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Differential effects of Type 2 (non-insulin-dependent) diabetes mellitus on pentosidine formation in skin and glomerular basement membrane

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Summary. Pentosidine is an advanced Maillard/glycation reaction product the formation of which in human skin is significantly increased in Type 1 (insulin-dependent) diabetes mellitus and correlates with the severity of diabetic complications. Preliminary data in a limited number of Type 2 (non-insulin-dependent) diabetic individuals showed that skin pentosidine was not significantly elevated, raising the question of whether statistical power was insufficient for differences to be revealed, or whether pentosidine did not form because biological factors intrinsic to Type 2 diabetes affected the advanced Maillard reaction altogether. To resolve this question, pentosidine levels were measured in 209 human skin samples obtained at autopsy and in purified glomerular basement membranes from 45 subjects of various ages, with and without Type 1 and Type 2 diabetes and uraemia. Pentosidine increased exponentially in skin but curvilinearly in glomerular basement membranes, and reached 75 and 50 pmol/mg collagen at projected 100 years, respectively. Skin levels were not significantly elevated in individuals with Type 2 diabetes ($p > 0.05$). In contrast, pentosidine levels in glomerular basement membranes were elevated above the 95% confidence interval in the majority of diabetic patients regardless of the type of diabetes and in all individuals on haemodialysis. These data clearly demonstrate that the advanced Maillard reaction is indeed accelerated in Type 2 diabetes and strongly suggest that differences in pentosidine accumulation rates may be due to differences in collagen turnover. In diabetes and uraemia, accelerated Maillard reaction mediated protein crosslinking, as reflected by pentosidine, may contribute to decreased turnover of the extracellular matrix, sclerosis and thickening of basement membranes.

Key words: Aging, uraemia, glycation, diabetes nephropathy, oxidation.

Pentosidine is a fluorescent glycoxidation product of the advanced glycation/Maillard reaction product which was originally discovered in human dura mater from elderly individuals [1]. Pentosidine can be synthesized from glucose, fructose and ascorbate upon reaction with protein, but at a much slower rate than with pentoses. Previous data indicated that pentosidine increases exponentially with age in human skin and that its skin and plasma levels were elevated in individuals with Type 1 (insulin-dependent) diabetes mellitus and particularly in uraemia [2]. Furthermore, recent data indicated that pentosidine levels in skin biopsies correlate with the severity of complications in Type 1 diabetes suggesting an association with cumulative glycaemia or the presence of tissue-specific effects such as variability in turnover rate of the extracellular matrix [3–5]. These results, however, did not address the question of whether findings made in Type 1 diabetes are essentially applicable to Type 2 (non-insulin-dependent) diabetes. Indeed, preliminary data with small sample size suggested that pentosidine was not increased in skin of individuals with Type 2 diabetes. This raised the important question of whether the advanced Maillard reaction leading to pentosidine formation was inhibited for reasons intrinsic to Type 2 diabetes.

As a preamble to this question, we have both greatly expanded the preliminary study in skin and extended pentosidine determination to highly purified glomerular basement membrane from individuals who died from Type 2 diabetes. The data are compared with similar data from control subjects and deceased subjects with Type 1 diabetes or uraemia. In skin, the data confirm previous associations between pentosidine levels and presence of Type 1 diabetes or uraemia, but pentosidine was not significantly elevated in skin from individuals with Type 2 diabetes. In contrast, pentosidine levels in glomerular basement membranes were elevated in most diabetic and uraemic individuals regardless of the type of diabetes.

Table 1. Summary of patient data for skin study[a]

Category	No. of subjects	Major diagnosis[b]
Non-diabetic		
< 80 years	75	Cancer (29), congestive heart failure (12), leukaemia (10), cirrhosis (7), all others (17)
> 80 years	31	Cardiovascular disease/congestive heart failure (19), pneumonia (3), cancer (1), urinary tract infection (1)
with CRF	10	Cardiovascular disease/congestive heart failure (7), emphysema/aortic aneurysm (1), polyarteritis nodosa (1), lupus erythematosus (1)
with ESRD	10	Hypertensive cardiovascular disease (7), endocarditis (1), emphysema (1), post-streptococcal glomerulonephritis (1), [haemodialysis (10), polycystic kidney disease (3), kidney transplant (4), septicaemia (1), aneurysm (1)]
Diabetic		
Type 1 without CRF	8	Cardiovascular disease/congestive heart failure (4), cerebral oedema with haemorrhage/pneumonia (1), seizure disorder (1), insulin over-dose (1), trauma (1)
Type 1 with ESRD	8	Heart failure (8) [renal transplant (4), amputation (1), retinopathy (8), neuropathy (5), haemodialysis (5), gangrene (1)]
Type 2 without CRF	44	Cardiovascular disease/congestive heart failure (26), cirrhosis (3), cancer (4), emphysema (3), brain aneurysm (1), stroke (1), leukaemia (2), pneumonia (2), rheumatic heart disease (1), meningitis (1) [gangrene (3)]
Type 2 with CRF	8	Cardiovascular disease/congestive heart failure (6), cirrhosis (1), cancer (1) [gangrene (1)]
Type 2 with ESRD	10	Cardiovascular disease/congestive heart failure (8), complications due to cholecystectomy (1), cancer (1) [haemodialysis (8), amputation (2), retinopathy (3), emphysema (1), polycystic kidney disease (1), neuropathy (2), gangrene (2)]
Other[c]	5	Cystic fibrosis (5)

[a] Pentosidine levels of 106 subjects were combined with levels from a further 103 subjects previously described [2] and presented together in this table and Figure 1.
[b] Primary diagnoses with further complications given in brackets.
[c] These individuals have neither Type 1 nor Type 2 diabetes as defined [23]
CRF, Chronic renal failure; ESRD, end-stage renal disease

Subjects and methods

A total of 106 human skin samples were collected for a follow-up study to a previous measurement of pentosidine in 103 human skin samples [2]. Samples were obtained from a collection of tissue acquired by our laboratory at autopsy and stored at −80 °C. Tissues were selected to include a broad age range of diabetic and non-diabetic tissues. Six of these samples, all from diabetic donors, were provided by the National Disease Research Interchange (NDRI, Philadelphia, Pa., USA). The final selection comprised 55 males, 52 females, 60% Caucasian, 40% African-American individuals. Autopsy records stating causes of death and major disease diagnoses were obtained for all subjects and are summarized in Table 1. The diagnosis of diabetes was based on information available from the chart.

For the determination of pentosidine levels in isolated glomerular basement membranes, 22 human kidneys were obtained at autopsy from the Institute of Pathology, Cleveland, Ohio, USA and 23 kidneys were obtained from autopsies performed by the Department of Pathology, University of North Dakota, Grand Forks, ND, USA. The final group consisted of 18 males and 26 females, of which 32 were Caucasian, 10 African-American, and 2 Native American individuals. One additional sample consisted of pooled tissue from 2.5- to 20-month-old Caucasian infants designated as 1-year-old tissue. Causes of death and major diagnoses especially in regard to diabetic complications and uraemia were obtained for all individuals and summarized in Table 2.

Processing of skin samples

Abdominal skin samples were prepared for pentosidine assay as previously described [2]. The subcutaneous fat and the epidermis were removed from approximately 10 cm^2 frozen portions of skin with a

Table 2. Summary of patient data used for the glomerular basement membrane study[a]

Category Major Diagnosis[a]	No. of subjects
Non-diabetic Congestive heart failure (10), cancer (4), leukaemia (2), pneumonia (2), carbon monoxide (1), trauma (2), aspiration (1), intracranial bleeding (1), pulmonary bleeding (1), pulmonary emboli (1), stroke (1), cirrhosis (1)	27[b]
Diabetic	
Type 1 without CRF congestive heart failure (3), pulmonary emboli (1)	4
Type 1 with CRF congestive heart failure (2), renal disease secondary to diabetic nephropathy (1) [ESRD (2)]	3
Type 2 without CRF congestive heart failure (5), pulmonary emboli (2), leukaemia (1), liver failure (1)	9
Type 2 with CRF cancer	1
Other[c] Diabetes secondary to steroid treatment for emphysema	1

[a] Primary diagnoses with further complications given in brackets. CRF, Chronic renal failure; ESRD, end-stage renal disease.
[b] One sample designated as 1 year in Figure 2 A consisted of pooled tissue from three children ranging in ages 2.5 to 20 months, death due to sudden infant death syndrome (SIDS), hydrocephalus and congenital heart disease.
[c] This individual has neither Type 1 nor Type 2 diabetes as defined [23]

sharp scalpel blade. The skin was minced into small pieces and, in turn, homogenized in 10 ml of phosphate buffered saline, pH 7.4, with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY, USA). The suspension was centrifuged in a Beckman Model J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, Calif., USA) at 31,000 × *g* for 20 min. The pellet was washed with distilled water, recentrifuged and extracted for 24 h with 2:1 chloroform/methanol. The organic solvents were removed through filtration under vacuum suction over a Büchner-type porcelain funnel fitted with a 4.25 cm diameter Whatman filter paper. The tissue was washed with methanol and left to dry on the filter paper under suction. The tissue (approximately 25 mg) was removed with forceps and placed in a 16 × 125 mm screw-capped tube. In turn, the tissue was washed two times with distilled water, centrifuged to remove the water, and lyophilized. A total of 2 ml deaerated 6 mol/l HCl was added to the tube. The tube was purged with nitrogen and sealed with a Teflon-faced rubber-lined cap.

Preparation of glomerular basement membranes

Preparations of acellular glomerular basement membranes were done in the laboratory of E.C.C. Fresh kidneys from the laboratory of V.M.M. were shipped by overnight express mail in ice to E.C.C. and glomerular basement membranes were prepared and purified by a combination of sieve and detergent treatments as described by Cohen and Carlson [3]. Briefly, the renal cortex was dissociated from the medulla by dissection and minced into 1 mm^2 or smaller pieces which were passed through a large diameter mesh screen of 250–350 μm to reduce the size of the material. The filtrate was centrifuged at low speed (350 × *g*) and the pellet was resuspended in phosphate buffered saline at pH 7.4. This procedure was repeated several times to remove cellular debris. Glomeruli were prepared from the resuspended pellet by passing the material through a series of graded nylon or stainless steel sieves.

The isolated glomeruli were subsequently made acellular with various treatments consisting sequentially of distilled water with protease inhibitors, 3% Triton X-100, 0.05% deoxyribonuclease in 1 mol/l NaCl all at 4 °C, and 4% sodium deoxycholate at room temperature. At each of these steps, the material was recovered by centrifugation and extensively washed in distilled water.

The purified glomerular basement membranes were stored at −80 °C. At the time of processing for hydrolysis, they were washed three times with 20-ml portions of phosphate buffered saline, lyophilized and extracted with 10-ml portions of 2:1 chloroform/methanol for 24 h. The residue was lyophilized and hydrolysed with 2 ml of deaerated 6 mol/l HCl.

HPLC assay for pentosidine in skin and glomerular basement membranes

Skin and glomerular basement membranes were acid-hydrolysed for 24 h at 110 °C. The acid was evaporated by a Speed-Vac (Savant Instruments, Inc., Farmingdale, NY, USA) and each sample was reconstituted with 1 ml of water containing 0.01 mol/l heptafluorobutyric acid. After filtering each sample with a 4-μm filter, collagen content was determined for all samples according to the method of Stegeman and Stalder [4] assuming a content of 14% hydroxyproline by weight [5].

Pentosidine was determined by reverse-phase HPLC as previously described [2]. Samples of 100 μl each, equivalent to 171 μg (skin), or 71 μg (glomerular basement membrane), of collagen were injected onto an HPLC (Waters Associates, Millipore Corp., Milford, Mass., USA) equipped with model 510 pumps, a model 712 WISP automatic injector, and a model 680 controller. For skin samples, the effluent from the HPLC was directed onto an on-line Varian Fluorichrom fluorescence detector (Varian Associates, Inc, Palo Alto, Calif., USA) using filters and instrument settings [2]. The output of the detector was directed to a strip chart recorder [2]. For glomerular basement membrane, Waters model 470 scanning fluorescence detector was used with excitation/emission at 335/385 nm and output directed to a Waters model 740 data module recorder.

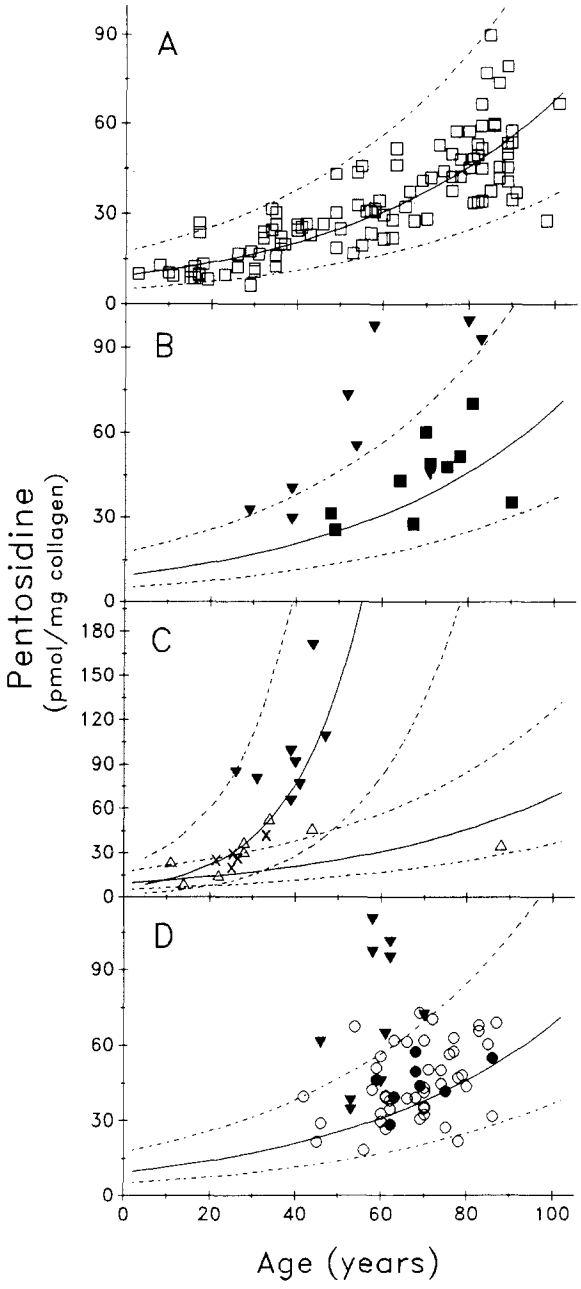

Fig. 1A–D. Pentosidine levels in human skin in relationship to age, diabetes, chronic renal failure (CRF) and end-stage renal disease (ESRD). **A** Non-diabetic subjects without CRF (□). Regression line and 95% confidence intervals are shown and reproduced in **B–D**: $y = 9.4e^{0.02x}$, $r = 0.86$, $n = 106$. **B** Non-diabetic patients with CRF (■) or with ESRD (▼). **C** Type 1 diabetic patients without CRF (△), or with ESRD (▼). Five patients with cystic fibrosis and diabetes, but without CRF, are indicated (X). Regression line equation and 95% confidence intervals were determined for levels of all patients as shown except the level for the 88-year-old patient: $y = 6.1e^{0.06x}$, $r = 0.82$, $p < 0.0001$. **D** Type 2 diabetic patients without (○) and with (●) CRF, or with ESRD (▼)

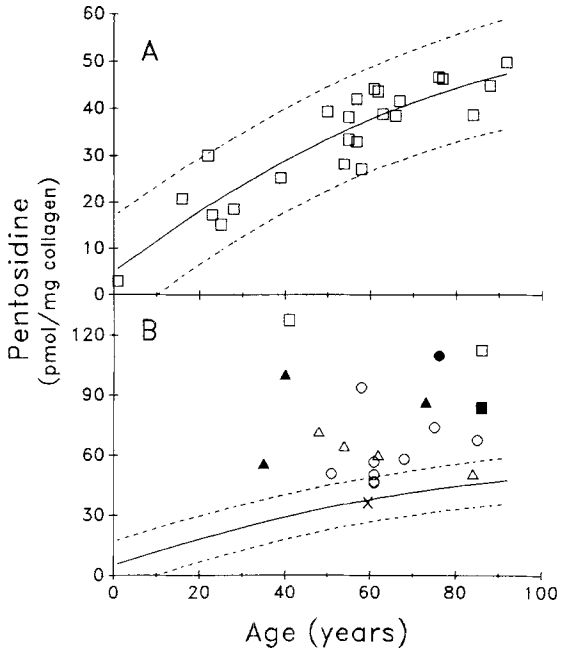

Fig. 2 A, B. Pentosidine levels in isolated glomerular basement membranes. **A** Pentosidine levels in non-diabetic subjects (□) as a function of age. Regression line and 95% confidence intervals are shown and reproduced in **B**: $y = 4.9 + 0.7x - 0.003x^2$, $r = 0.91$, n = 24. **B** Pentosidine in relationship to age, diabetes and renal disease. Non-diabetic patients without (□) and with (■) chronic renal failure (CRF), Type 1 diabetic patients without (△) and with (▲) CRF, and Type 2 diabetic patients without (○) and with (●) CRF. One patient with diabetes secondary to steroid use indicated (X)

Statistical analysis

Pentosidine levels of skin for subjects of the present study were combined with those from the previous study [2] and hence presented together in Table 1 and Figure 1. For both skin and glomerular basement membrane, the best fit model was determined for non-diabetic subjects without chronic renal failure by regression analyses [6]. Confidence intervals for the regression lines (Figs. 1 and 2) were computed using the error of prediction formula by Armitage [7]. All other data consisting of pentosidine levels in tissue samples of diabetic and/or uraemic individuals are presented in relationship to these intervals. Pentosidine levels for three individuals without diabetes in the glomerular basement membrane study were found well above the mean of non-diabetic subjects without chronic renal failure, and thus were not included in the regression analysis of Figure 2 A, but presented separately in Figure 2 B. This was justified by the fact that these individuals without diabetes had mean pentosidine values greater than three standard deviations above the mean of the regression line which were proven to be outliers by a statistical test [8].

Results

Pentosidine in human skin

Pentosidine levels in skin of non-diabetic individuals increased with age following an exponential pattern as determined by curve-fitting analysis with a large variation in levels occurring at late age (Fig. 1 A). Many subjects without diabetes, but with chronic renal failure due to other causes, had levels elevated above the regression line determined for non-diabetic individuals (Fig. 1 B) however, only those subjects with end-stage renal disease had levels above the 95% confidence intervals.

Similar relationships were noted for skin pentosidine levels for Type 1 diabetic patients. Levels were borderline elevated in five individuals with diabetes secondary to cystic fibrosis and were dramatically elevated in all Type 1 diabetic patients with end-stage renal disease (Fig. 1 C). One important observation is that when levels for all diabetic subjects of Figure 1 C were considered as a group without regard to the effects of uraemia, a strong exponential relationship ($r = 0.82$, $p < 0.0001$) was noted with age which was highly accelerated by end-stage renal disease (Fig. 1 C).

A large variation existed in the degree of severity, type of complications and methods of control in patients with Type 2 diabetes (Table 1). In general, many of these patients tended to have levels elevated above the regression line determined for non-diabetic control subjects (Fig. 1 D). However, again the general pattern was that only patients with end-stage renal disease, except for three, had levels outside the confidence intervals.

Pentosidine in isolated glomerular basement membranes

The isolated glomerular basement membranes used for pentosidine determination were acellular and contained both the peripheral basement membrane (epithelial and endothelial) and the basement membrane-like material of the mesangial matrix [9]. The isolated glomerular basement membranes had been stripped from other proteins by proteolytic treatment and further treatments using chaotropic agents with β-mercaptoethanol did not solubilize additional material.

Pentosidine determination in glomerular basement membrane revealed that levels increased in a asymptotic fashion with age in non-diabetic subjects ($r = 0.91$). The best-fitted curve was a polynomial equation (Fig. 2 A). Three individuals without diabetes had elevated pentosidine levels (Fig. 2 B). One of these individuals (age 41) who showed the highest level in the study (127 pmol/mg) had been treated for acute lymphoblastic leukaemia. High levels were also noted for two other individuals, one of whom died of chronic renal failure requiring haemodialysis 10 days before his death. The other individual was an 86-year-old patient with Alzheimer's disease who died from acute pneumonia and urinary tract infection which developed after a hip fracture. At the time of autopsy, he was diagnosed with chronic pyelonephritis, but apparently no chronic renal failure. In this case, the pentosidine levels were very high, reaching 112 pmol/mg collagen (Fig. 2 B).

Unlike the results for skin, pentosidine levels of glomerular basement membrane from diabetic kidneys in most cases were elevated above the 95% confidence intervals determined for non-diabetic subjects of Figure 2 A regardless of the type of diabetes and the presence or absence of chronic renal failure (Fig. 2 B). Only in four cases were levels within the confidence intervals. One of these individuals was an 84-year-old patient who had a mild case

of diabetes treated with insulin and who died from congestive heart failure. Two other individuals, both age 61, had Type 2 diabetes for either 2 or 20 years and died from congestive heart failure and congenital hepatic fibrosis, respectively. Diabetes in the latter individual was complicated by nephropathy without apparent chronic renal failure. An additional individual (age 59 years) with a level on the regression line of Figure 2B contracted diabetes secondary to prolonged steroid use for treatment of emphysema with documented chronic renal failure.

In the diabetic group, the highest levels tended to be associated with patients with chronic renal failure. Two were Type 1 diabetic subjects of 40 and 73 years of age with documented end-stage renal disease. Unexpectedly, a high level (94 pmol/mg) was found in the glomerular basement membrane of one patient (age 58) with Type 2 diabetes without nephropathy (Fig. 2B). Death in this case was attributable to congestive heart failure.

Discussion

The primary goal of this investigation was to resolve the question of whether the preliminary data indicating lack of increase of pentosidine in skin of individuals with Type 2 diabetes meant that advanced Maillard reaction was not occurring for reasons intrinsic to Type 2 diabetes. This was done by greatly expanding the preliminary data in skin and by comparing patterns of pentosidine formation in skin and human glomerular basement membranes. The results of this study show unequivocally that skin pentosidine levels are not increased in Type 2 diabetes, whereas 60% of values are increased in purified glomerular basement membranes. It can therefore be concluded that Type 2 diabetes exerts a differential effect on pentosidine formation in these two tissues, and that the advanced Maillard reaction does indeed proceed at a higher rate not only in Type 1, but also in Type 2 diabetes.

Several reasons may explain why pentosidine levels were not significantly elevated in skin from individuals with Type 2 diabetes. First, a close inspection of the data reveals that 73% of pentosidine values in Type 2 diabetic patients without chronic renal failure were above the regression line for control subjects, suggesting thereby, a clear trend toward pentosidine elevation in diabetic skin. Interestingly, the progressive widening of the confidence intervals for control subjects could itself be linked to an age-related increase in glucose intolerance, thereby leading to accelerated pentosidine formation and decreased skin collagen turnover rate in aging. Secondly, it is also possible that normal skin values in Type 2 diabetic individuals might be related to the severity of hyperglycemia which is usually less in Type 1 diabetes, or to differences in skin collagen turnover rates, or both. Although collagen turnover of skin and glomerular basement membrane has not been studied in depth in humans, various studies in rodents show skin collagen turns over more readily than that for glomerular basement membrane. The maturation rate of rat skin collagen is slow [10] although the half-life of skin collagen increases with age [11]. Studies with collagen labelled with ^{14}C-proline in rats showed turnover time to be much greater for skin than for kidney or tail tendon [12]. Similarly, studies with glomerular basement membrane in rats using labelled proline, hydroxyproline and glycine showed turnover time to be very slow and equivalent to that of tail tendon [13].

Changes in the composition of proteins in the extracellular matrix could potentially explain variations in skin and glomerular basement membrane pentosidine levels. For example, the ratio of type III to type I collagen is increased in diabetic rodent skin [14]. In human kidney, on the other hand, it has been shown that the expansion of the mesangial matrix and thickening of the glomerular basement membrane involves distinct collagen components [15]. However, it is not known whether pentosidine forms preferentially on some but not other proteins.

The finding of elevated pentosidine levels in glomerular basement membranes of the two non-diabetic and non-uraemic individuals is puzzling. One individual was treated with a chemotherapeutic regimen which is expected to lyse cells and possibly release pentose sugars which may act as pentosidine precursors. Such sugars are likely to accumulate in uraemia [16]. More difficult to explain, however, is the dramatic increase in glomerular basement membrane pentosidine in the 83-year-old patient with Alzheimer's disease. In absence of a plausible explanation based on increase in tissue levels of a biochemical pentosidine precursor, one would have to invoke an impairment of glomerular basement membrane turnover. Evidently, the presence of elevated levels of pentosidine in glomerular basement membranes from non-diabetic and non-uraemic individuals may also indicate that pentosidine formation is unrelated to the pathogenesis of diabetic renal disease. The marked elevation of skin pentosidine in haemodialysis patients suggests that the metabolic complication of end-stage renal disease is not corrected by dialysis therapy and may explain some of the clinical manifestations of renal failure that persist even after apparently "adequate" dialysis. Since dialysis membranes are virtually impermeable to large molecules, it has been speculated that the toxic products accumulating in uraemia have molecular weights below 5 kDa [17]. Also, since small molecules such as urea, creatinine, and uric acid pass through these membranes readily, it was also thought that these toxic molecules were of this size. However, failure to correlate the plasma levels of these particular small molecules to the manifestations of uraemia has led to speculation that the toxic molecules exist between these two sizes, the uraemic middle molecule hypothesis [17, 18]. Evidence to support this hypothesis includes the correlation between successful removal of these molecules with the efficiency of dialysis in preventing complications of uraemia such as neuropathy [18]. Because of the association, a large effort has been made in the determination and characterization of these molecules. Many of these compounds were proposed to be peptide in origin with a chromophoric and ionic character [18]. However, most studies have not established a clear relationship between specific uraemic symptoms and the accumulation of any specific middle molecule fraction, although patients with

uraemic complications tend to have higher plasma levels than patients without serious symptoms.

Because of the observed increase in pentosidine levels in end-stage renal disease, it is tempting to speculate that sugar-derived glycation and advanced glycation of proteins may play a role in the progression of both diabetic and non-diabetic renal disease. Evidence for the potential of such reactions is the known hyperglycaemic effects of uraemia [16]. Although adequate dialysis corrects the serum profile concentrations for many of the sugars which are elevated during uraemia, the correction is only partial for some of the sugars while variable for others, particularly the polyols [16].

Recently, Makita et al. [19], using a competitive whole-cell radioreceptor assay for advanced glycation end-product (AGE) on proteins, demonstrated that these products are significantly elevated in human serum during end-stage renal disease with most of this elevation occurring in peptides of low-molecular weight of less than 10 kDa. Haemodialysis significantly alleviated, but did not totally correct, the elevation, particularly in diabetic patients. Their data suggests that some of these uraemic middle molecules, if not all, may represent Maillard-modified proteins and peptide fragments.

The possibility exists that some of the products measured by Makita et al. [19] could be pentosidine, although a recent study with an AGE antibody did not cross-react with pentosidine [20]. This suggests that pentosidine, which is a marker for the greatly accelerated Maillard reaction in end-stage renal disease, was elevated 23-fold in human plasma proteins during uraemia [21]. Both kidney and kidney-pancreas transplantations were accompanied by a dramatic, but incomplete, reduction of plasma pentosidine concentrations in diabetic patients with nephropathy, and remained above normal more than 2 years after transplant [22]. Such findings suggest that some degree of metabolic abnormalities persists despite transplant which may explain why transplantation fails to fully reverse the complications of diabetes in some patients [22].

In summary, patterns of formation of the advanced glycation product pentosidine are very different in aging skin and glomerular basement membranes. Similarly, whereas pentosidine is elevated in glomerular basement membranes in Type 1 and 2 diabetes and in uraemia, no increase was found in skin of individuals with Type 2 diabetes. These results suggest that additional factors besides cumulative glycaemia, such as e. g. tissue turnover rate play a role in regulating levels of advanced Maillard reaction products.

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