collagen (28 μg of hydroxyproline) was pipetted into 10 × 1-cm test tubes and the volume was brought to 0.5 ml with buffer H. Preliminary experiments revealed that Tris buffer could not be used for borohydride reduction because it is covalently incorporated into Amadori products. Standards containing 0, 0.1, 0.5, 1.0, and 5.0 nmol of α-formyl-ε-fructoselysine in buffer H containing collagenase were included. Borohydride reduction was performed with ~200 M excess borohydride over the total number of amino groups per mole of collagen α-chain. To each tube was added 50 μl of 0.001-N NaOH containing 238 μCi of [^{3}H]$NaBH_4$ (ICN lot 2038122, Covina, CA; specific activity 50 mCi/mmol). Reduction was carried out for 10 min at room temperature, 50 min at 4°C, and interrupted with 50 μl of 6 N HCl after addition of 50 μl *n*-pentanol to prevent frothing. One milliliter of distilled water was added and the aqueous solution was evaporated with a Speed Vac concentrator (Savant, Hicksville, NY) equipped with a −70°C trap. This procedure was repeated four times. Because of the large amounts of radioactivity escaping in the form of tritium gas or tritiated water, all steps described above were performed under the hood. The dry residue was acid hydrolyzed with 6 N HCl for 12 h at 110°C, and HCl was evaporated. Exhaustive tritium exchange was performed by repeating five times the evaporation sequence described above. Failure to perform at least four evaporations led to high background levels of radioactivity and poor re-

producibility. The residue was dissolved in 0.5 ml of 0.25-M ammonium acetate (Pierce buffer) and loaded onto a Glycogel minicolumn (Pierce). The column was washed with 20 ml of buffer, and Amadori products were eluted with 5 ml of 0.2-M sorbitol (buffer). Three milliliters were mixed with 17 ml of Hydrofluor (National Diagnostics, Sommerville, NJ), and tritium activity was counted with a Beckman LS liquid scintillation counter. The calibration line obtained with borohydride-reduced and acid-hydrolyzed samples of α-formyl-ε-fructoselysine is shown in Fig. 1. Nonspecific incorporation of tritium into normal and diabetic collagen, as estimated by the radioactivity not retained by the column, was on the average 93.7 (range 91.7–94.6%) and 88.6% (range 84.9–91.8%), respectively. To estimate and correct for background activity due to nonspecific binding of tritiated non-Amadori products onto the affinity support, the calibration line was established in presence of collagenase as described above. Although mean background activities were as high as 54 and 25% of total counts incorporated into normal and diabetic samples, respectively, it was constant throughout the preliminary experiments (CV < 10%, $N = 6$). Interassay coefficient of variation (CV) for diabetic skin collagen obtained at autopsy from a single donor was 14.8% ($N = 9$).

Collagen content assay. Collagen content was assayed as described earlier.[18] Fifty milliliters of digest was acid hydrolyzed in 1.0 ml of 6-N HCl for 24 h at 110°C. Hydrochloric acid was evaporated and 4-hydroxyproline was assayed in the dry residue. Quantitative data are expressed per milligram of collagen, assuming a 14% content of hydroxyproline.[1]

Glycohemoglobin assay. Glycohemoglobin (GHb) was measured in the Laboratory of Clinical Chemistry by boronate affinity chromatography with the Glycogel minicolumn system (Pierce). Blood specimens were stored at 4°C until assayed. Intra- and interassay CVs were 3.2 and 4.1%, respectively. Reference values were 5.2–7.8%. Glycohemoglobin values were found to correspond to HbA_1 values (Isolab method) according to the formula $HbA_1 = 0.7\ GHb + 1.3$.

Statistical analysis. All assays were performed without knowledge of the clinical status of individual subjects. Linear equations, regression coefficients, confidence limits, plot diagrams, and *P* values from the two-tailed Student's *t* test were calculated with the Stats Plus Program (Human Systems Dynamics, Northrich, CA) and an Apple IIe computer. The data are presented as mean ± SD.

RESULTS

To allow meaningful comparison of the degree of glycation of skin collagen with the severity of late complications of diabetes, only diabetic subjects with at least 20 yr of diabetes duration and, with three exceptions, only subjects between the ages of 29 and 45 yr were included. Because previous reports have described an age-related increase in glycation of collagen,[2] we first investigated the effects of age and diabetes duration on the level of skin glycation. No correlation was found between extent of glycation and age of the subject in the diabetic ($r = .10$, $P > .05$) or nondiabetic group ($r = .18$, $P > .05$). Similarly, there was no correlation between the duration of diabetes and the amount of glycated collagen (not shown). Thus, adjustment for age and duration of diabetes was not required before investigating the correlation between severity of diabetic complication and the level of glycated collagen. These latter data are shown in Fig. 2 and Table 1. Mean level (±SD) of glycation in equivalent of ε-glucitolyllysine was 3.34 ± 1.06 and 7.85 ± 1.78 nmol/mg collagen in control and diabetic subjects, respectively. The increase in glycation in diabetic subjects was highly significant ($P < .001$). However, mean level of collagen-linked Amadori products was not significantly different ($P > .05$) between grades 0, 1, and 2 retinopathy, nephropathy, arterial stiffness, and joint stiffness. It should be noted that mean age of subjects and mean diabetes duration among the subgroups did not differ significantly, with the exception of the "arterial stiffness" group in which mean age of subjects with grade-2 stiffness was significantly higher than mean age of subjects with normal stiffness (grade 0).

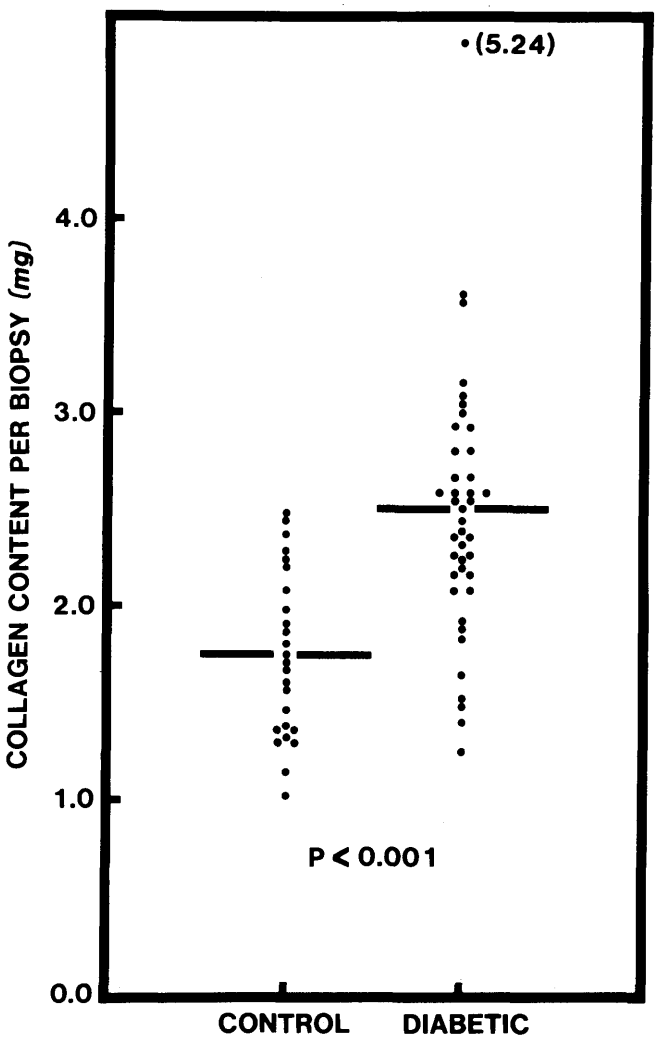

FIG. 3. Collagen content per biopsy in control and diabetic subjects. Mean levels are significantly different ($P < .001$) but do not correlate with severity of complication (not shown).

Because of a possible effect of glycation on collagen turnover, we also measured total collagen content per biopsy. In diabetic subjects, mean collagen content per biopsy was increased 42% over control values (Fig. 3, Table 1), but it did not correlate with the severity of diabetic complications. In addition, collagen content did not correlate with age

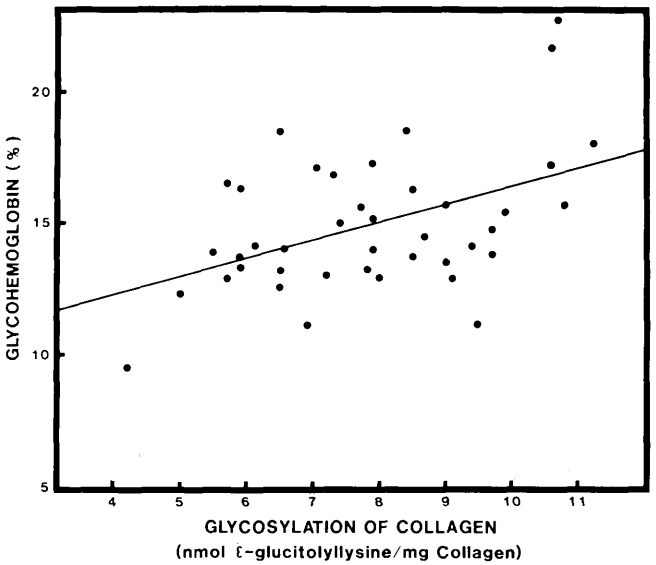

FIG. 4. Glycohemoglobin (GHb) level vs. collagen-linked Amadori products in diabetic subjects. y = 0.69x + 9.55 (r = .47, P < .01).

(r = .12, P = .45) and duration of diabetes (r = .23, P = .71). Similarly, no correlation was observed between extent of collagen glycation and collagen content (r = .14, P = .36).

Finally, a weak but significant correlation between glycation of skin collagen and GHb level at the time of biopsy was found (Fig. 4). This confirms a similar observation by Lyons and Kennedy.[20]

DISCUSSION

Our data indicate that although glycation of collagen and collagen content per biopsy are significantly increased in skin of subjects with type I diabetes, neither of these parameters correlate with the severity of retinopathy, nephropathy, arterial stiffness, or joint stiffness. These results confirm a similar study by Lyons and Kennedy,[20] who found no correlation between the extent of skin-collagen-linked Amadori products and the degree of limited joint mobility in type I diabetes. If it is assumed that organs and tissues particularly affected by diabetes, such as retina, kidney, nerves, arteries, and joints, are exposed to similar glucose concentrations as the skin, it can be concluded that glycation, i.e., the amount of collagen-linked Amadori products, does not correlate with the presence or absence of a given complication. Thus, a pathogenetic role for glycation in increasing arterial or joint stiffness and causing retinopathy and nephropathy appears to be unlikely. Another conclusion that is warranted, provided the turnover of Amadori products is identical in all diabetic subjects, is that diabetes control is equally poor in subjects with and without complications.

Caution should be exerted when considering these statements. First, it is possible that excess glycation could affect collagen fibril formation but that tissue response to impaired assembly of collagen would differ among sites in the body and among diabetic subjects. Evidence for disturbed fibril formation in the presence of high glucose concentrations in vitro has been reported.[22-24] Although Lien et al.[24] did not attribute their findings to glycation, a role for the latter cannot be excluded because they used, even at the lowest glucose concentration, a 1000-M excess of glucose over collagen. Such an excess could result in the formation of labile Schiff bases, the amount of which is strongly dependent on the ambient glucose concentration.[25] An example of selective tissue response to metabolic insult can be found in diabetic patients with scleredema, which is characterized by a diffuse thickening of the skin that is limited to the dorsum.[26] Thus, although mean level of Amadori products are identical in subjects with and without complications, glycation could have deleterious effects depending on the genetic predisposition of a particular individual. Second, there is firm evidence that glycation, i.e., the formation of Amadori products, represents only an intermediate stage of the Maillard or nonenzymatic browning reaction.[27] Therefore, the amount of collagen-linked Amadori products represents a steady state, the mean of which is identical in all categories and degrees of complications. Factors that could influence the steady-state level include collagen turnover, rate of dissociation, and browning of Amadori products. It is not known whether the increased amount of collagen noted in many diabetic subjects results from increased synthesis or decreased degradation. The absence of a linear correlation between glycation and collagen contents suggests that Amadori products are not involved in decreasing collagen turnover. However, it is conceivable that the late steps of the Maillard reaction could reduce collagenase digestibility by extensive cross-linking.

Because the turnover of Amadori products in skin is not known, it is not possible to evaluate the period over which metabolic status is represented by collagen-linked Amadori products. However, the positive correlation between glycation of collagen and hemoglobin points to the presence of Amadori products that have been synthesized in the recent past, perhaps less than 6 mo ago. Little is known about the factors that affect the browning rate of Amadori products in vivo. Recent observations suggest that the presence of oxygen may prevent nonenzymatic browning of Amadori products by forming ε-carboxymethyllysine residues[28] and potentially explain why those diabetic subjects who have no complications have less browning of collagen than those subjects with severe complications.[29]

In summary, it is likely that Amadori products have, by themselves, few pathogenetic effects in the long-term complications of diabetes. In conjunction with a genetic predisposition factor, as yet undefined, Amadori products may, however, have a significant effect on tissue structure and function. Thus, if Amadori products play a role in the initial lesion leading to diabetic complications, why some diabetic subjects are resistant to the deleterious effects of blocked amino groups in biological molecules will need to be explained. Whether or not the early stages of the Maillard reaction play a role in the pathogenesis of diabetic complications, it would appear reasonable to postulate that excessive protein cross-linking and covalent trapping of molecules[30] during the late stages of the reaction would drive tissue lesions beyond reversibility.

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