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Formation of Advanced Glycosylation End Products and Oxidative Stress in Young Patients with Type 1 Diabetes

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ABSTRACT

Increased production of advanced glycosylation end products (AGEs) and augmented oxidative stress may contribute to vascular complications in diabetes. Little is known about the formation and accumulation of AGEs in young patients with type 1 diabetes. The aim of the present study was to investigate whether AGE production and oxidative stress are augmented in young patients with type 1 diabetes at early clinical stages of the disease. Urine samples of 38 patients with type 1 diabetes [mean age (±SD), 12.8 ± 4.5 y; diabetes duration, 5.7 ± 4.3 y; HbA_{1c}, 8.0 ± 1.6%; urinary albumin excretion, 12.6 ± 14.4 mg/g creatinine (Cr)] and those of 60 age-matched healthy control subjects were assayed for AGEs, pentosidine and pyrraline, and markers of oxidative stress, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and acrolein-lysine. Of these four markers, urinary concentrations of pentosidine, 8-OHdG, and acrolein-lysine were significantly higher in the patients with diabetes than in the healthy control subjects. For the patient group, pentosidine correlated significantly with 8-OHdG and acrolein-lysine, and pyrraline correlated significantly with acrolein-lysine. Urinary pentosidine, 8-OHdG, and acrolein-lysine but not pyrraline correlated significantly with urinary albumin excretion. Patients with microalbuminuria (≥15 mg/g Cr) showed significantly higher levels of all four markers than did normoalbuminuric patients and control subjects. The present study indicates that accumulation of AGEs, whose formation is closely linked to oxidative stress, and resultant endothelial dysfunction may start early in the course of type 1 diabetes. This means that the risk of vascular complications may be present at an early age and that the best possible glycemic control should be emphasized from the diagnosis of diabetes. **(*Pediatr Res* 54: 419–424, 2003)**

Abbreviations

AGE, advanced glycosylation end product
RAGE, advanced glycosylation end product-specific receptor
8-OHdG, 8-hydroxy-2'-deoxyguanosine
Cr, creatinine
ROI, reactive oxygen intermediate

As first described by Maillard, glucose and reducing sugars react nonenzymatically with protein amino groups to form reversible Schiff base adducts, which, upon molecular rearrangement, convert *in vivo* into the more stable Amadori products such as HbA_{1c} (1, 2). A small proportion of Amadori products are then irreversibly transformed, over several weeks to months, into advanced glycosylation end products (AGEs). The latter process is slow and affects mainly proteins with a slow turnover, such as matrix tissue proteins.

Generation of AGEs was first described as an epiphenomenon of aging. AGE contents slowly increase with age in a variety of collagenous structures, such as vascular wall collagen and basement membranes. Subsequent studies showed accumulation of AGEs during the progression of diabetes (1–3), whereas others identified AGEs in the blood and tissues of patients with chronic renal failure (4, 5). In the latter group, AGE generation was not linked to the glycemic status. AGE formation is accompanied by a cross-linking of proteins caus-

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ing alterations of structural and functional properties of macromolecules. Furthermore, AGEs have been shown to display diverse biologic activities, such as increase in endothelial permeability; binding to AGE-specific receptors (RAGEs) on macrophages, endothelial cells, vascular smooth muscle cells, and glomerular mesangial cells; activation of these cells with secretion of cytokines and growth factors, which in turn accelerate chronic inflammation; quenching of the vasodilator and antiproliferative effects of nitric oxide; enhancing oxidative stress; and oxidation of LDL (1–3, 5).

It has been hypothesized that AGEs play a causal role in the development of a variety of diabetic complications (1–3, 5). AGEs accumulate in plasma and tissue proteins of patients with diabetes(6–8). Their accumulation correlates with the severity of diabetic complications. Miyata and colleagues (3, 9) recently demonstrated that carboxymethyl-lysine and pentosidine, which are AGE compounds, accumulate in expanded mesangial matrix and nodular lesions in diabetic nephropathy, in co-localization with lipid peroxidation end products such as malondialdehyde-lysine, 4-hydroxynonenal-protein adduct, and acrolein-protein adduct. However, pyrraline, another AGE structure whose deposition is independent of oxidative stress, was not found within diabetic glomeruli. Animal studies suggest that inhibitors of advanced glycosylation such as aminoguanidine and OPB-9195 prevent diabetic complications and thus support the above hypothesis (10, 11).

Because measurement of AGEs in tissue specimens is not useful clinically, analyses of blood and urine samples have been pursued. Recently, we developed a rapid and accurate method for the determination of urinary and serum pentosidine (12) and urinary pyrraline (13) by HPLC with fluorometric detection. Using this method, we found that urinary and serum concentrations of pentosidine in control subjects (aged 22 to 70 y) gradually increased with age, whereas urinary pyrraline did not increase with aging (from 20 to 77 y), and that elder patients with cerebral infarction showed higher levels of pentosidine compared with their control subjects. In contrast to numerous investigations in adults, little is known about the formation and accumulation of AGEs in young patients. Furthermore, urinary levels of AGEs have not been previously examined in young patients with type 1 diabetes and even in healthy children and adolescents.

The objective of the present work was to study markers of reactive AGE formation, urinary pentosidine and pyrraline, as well as markers of oxidative stress, 8-hydroxy-2'-deoxyguanosine (8-OHdG) (14) and acrolein-lysine (15, 16), in a group of young patients with type 1 diabetes with normal renal function and to compare the findings with those of the control group of similar age. In the present study, we provide evidence for the first time of both increased AGE formation and augmented oxidative stress in young patients with type 1 diabetes.

METHODS

Patients and control subjects. We examined 38 young Japanese patients with type 1 diabetes (17 male and 21 female). The mean age (±SD) was 12.8 ± 4.5 y (range, 3.8–21.3 y), diabetes duration was 5.7 ± 4.3 y (0.3–16.8 y), HbA_{1c} was 8.0 ± 1.6% (4.4–12.6%), and body mass index (kg/m^2) was 18.9 ± 2.3 (14.8–25.6). All patients were on a diabetes diet and were treated with insulin (0.98 ± 0.40 IU · kg^{-1} · d^{-1}; range, 0.23–2.05 IU · kg^{-1} · d^{-1}). They were not taking other medications. All patients were normotensive and had normal (defined as <1.2 mg/dL) serum creatinine (Cr) concentrations (0.53 ± 0.19 mg/dL; range, 0.20–1.09 mg/dL). Their urinary albumin excretion was 12.6 ± 14.4 mg/g Cr (1.4–76.2 mg/g Cr), and 11 had microalbuminuria (defined as ≥15 mg/g Cr). None had any clinical evidence of retinopathy or neuropathy. We also recruited 60 healthy Japanese subjects (28 male and 32 female) as the control group, with a broad age range (11.4 ± 4.5 y; range, 4.2–21.0 y) to encompass the ages of the patients with diabetes. None of the control subjects was taking any medication.

All subjects were nonsmokers. None of them had any acute illness or chronic condition at the time of study. The nature and purpose of the study were explained to the subjects and/or their parents, and informed consent was obtained from them before enrollment. Approval of the project was obtained from the local medical ethics committee.

Sample collection. Early-morning void urine samples were obtained from each subject. The samples were centrifuged, and the supernatants were stored at −20°C until analysis. All analyses were performed in duplicate, and the examiner was blinded to the clinical and laboratory results.

Determination of urinary pentosidine. The washing solution was a mixture of *n*-butanol-acetic acid:hydrochloric acid (8:1:1, vol/vol). CF-1 slurry was prepared by making a 5% (wt/vol) suspension of CF-1 cellulose powder in the washing solvent. The pretreatment column was prepared by adding 8 mL of CF-1 slurry to a Poly-Prep chromatography column (0.8 mm × 40 mm, internal diameter (i.d.); Bio-Rad, Hercules, CA, U.S.A.). A total of 250 μl of urine was hydrolyzed with an equal volume of concentrated hydrochloric acid at 108°C for 18 h. The cooled hydrolysate (250 μL) was mixed with 250 μL of CF-1 slurry, 250 μL of acetic acid, and 2 mL of *n*-butanol, then loaded to the pretreatment column. After the column was washed with 35 mL of the washing solvent, pentosidine was eluted from the column with 9 mL of 50 mM of hydrochloric acid and blown up to dryness under N_2 gas flow. The dry residue was then dissolved in 250 μL of 1% *n*-heptafluorobutyric acid (vol/vol), and an aliquot (10 μL) of each sample was applied to analytical HPLC. The HPLC eluent was 7% acetonitrile (vol/vol) containing 0.1% *n*-heptafluorobutyric acid. The HPLC system was equipped with an L-6200 intelligent pump (Hitachi, Ibaragi, Japan), an F-1050 fluorescence detector set at excitation and emission wavelengths of 335 nm and 385 nm, respectively (Hitachi), and Symmetry RP18 column (3.5 μm, 4.6 mm × 150 mm, i.d.; Waters, Milford, MA, U.S.A.). The flow rate was maintained at 0.8 mL/min, and the column was kept at 30°C. Standard pentosidine was synthesized and purified, as described in detail by our laboratories previously (12). Values obtained for healthy adults (n = 64) are 28.2 ± 12.0 pmol/mg Cr.

Determination of urinary pyrraline. The solid-phase extraction cartridge, Oasis HLB (3 mL; Waters), was used for pretreatment of urine samples. The cartridge was precondi-

tioned with 1 mL of methanol and equilibrated with 1 mL of distilled water before loading the sample. A total of 500 μl of urine sample was applied to the cartridge, followed by washing the cartridge with 1 mL of 0.1% acetic acid (vol/vol). Pyrraline was eluted from the cartridge with 3 mL of 60% acetonitrile, and the eluent was blown up to dryness under N_2 gas flow. The dried residue was then dissolved in 500 μL of 0.1% trifluoroacetic acid (vol/vol), and an aliquot (20 μL) of each sample was applied to the analytical HPLC system. The HPLC eluent was 7% acetonitrile (vol/vol) containing 0.1% trifluoroacetic acid (vol/vol). The HPLC system was equipped with an L-7100 intelligent pump, an L-7400 UV detector set at 298 nm, and Capcellpak UG120 column (3 μm, 4.6 mm × 150 mm, i.d.; Shiseido, Tokyo, Japan). The flow rate was maintained at 0.8 mL/min, and the column was kept at 35°C. Standard pyrraline was synthesized and purified (13). Values obtained for healthy adults ($n = 27$) are 25.3 ± 30.0 nmol/mg Cr.

Determination of urinary markers of oxidative stress. Currently, 8-OHdG is one of the most popular markers for oxidative DNA damage and oxidative stress *in vivo* (14). Acrolein ($CH_2 = CH - CHO$) is one of the major lipid peroxidation products with cytotoxic and mutagenic activities (15, 16). Among α,β-unsaturated aldehydes, acrolein is by far the strongest electrophile and reacts with nucleophiles, such as the sulfhydryl group of cysteine, imidazole group of histidine, and amino group of lysine. Acrolein-lysine adduct can be used as a reliable index of lipid peroxidation *in vivo*.

The concentrations of 8-OHdG and acrolein-lysine were determined using competitive ELISA kits (8-OHdG Check, Institute for the Control of Aging, Shizuoka, Japan (17); ACR-Lysine Adduct ELISA, NOF Corporation, Tokyo (18), respectively). The assay variances of all methods described above were <10%.

Measurement of urinary Cr. All urinary markers were expressed relative to urinary Cr concentration, which was measured enzymatically by using the Creatinine HR-II Test kit (Wako Pure Chemicals, Osaka, Japan).

Statistical analysis. Data are presented as mean ± SD and range. Comparisons between groups were analyzed by two-tailed *t* test. Correlations between variables were assessed by linear regression. $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the concentrations of pentosidine, pyrraline, 8-OHdG, and acrolein-lysine adduct in urinary samples of 60 control subjects and 38 patients with type 1 diabetes. Of these four markers, urinary levels of pentosidine, 8-OHdG, and acrolein-lysine were significantly higher in patients with type 1 diabetes than in control subjects. For the diabetic group, urinary pentosidine levels correlated significantly with those of urinary pyrraline. Furthermore, pentosidine levels correlated significantly with those of 8-OHdG and acrolein-lysine, and pyrraline levels correlated significantly with those of acrolein-lysine (Fig. 1). Of these four markers, urinary levels of pentosidine ($r = 0.32$, $p < 0.05$), 8-OHdG ($r = 0.57$, $p < 0.001$), and acrolein-lysine ($r = 0.38$, $p < 0.05$) but not pyrraline ($r = 0.11$) correlated significantly with urinary albumin creatinine ratio. Only urinary levels of 8-OHdG significantly correlated with HbA_{1c} values ($r = 0.41$, $p < 0.05$). Urinary albumin creatinine ratio correlated with HbA_{1c} ($r = 0.44$, $p < 0.01$).

When the patients with diabetes were divided into two subgroups according to the presence of microalbuminuria (≥15 mg/g Cr), significantly higher urinary concentrations of pentosidine, pyrraline, 8-OHdG, and acrolein-lysine were noted in patients with microalbuminuria ($n = 11$) than in normoalbuminuric patients ($n = 27$) and the control subjects (Table 1). Microalbuminuric patients also had significantly high HbA_{1c} values compared with normoalbuminuric patients (8.9 ± 1.7% *versus* 7.6 ± 1.5%; $p < 0.05$). There was no significant difference in age and duration of diabetes between these two subgroups of patients (11.8 ± 6.1 *versus* 13.3 ± 3.6 y and 5.0 ± 3.7 *versus* 6.0 ± 4.6 y, respectively).

DISCUSSION

With the rapid development of therapy for diabetes, the mortality rate associated with acute complications has decreased but that associated with chronic complications of diabetes has increased (3). Diabetic nephropathy is one of the major chronic complications of both type 1 and type 2 diabetes and also an important cause of increased morbidity and mortality among these patients. Therefore, elucidation of the molecular pathogenesis of diabetic nephropathy is critical for the

Table 1. *Urinary markers of advanced glycosylation and oxidative stress in control subjects and patients with type 1 diabetes*

			Diabetic group	
	Control group ($n = 60$)	Diabetic group ($n = 38$)	Normoalbuminuria ($n = 27$)	Microalbuminuria ($n = 11$)
Pentosidine (pmol/mg Cr)	23.0 ± 7.5 (8.0–53.1)	32.8 ± 13.8*** (14.7–78.0)	29.4 ± 9.2*** (14.7–52.8)	41.0 ± 19.4***† (15.3–78.0)
Pyrraline (nmol/mg Cr)	32.9 ± 27.1 (7.7–152.0)	39.4 ± 33.3 (3.3–146.0)	24.4 ± 17.7 (3.3–73.0)	59.0 ± 48.3*††† (5.3–146.0)
8-OHdG (ng/mg Cr)	11.9 ± 5.2 (5.0–27.2)	15.4 ± 6.7** (4.7–32.1)	13.5 ± 4.9 (4.7–23.4)	19.9 ± 8.4***†† (8.6–32.1)
Acrolein-lysine adduct (nmol/mg Cr)	152.4 ± 60.6 (61.8–319.0)	201.8 ± 95.1** (79.4–509.3)	167.4 ± 56.6 (79.4–285.8)	286.3 ± 118.8***†††† (107.4–509.3)

Data are presented as mean ± SD and range. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ *vs* control; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.005$, †††† $P < 0.001$ *vs* Normoalbuminuria.

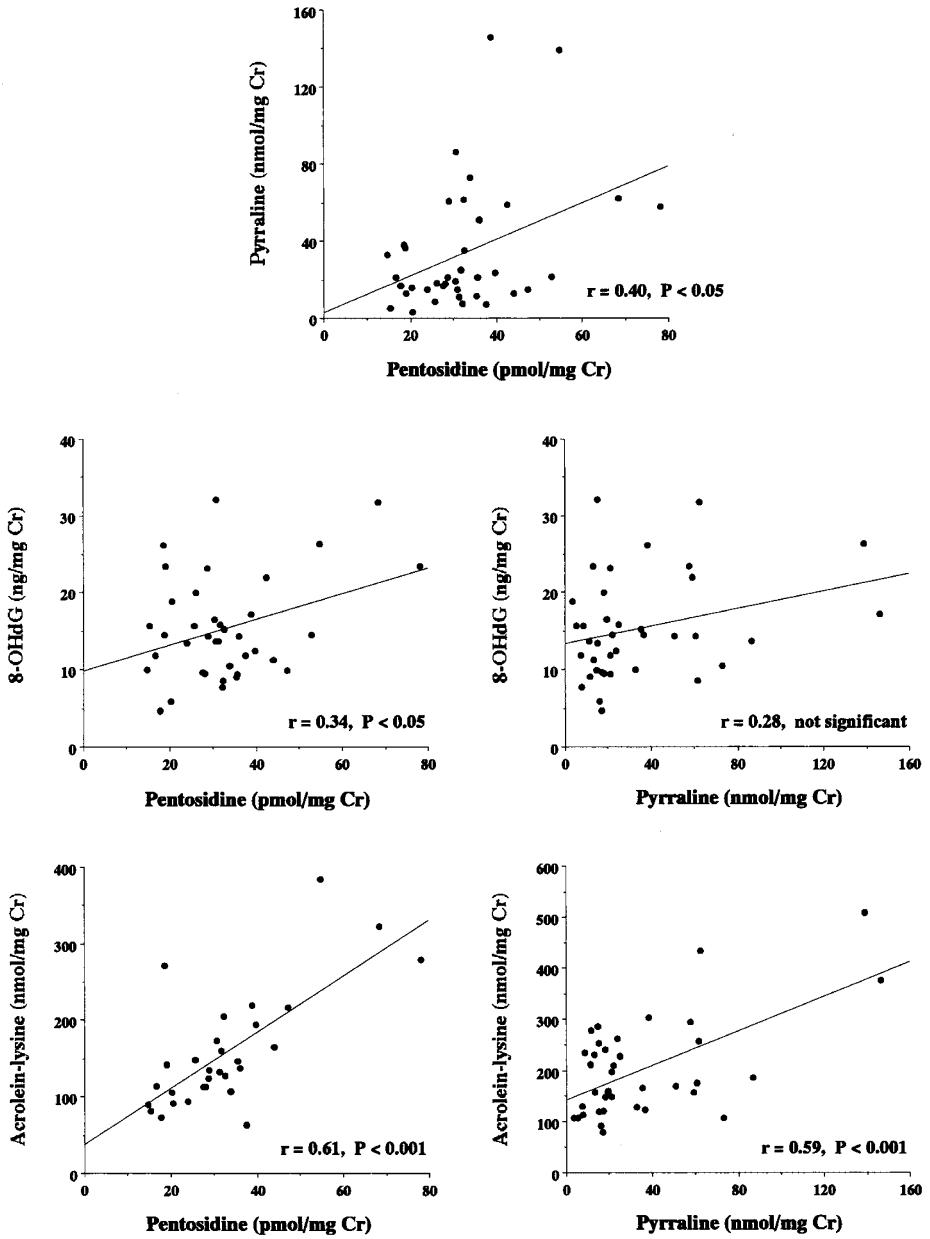

Figure 1. Relationships between urinary concentrations of pentosidine, pyrraline, 8-OHdG, and acrolein-lysine adduct in patients with type 1 diabetes.

design of new and effective management protocols to prevent the onset of or to halt the development of diabetic nephropathy.

Although the Diabetes Control and Complications Trial has identified sustained hyperglycemia, as measured by HbA_{1c}, as a risk factor for the development of diabetic complications (19), there is no consensus regarding the pathogenic link between hyperglycemia and complications. There are a number of equally tenable hypotheses on the origin of complications, including but not limited to the Maillard or AGE hypothesis, the aldose reductase hypothesis, oxidative stress, reductive stress, altered lipoprotein metabolism, increased protein kinase C activity, and altered growth factor or cytokine activities (20). These various hypotheses seem to overlap and intersect with one another.

The objective of our work was to study one of the above hypotheses, the AGE hypothesis, in young patients with type 1 diabetes and normal renal function. Because the AGE hypothesis and oxidative stress are inextricably intertwined, we also examined the status of oxidative stress in these patients. For these purposes, we determined the urinary concentrations of pentosidine and pyrraline, representing AGEs, as well as those of 8-OHdG and acrolein-lysine adduct, representing oxidative cellular damage. Measurement of urinary specific markers enables repeated monitoring of AGE formation and oxidative stress, which is otherwise not possible with invasive tests.

In the present study, we found significantly higher urinary concentrations of pentosidine in young patients with type 1 diabetes as compared with age-matched healthy control subjects, whereas pyrraline levels were not increased. Our results support the findings of the recent studies of Berg *et al.* (21, 22), Chiarelli *et al.* (23, 24), and Misselwitz *et al.* (25). These investigators found that serum concentrations of AGEs, includ-

ing carboxymethyl-lysine and pentosidine, were significantly increased in young patients with type 1 diabetes compared with control subjects. Berg *et al.* (21) performed serial renal biopsies in 10- to 30-year-old patients who had type 1 diabetes and microalbuminuria with normal renal function. They found a significant correlation between AGE levels but not HbA_{1c} at the start of the study and the progression of early morphologic renal damage at the follow-up biopsy 26 to 34 mo later. Chiarelli *et al.* (24) found a close correlation between serum AGE concentrations and the severity of diabetic retinopathy and microalbuminuria. A highly significant relationship between glycemic control (based on HbA_{1c}) and collagen-linked AGEs was also demonstrated in skin biopsy specimens from adults with type 1 diabetes (26). These findings support the view that serum levels of AGEs are equivalent to AGEs in the tissues and organs. Previously, we found a close correlation between serum and urinary concentrations of pentosidine not only in 26 subjects with normal renal function (12) but also in 44 patients with impaired renal function (unpublished observations). These results indicate that pentosidine in the circulation is duly excreted into the urine according to its serum concentration. Therefore, we assume that the increased excretion of pentosidine observed in our patients with diabetes reflects a greater synthesis and accumulation in the serum, tissues, and organs.

We also found significantly higher concentrations of urinary 8-OHdG and acrolein-lysine in our patients with diabetes compared with the healthy control subjects. The results indicate the presence of greater oxidative damage to DNA and lipid peroxidation in young patients with type 1 diabetes. The metabolic disturbances in diabetes may favor generation of excess reactive oxygen intermediates (ROIs), and this might be associated with reduced activity of antioxidants (20). Evidence indicating that systemic oxidative stress occurs in young patients with type 1 diabetes and may play an important role in the pathogenesis of endothelial perturbation and diabetic complications has accumulated (27–29). Specifically, high levels of malondialdehyde and protein carbonyl groups and low levels of thiols in plasma, low erythrocyte glutathione levels, and glutathione peroxidase activities, as well as high levels of E-selectin and intercellular cell adhesion molecule in plasma, have been reported. We now extend these observations to urinary specific markers of oxidative DNA damage (*i.e.* 8-OHdG) and lipid peroxidation (*i.e.* acrolein-lysine adduct) and provide additional evidence that ROIs may exert their cytotoxic effects at early clinical stages of type 1 diabetes.

In our diabetic group, both AGE products significantly correlated with each other, and there were significant correlations between pentosidine and either 8-OHdG or acrolein-lysine and between pyrraline and acrolein-lysine. The present study is the first demonstration of a possible link between reactive AGE formation and oxidative stress in young patients with diabetes. AGEs exert their cellular effects on several cell types, including glomerular mesangial cells, by interacting with specific receptor or RAGE (1). A key consequence of the interaction of AGEs with RAGEs is the generation of ROIs. *In vitro* and/or *in vivo* AGE-RAGE interaction results in the generation of ROIs, activation of NADPH oxidase, stimulation of Ras/Raf/MEK/ERK1/2, enhancement of nuclear translocation of nuclear factor-kappa B and activator protein-1, induction of heme oxygenase-1, and increased endothelial permeability and expression of vascular cell adhesion molecules (30–34). These studies revealed that generation of ROIs and enhanced oxidative stress is a potent factor capable of initiating signal transduction and altered gene expression, because the AGE-RAGE–mediated effects were inhibited in the presence of antioxidants such as *N*-acetylcysteine, probucol, vitamin E, or nitecapone. However, ROIs are produced in the Maillard reaction and also help to form AGEs (3, 5). Support for the causal role of high oxidative stress in AGE formation may be the correlation present in the serum of uremic patients between pentosidine and oxidative markers such as advanced oxidation protein products (35) and dehydroascorbate (36). Furthermore, the co-localization of AGEs with markers of lipid peroxidation in vascular and renal tissues suggests that both glycosylation and oxidation reactions contribute to pathologic lesions in diabetic atherosclerosis and nephropathy (9, 37). Indeed, AGE formation pathways consist of two types; one requires oxidative conditions, and the other requires nonoxidative conditions (3, 5, 20). Pentosidine is produced by the combined processes of glycosylation and oxidation (thereby termed glycoxidation) (38), and pyrraline is derived from nonoxidative chemistry (39). A recent study by Rodriguez-Garcia *et al.* (40) showed not only that hyperglycemia is a factor in pentosidine formation but also that oxidative reactions cause accumulation of pentosidine in rheumatoid arthritis with normoglycemia and normal renal function. Therefore, our findings of increased concentrations of pentosidine, 8-OHdG, and acrolein-lysine and their close interrelationship, together with seemingly normal pyrraline concentrations, in patients with diabetes are interpreted as evidence that pentosidine levels are partly determined by the severity of prevailing oxidative stress in these patients.

Another important demonstration of the present study was that in our patients with diabetes and normal renal function, microalbuminuria was closely linked with higher urinary concentrations of pentosidine, pyrraline, 8-OHdG, and acrolein-lysine adduct, in addition to the poorer quality of glycemic control (expressed by higher HbA_{1c} values). Of interest are data showing a significant increase of urinary pyrraline in the patients with diabetes and microalbuminuria relative to the healthy control subjects. Because albuminuria may represent disruption of the endothelial barrier in the kidney, our findings suggest that both AGE formation and enhanced oxidative stress are involved in the progression to the earliest detectable clinical stages of microvasculopathy in type 1 diabetes. We did not see any correlation between urinary concentrations of both AGEs and HbA_{1c} levels. This finding is consistent with the results of Berg *et al.* (22) and Misselwitz *et al.* (25) but not with those of Chiarelli *et al.* (23, 24). The difference in the results may be explained by the fact that glycosylated HbA_{1c} is an indicator of the glycemic status of the preceding several weeks, whereas AGEs reflect a process over a longer period.

Children and adolescents with type 1 diabetes were previously considered to be protected from vascular diseases, which become clinically apparent only by early adulthood. However,

this concept has been challenged recently. There is an increasing body of evidence supporting the hypothesis that endothelial dysfunction, a forerunner of diabetic vasculopathy, is present early in the course of diabetes (20, 28, 41, 42). The present study shows that accumulation of AGEs, whose formation is closely linked to oxidative stress, and resultant endothelial dysfunction may start early in the course of type 1 diabetes and supports the contention that good glycemic control should be emphasized from the diagnosis of diabetes to prevent the development or to slow the progression of vascular complications.

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