Accumulation of Albumin-Linked and Free-Form Pentosidine in the Circulation of Uremic Patients with End-Stage Renal Failure: Renal Implications in the Pathophysiology of Pentosidine[1]

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ABSTRACT

Pentosidine is an advanced glycation end product and its formation is shown to be closely related to oxidative processes. Recent studies have shown that pentosidine levels are increased not only in plasma and matrix proteins from diabetic patients, but also markedly in nondiabetic hemodialysis patients. Currently, the mechanism of accumulation and kinetics of pentosidine formation in hemodialysis patients remain unknown. Gel filtration of uremic plasma revealed that plasma pentosidine exists in the albumin fraction (approximately 90%) and, interestingly, in free form (approximately 5%) as well. Plasma free pentosidine was undetectable in subjects with normal renal function. There was a significant correlation between the plasma levels of albumin-linked and free pentosidine in hemodialysis patients. Kinetic studies indicated that dietary pentosidine was absorbed into the circulation and that, after either oral or intravenous administration of pentosidine to intact or nephrectomized rats, the plasma free pentosidine level was closely linked to the level of renal function. These findings demonstrate that: (*1*) Pentosidine accumulates as albumin-linked and in free form in the circulation of uremic patients; (*2*) dietary pentosidine can be absorbed into the circulation, thus being one possible origin of circulating free pentosidine; (*3*) free pentosidine may accumulate as a result of decreased glomerular filtration; and (*4*) the mechanism of accumulation of albumin-linked pentosidine is not related to high glucose levels. It suggests the simultaneous accumulation, during renal failure, of either unknown pentosidine precursor(s) or catalyst(s) of glycoxidation, independent of glucose.

Key Words: *Advanced glycation end products, glycoxidation, pentosidine, hemodialysis, diabetes*

Advanced glycation end products (AGE) are formed over months by a nonenzymatic reaction between aldoses and proteins, known as the Maillard reaction (1,2). They constitute a heterogeneous class of structures that are brown in color, fluoresce, and form crosslinks. Recent advances in the understanding of the pathophysiology of AGE have shed new light on the pathogenesis of atherosclerosis (3–6), diabetic nephropathy (7–9), dialysis-related amyloidosis (10–15), and Alzheimer's disease (16–18). AGE-modified proteins are thought to play a role in normal tissue remodeling, *i.e.*, the removal and replacement of senescent extracellular matrix components. Under pathological conditions, however, the accumulation of AGE-modified proteins might lead to tissue damage through a variety of mechanisms: through an alteration of the structure and function of tissue proteins (1), by the stimulation of cellular responses (3,4,7,8,11–13) via receptors specific for AGE-modified proteins (5,6,19), or by the generation of reactive oxygen intermediates (18,20,21).

Pentosidine is a fluorescent crosslink (22) postulated to be a glycoxidation marker for AGE in proteins (21). Recent studies have demonstrated the presence of pentosidine in skin collagen (9,22–24), glomerular basement membrane (22,25), and plasma proteins of diabetic patients (26,27). The pentosidine level in skin correlates well with the severity of diabetic complications (28,29). Also, in hemodialysis patients with end-stage renal failure, a dramatic increase in pentosidine was demonstrated in the plasma proteins (26,27), β2-microglobulin amyloid deposits (14), and skin collagens (30), irrespective of the presence or absence of diabetes. Pentosidine, thereby, has been implicated in the tissue damage not only of diabetic patients but

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also of hemodialysis patients with end-stage renal failure.

Elucidation of the mechanism by which pentosidine accumulates should help in understanding its pathological role. In diabetic patients, the increase of pentosidine levels has been ascribed to high glucose levels. In nondiabetic hemodialysis patients with normal glucose levels, however, it remains poorly understood. The source, the circulating form, and the kinetics of formation of plasma pentosidine are yet to be described. Whether glucose is the sole precursor of pentosidine in these patients remains to be established. To address these issues, we have quantitated plasma pentosidine level by HPLC assay in plasma of normal subjects, non-insulin dependent diabetic patients with normal renal function, and patients with ESRD requiring hemodialysis. During the studies, we found that a small fraction of pentosidine in the circulation of hemodialysis patients exists in free form, which was undetectable in the plasma of either healthy subjects or diabetic patients with normal renal function. We also found that protein-linked pentosidine is exclusively present in the albumin fraction. The work presented here demonstrates that pentosidine accumulates in albumin-linked and free form in the circulation of hemodialysis patients. The origin and kinetics of plasma free pentosidine was also investigated in experimental animals to elucidate the mechanism of pentosidine accumulation.

METHODS

Samples

Fresh heparinized plasma samples were obtained from healthy subjects (Group I in Table 1), non-insulin dependent diabetic patients with normal renal function and no proteinuria (Group II), and non-insulin dependent diabetic patients with end-stage renal failure requiring hemodialysis (Group III). Plasma samples were also obtained from non-diabetic patients with elevated serum creatinine levels but no symptoms of uremia (Group IV) and from non-diabetic patients undergoing hemodialysis (Group V) because of chronic glomerulonephritis. Informed consent was obtained from all patients. Normal renal function was defined as a serum creatinine level below 1.0 mg/dL. Proteinuria was considered to be absent if no protein had ever been detected in the urine with Albustix (Bayer-Sankyo Co. Ltd., Tokyo, Japan). Hemodialysis was performed with a cellulosic dialyzer and dialysate containing 30 mEq/L of bicarbonate and 8 mEq/L of acetate. The plasma protein concentration was determined using Bio-Rad protein assay reagents (Richmond, CA) with human serum albumin (HSA) as a standard. Separation of plasma proteins was performed by gel filtration on a Sephacryl S-200 column (5.0 × 67 cm) (Pharmacia, Uppsala, Sweden).

Synthesis of Pentosidine

Pentosidine was prepared by the method of Grandhee and Monnier (31) with some modification. A suspension of 0.01 mol of N^{α}-*t*-butoxycarbonyl-L-lysine and 0.08 mol of D-ribose in 100 mL of methanol was stirred for 3 h at 30°C. Methanol was evaporated under reduced pressure, resulting in a dark

TABLE 1. Profile of subjects examined in this study[a]

	Group	Number of Subjects	Sex		Age (yr)	Plasma Protein (mg/mL)	Plasma Pentosidine (pmol/mg)	Plasma Free Pentosidine (pmol/mL)	Plasma Creatinine (mg/dL)	Plasma fructoselysine (nmol/mg)	Duration of Hemodialysis (yr)
			Male	Female							
I	Healthy Subjects	20	14	6	58.20 ± 5.93	70.91 ± 2.09	1.57 ± 0.23	ND	0.68 ± 0.13	3.64 ± 0.33	
II	Diabetic Patients with Normal Renal Function	26	8	18	61.96 ± 11.59	71.94 ± 5.64	1.92 ± 0.72	ND	0.68 ± 0.18	7.59 ± 3.26	
III	Diabetic Hemodialysis Patients	36	22	14	58.0 ± 11.65	70.35 ± 5.50	21.74 ± 9.46	59.84 ± 24.27	10.99 ± 2.20	6.17 ± 1.40	5.07 ± 2.76
IV	Predialysis Nondiabetic Patients with Elevated Serum Creatinine	52	29	23	55.67 ± 10.09	68.03 ± 7.19	2.82 ± 2.10	3.61 ± 6.33	4.40 ± 1.80	3.86 ± 1.08	
V	Nondiabetic Hemodialysis Patients	46	29	17	59.17 ± 12.72	70.28 ± 5.41	20.62 ± 8.44	53.67 ± 19.33	12.10 ± 2.47	4.39 ± 0.67	7.39 ± 5.02

[a] Data are expressed as means ± SD. ND, not detected.

brown syrup. The residue was purified by column chromatography on Dowex 50 W × 2 (Aldrich, Milwaukee, WI), using a linear gradient from 0.2 M pyridine acetate (pH 3.1) to 2 M pyridine acetate (pH 5.0). The main fraction (N^{α}-*t*-butoxycarbonyl-N^{ϵ}-(1-deoxy-D-ribulos-1-yl)-L-lysine) was collected, concentrated *in vacuo* and lyophilized to give a partially purified product (4.15 g). The product thus obtained (0.01 mol) and N^{ω}-*t*-butoxycarbonyl-L-arginine (0.042 mol) were dissolved in 100 mL of sodium phosphate buffer (pH 9.4), and the pH of the solution was adjusted to 11 to 12 with 1 N NaOH. After being stirred for 20 h at room temperature, the solution was acidified to pH 2 with 6 N HCl and concentrated *in vacuo* to give an oily residue. The *t*-butoxycarbonyl groups were removed by treatment with 300 mL of trifluoroacetic acid for 1 h at room temperature. After removal of excess trifluoroacetic acid *in vacuo*, the residue was purified on HPLC using a reverse-phase column (YMC-Pack ODS; YMC Corp., Kyoto, Japan) to give a homogeneous product (70 mg). The identity of the final product was confirmed as pentosidine by nuclear magnetic resonance and fast atom bombardment-mass spectrometry.

Pentosidine Measurement by HPLC Assay

For quantitation of total pentosidine in plasma or plasma fractions, plasma containing approximately 4 mg of proteins or each plasma fraction was lyophilized, hydrolyzed by 100 μL of 6 N HCl for 16 h at 110°C under nitrogen, followed by neutralization with 100 μL of 5 N NaOH and 200 μL of 0.5 M phosphate buffer (pH 7.4), then filtered through a 0.5-μm pore filter, and diluted with phosphate-buffered saline. For quantitation of plasma free pentosidine, the plasma was mixed with an equal volume of 10% trichloroacetic acid and centrifuged at 5000 × *g* for 10 min. The supernatant was filtered through a 0.5-μm filter and diluted with distilled water. Pentosidine in these specimens was analyzed by reverse-phase HPLC according to the method of Odetti *et al.* (26). In brief, a 50-μL solution of plasma acid hydrolysate (corresponding to 25 μg of plasma proteins) or diluted protein-free plasma (corresponding to 6.25 μL of plasma) was injected into an HPLC system and separated on a C18 reverse-phase column (Waters, Tokyo, Japan). The effluent was monitored using a fluorescence detector (RF-10A: Shimadzu) and an excitation-emission wavelength of 335/385 nm. Synthetic pentosidine was used to obtain a standard curve. The substance in the specimens, detected at the same retention time as authentic pentosidine, was confirmed as pentosidine by fast atom bombardment-mass spectrometry. Plasma total pentosidine and free pentosidine levels were expressed as pmol per mg of plasma protein and pmol per mL of plasma, respectively. Limits of detection were 0.2 pmol/mg for plasma total pentosidine and 2 pmol/mL for plasma free pentosidine.

Fructoselysine Measurement

Fructoselysine was determined by colorimetric assay using a kit (Fructosamine Test Roche-II; Nihon Roche Ltd., Tokyo, Japan) according to the manufacturer's instruction (13). The assay was based upon the property of fructoselysine to act as a reducing agent in alkaline solution. Poly-L-lysine, provided by the manufacturer, was used as a standard, and the amount of fructoselysine in each sample was expressed as mol per mg of protein.

Kinetic Study of Plasma Free Pentosidine in Normal and Uremic Rats Given Pentosidine

Eight-week-old Wistar rats (Charles River Japan Inc., Yokohama, Japan) were fed a casein-based diet (AIN-76; Clea Japan Inc., Tokyo, Japan) with low pentosidine content as estimated by HPLC assay (0.3 nmol/g) for 1 wk. After a 24-h fast, a bilateral nephrectomy was performed in some rats. Both nephrectomized and intact rats were then given an oral (400 nmol/rat) or an intravenous (10 nmol/rat) load of synthetic pentosidine. Seven-week-old Wistar rats underwent a 6/7 nephrectomy according to the method of Platt *et al.* (32). After 6 wk on a casein-based diet and a 24-h fast, they were given synthetic pentosidine, either orally or intravenously, as described above. To examine the kinetics of clearance of plasma free pentosidine in each group, heparinized blood was taken at intervals and assayed for plasma free pentosidine. All studies were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Institutional Animal Care and Use Subcommittee.

Statistical Analysis

Data are expressed as means ± SD. The *t* test was used for a statistical evaluation of significant difference between two groups. Correlation was assessed by linear or quadratic regression analysis.

RESULTS

Accumulation of Pentosidine in Plasma of Hemodialysis Patients

Pentosidine contains lysine and arginine residues linked in an imidazo-(4,5b)-pyridinium ring (inset in Figure 1). The pentosidine levels in acid hydrolysates of plasma were measured by HPLC assay (Figure 1). Mean (± SD) pentosidine levels were significantly higher in diabetic patients with normal renal function (Group II) than that in healthy subjects (1.92 ± 0.72 *versus* 1.57 ± 0.23 pmol/mg, $P < 0.05$), in good agreement with previous reports (27,28). This difference in the pentosidine levels between diabetics and nondiabetics disappeared in patients on hemodialysis. These plasma pentosidine levels (21.74 ± 9.48 pmol/mg for diabetics [Group III] and 20.62 ± 8.44 pmol/mg for nondiabetics [Group V]) were over 10-fold higher than in healthy and diabetic subjects with normal renal function ($P < 0.0001$). Even when adjusted for albumin levels and duration of hemodialysis, analysis of variance failed to detect a significant difference between diabetic and nondiabetic hemodialysis patients.

Plasma pentosidine was significantly correlated with creatinine in nondiabetic patients with renal dysfunction (Group IV) ($P < 0.0001$, $r^2 = 0.57$) (Figure 2), indicating that the plasma pentosidine level is closely related to the level of renal function. Of particular interest, in diabetic hemodialysis patients, plasma pentosidine did not correlate with fructoselysine, *i.e.*, a glucose-derived Amadori adduct (Figure 3). Plasma fructoselysine was not correlated with cre-

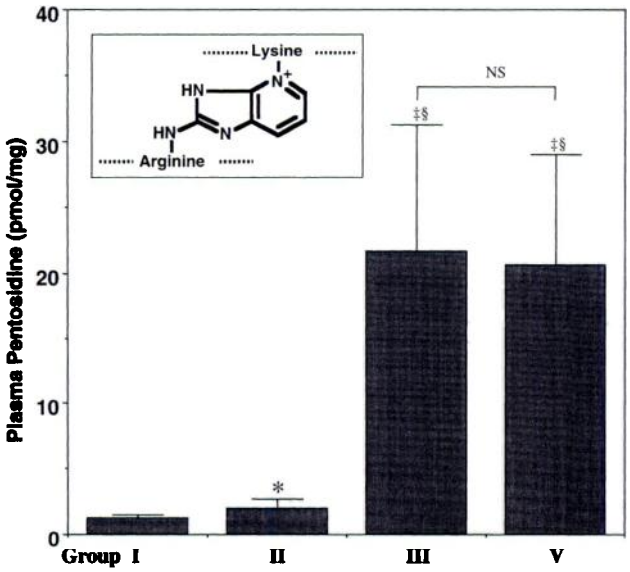

Figure 1. Plasma pentosidine levels in healthy subjects (Group I: $N = 20$), diabetic patients with normal renal function (Group II: $N = 26$), diabetic hemodialysis patients (Group III: $N = 36$) and nondiabetic hemodialysis patients (Group V: $N = 46$). Inset, structure of pentosidine. The pentosidine level in acid hydrolysate of plasma was determined by HPLC assay (see the Methods section). Data are expressed as means ± SD. $^{*}P < 0.05$ *versus* normal subjects, $^{\ddagger}P < 0.0001$ *versus* healthy subjects, and $^{\S}P < 0.0001$ *versus* diabetic patients with normal renal function.

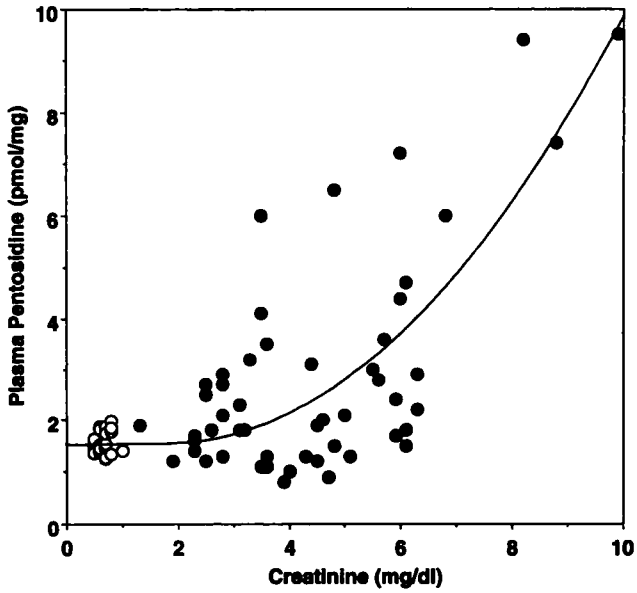

Figure 2. Correlation between plasma pentosidine and creatinine. Plasma pentosidine increases with deterioration of renal function ($N = 72$, $P < 0.0001$, $r^2 = 0.57$). Open squares and circles denote healthy subjects (Group I: $N = 20$) and nondiabetic predialysis patients with renal dysfunction (Group IV: $N = 52$), respectively. Quadratic regression curve equation: $y = 0.125X^2 - 0.461X + 1.974$.

atinine in nondiabetic patients with renal dysfunction ($P > 0.05$).

Accumulation of Albumin-Linked and Free-Form Pentosidine in Hemodialysis Plasma

To determine the plasma protein fraction(s) that was post-translationally modified with pentosidine, plasma proteins were separated by gel filtration and the pentosidine content was determined in each fraction. Figures 4A and B show representative profiles of pentosidine levels in the plasma fractions from diabetic and nondiabetic hemodialysis patients, respectively. In both groups, most pentosidine was found within the albumin fraction (indicated by B in Figure 4). We subsequently purified plasma albumin from these patients and attempted to separate pentosidine from the albumin. However, even with vigorous vortexing, ultrafiltration, dialysis against distilled water, and heparin or sodium-dodecyl sulfate treatment, separation was impossible (data not shown), suggesting a covalent interaction between pentosidine and albumin.

We assumed that free pentosidine is present in the plasma of end-stage renal failure patients, originating from the catabolism of body proteins linked with pentosidine and/or from the intestinal absorption of dietary pentosidine-linked proteins. In the chromatogram, we noted a small peak indicated by arrowheads

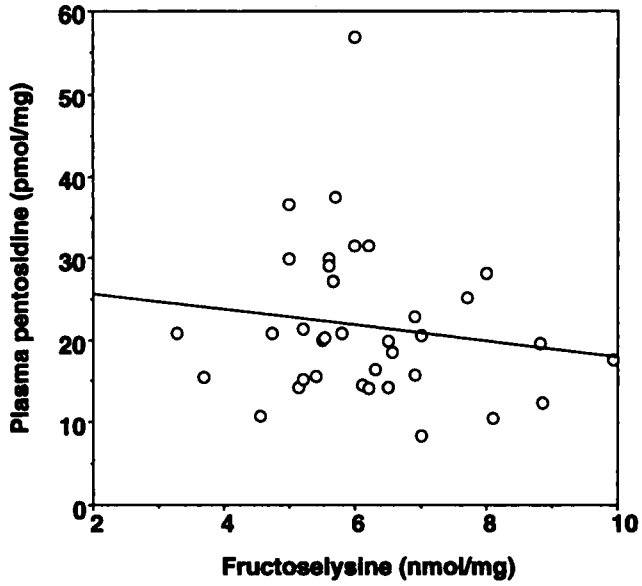

Figure 3. Correlation between plasma pentosidine and fructoselysine in diabetic hemodialysis patients (Group III: $N = 36$). Plasma pentosidine did not correlate with fructoselysine ($P > 0.05$). The equation of the line for diabetic patients with normal renal function is $y = 0.053X + 1.319$.

in Figure 4. This substance had a fluorescence typical of pentosidine with excitation-emission fluorescence maxima at 335/385 nm. Furthermore, fast atom bom-

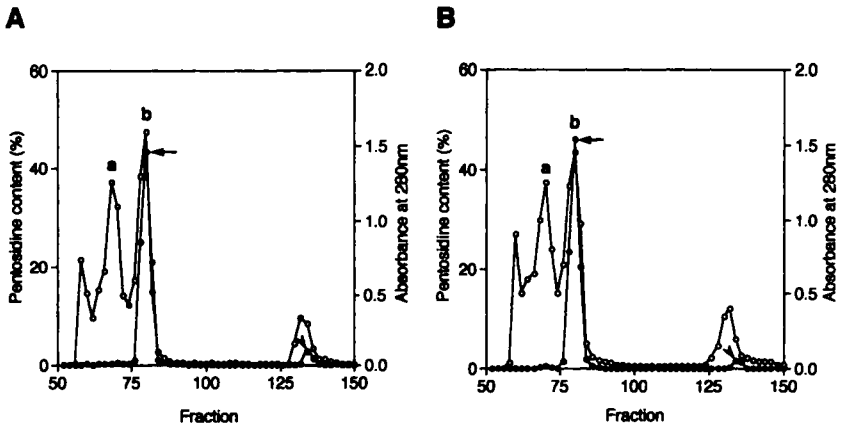

Figure 4. Representative profiles of pentosidine level in the plasma fractions of diabetic hemodialysis patient (A) and nondiabetic hemodialysis patients (B). Separation of plasma proteins was performed by gel filtration on a Sephacryl S-200 column chromatograph. The proteins in each fraction were detected by their absorbance at 280 nm. The pentosidine content in acid hydrolysate of each fraction was measured by HPLC assay and expressed as percentage of total plasma pentosidine. Closed circles, pentosidine level; open circles, absorbance at 280 nm. A, immunoglobulin G (150 kd); B, albumin (67 kd). Albumin-linked and free-form pentosidine are indicated by arrows and arrowheads, respectively.

bardment-mass spectrometry showed a measured mass of m/z 379.4 (data not shown), in good agreement with the theoretical value for pentosidine (379.4394 d for chemical mass). These observations indicate that pentosidine also exists in free form in the plasma of hemodialysis patients. As mentioned above, various treatments of albumin modified with pentosidine failed to release free pentosidine. This finding suggests that plasma free pentosidine is not a byproduct released from albumin modified with pentosidine during sample preparation.

By HPLC assay (Figure 5), a significant amount of free pentosidine was detected in the plasma of hemodialysis patients with (Group III: 59.84 ± 24.27 pmol/mL) and without diabetes (Group V: 53.67 ± 19.33 pmol/mL). The difference between the two groups was not statistically significant. Free pentosidine was not detected in the plasma of healthy and diabetic subjects with normal renal function.

Free pentosidine levels were statistically correlated with plasma total pentosidine levels in hemodialysis patients ($P < 0.0001$, $r^2 = 0.36$ and $r^2 = 0.54$ for diabetic and nondiabetic patients, respectively) (Figure 6). Free pentosidine levels, just as albumin-linked pentosidine, were not correlated with fructoselysine levels in diabetic hemodialysis patients ($P > 0.05$).

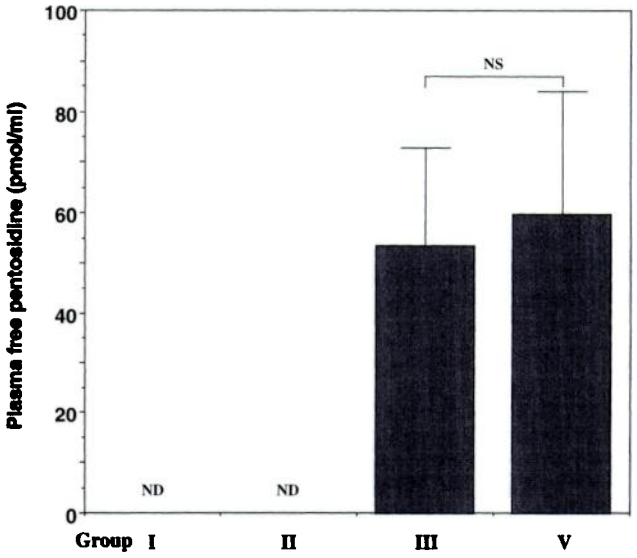

Figure 5. Plasma free pentosidine level in healthy subjects (Group I: $N = 20$), diabetic patients with normal renal function (Group II: $N = 26$), diabetic hemodialysis patients (Group III: $N = 36$) and nondiabetic hemodialysis patients (Group V: $N = 46$). Free pentosidine in the plasma was determined by HPLC assay after removing plasma proteins (see the Methods section). Data are expressed as means ± SD. ND, not detected.

Kinetics of Plasma Free Pentosidine in Healthy and Uremic Rats Given Pentosidine

In healthy rats fed a casein-based diet, neither albumin-linked nor free pentosidine was detectable in the plasma. After intravenous injection, free pentosidine was eliminated rapidly from the plasma and had almost completely disappeared within 2 h (closed squares in Figure 7). The calculated biological half-life averaged 0.46 ± 0.05 h. By contrast, in uremic rats, free pentosidine given intravenously had an appreciably prolonged half-life: 2.05 ± 0.68 h and 25.7 ± 2.76 h, respectively, in rats with a 6/7 (closed triangles) and a bilateral nephrectomy (closed circles). In the latter group, plasma free pentosidine level were mildly elevated (43.4 ± 8.2 pmol/mL) 48 h after administration. These findings suggest that the plasma free pentosidine clearance is closely linked to the level of renal function.

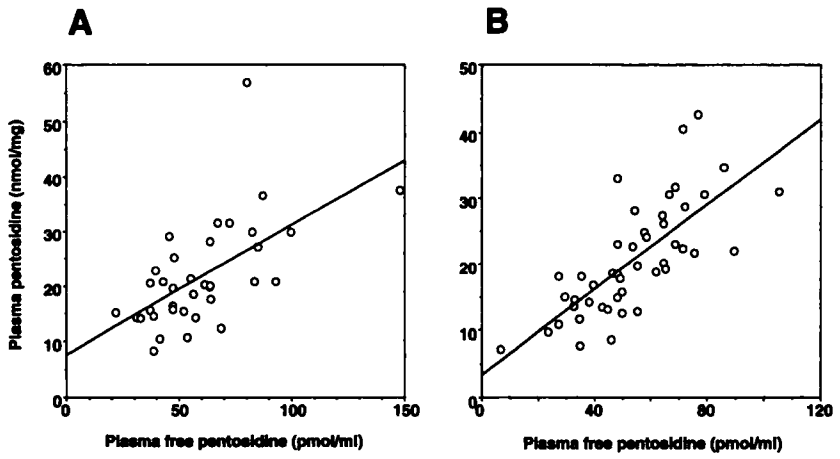

Figure 6. Correlation between albumin-linked pentosidine and free pentosidine in hemodialysis patients with diabetes (A, $N = 36$) or without diabetes (B, $N = 46$). There was a positive correlation between albumin-linked pentosidine and free pentosidine in hemodialysis patients with diabetes ($P < 0.0001$, $r^2 = 0.36$) and without diabetes ($P < 0.0001$, $r^2 = 0.54$). The equation of the line for hemodialysis patients with diabetes is: $y = 0.236X + 7.626$; and for hemodialysis patients without diabetes: $y = 0.322X + 3.347$.

On the basis of the assumption that dietary pentosidine could be absorbed into the circulation, pentosidine was given orally to healthy and uremic rats. In intact rats, plasma free pentosidine rose slightly and transiently to become undetectable at 6 h (calculated biological half-life of 1.32 ± 0.21 h: closed squares in Figure 8). In uremic rats, elimination was markedly delayed. In rats with 6/7 nephrectomy (closed triangles), plasma free pentosidine peaked at 3 h (121.0 ± 43.8 pmol/mL) and fell thereafter (calculated biological half-life of 4.08 ± 1.68 h). In bilaterally nephrectomized rats (closed circles), plasma free pentosidine level peaked at 12 h (149.0 ± 28.4 pmol/mL) and decreased subsequently (calculated biological half-life of 47.3 ± 10.2 h). These observations demonstrate that dietary pentosidine is absorbed by the gastrointestinal tract and confirm the relationship between renal function and pentosidine half-life.

In control animals that were not administered pentosidine, free pentosidine was detectable in plasma, neither in bilaterally nephrectomized rats at 50 h nor in 6/7 nephrectomized rats at 6 wk, despite marked increases in BUN. This finding suggests that, in nephrectomized rats, plasma free pentosidine was almost completely derived from dietary intake.

DISCUSSION

This article is the first to report that pentosidine accumulates in free form in the circulation of hemodialysis patients with end-stage renal failure, irrespective of the presence or absence of diabetes. One of the causes of plasma free pentosidine accumulation in these patients may be the loss of glomerular filtration, because the molecular weight of pentosidine (379 D) is low enough to allow complete filtration through the glomerular basement membrane. The results of the present *in vivo* kinetic study suggest indeed that the elimination of free pentosidine occurs through glomerular filtration. This assumption is further supported by the finding in this study that plasma free pentosidine was not detected in either healthy subjects or diabetic patients with normal renal function.

The mechanism of accumulation of albumin-linked pentosidine in hemodialysis patients, on the other hand, remains unclear. In diabetic patients with normal renal function, high plasma glucose is thought to accelerate the Maillard reaction and increase generation of albumin-linked pentosidine. By contrast, albumin-linked pentosidine levels increase by an order of magnitude (almost 10 times normal level) when renal failure develops, at which time there are no longer differences between diabetic and nondiabetic hemodialysis patients, meaning that whatever the effect of glucose, it is blurred or lost in the renal failure effect. Indeed, there was no statistical correlation between albumin-linked pentosidine and fructoselysine in diabetic hemodialysis patients. Because albumin-linked pentosidine is not thought to be filtered through the glomerular basement membrane, diminished glomerular filtration is unlikely to cause an increase in albumin-linked pentosidine. Thus, it is likely that, in addition to glucose, other substance(s) accumulating in uremic plasma might be a precursor of albumin-linked pentosidine as Odetti *et al.* suggested (26), or that some factor(s) might catalyze the glycoxidation and accelerate pentosidine formation on albumin even in the absence of high glucose levels. These contentions are supported by our unpublished observation that incubation of HSA in plasma ultrafiltrate resulted in a more rapid increase of albumin-linked pentosidine, if the plasma was obtained from hemodialysis patients rather than from subjects with normal renal function, irrespective of presence or absence of diabetes. Although the pentosidine precur-

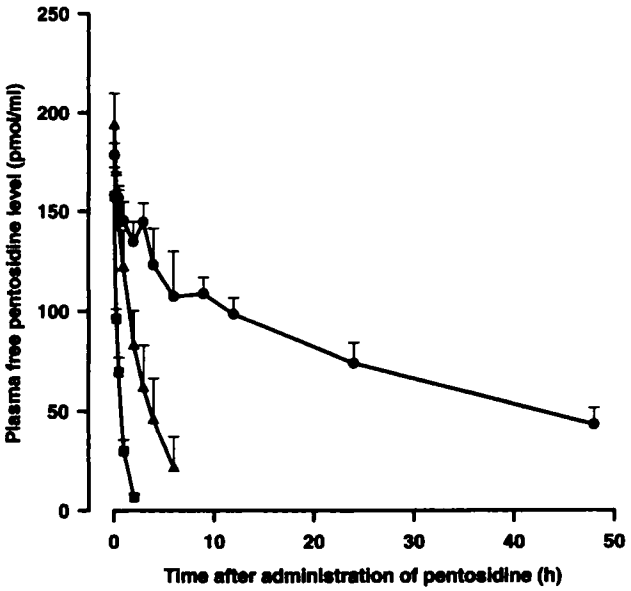

Figure 7. Elimination of plasma free pentosidine in normal and uremic rats with intravenous pentosidine administration. Bilaterally nephrectomized (closed circles: $N = 5$), 6/7 nephrectomized (closed triangles: $N = 5$), and intact rats with normal renal function (closed squares: $N = 5$) were intravenously given pentosidine (10 nmol/rat). Blood was taken at several points and plasma free pentosidine levels were determined by HPLC assay. The BUN levels in intact rats with normal renal function, 6/7 nephrectomized rats, and bilaterally nephrectomized rats were 15.5 ± 3.0, 69.2 ± 14.7, and 17.4 ± 6.32 mg/dL at the time of pentosidine administration, respectively, and 12.1 ± 1.5, 56.2 ± 19.3, and 106.0 ± 11.8 mg/dL at 24 h after administration, respectively. BUN level in bilaterally nephrectomized rats was 163.5 ± 6.34 mg/dL at 48 h after administration. Plasma free pentosidine was not detectable in all of the three control groups ($N = 5$, respectively) without pentosidine administration.

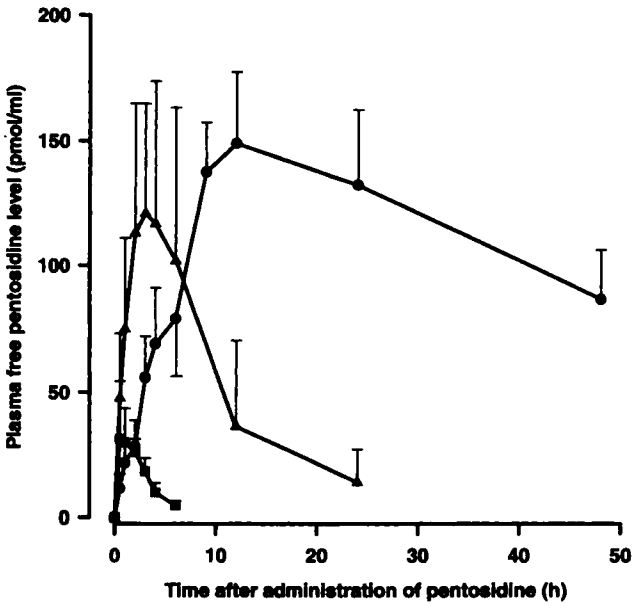

Figure 8. Absorption and elimination of free pentosidine in healthy and uremic rats with oral pentosidine administration. Bilaterally nephrectomized (closed circles: $N = 5$), 6/7 nephrectomized (closed triangles: $N = 5$), and intact rats with normal renal function (closed squares: $N = 5$) were orally given pentosidine (400 nmol/rat). Blood was taken at several points and plasma free pentosidine levels were determined by HPLC assay. BUN levels in intact rats with normal renal function, 6/7 nephrectomized rats, and bilaterally nephrectomized rats were 13.5 ± 2.5, 67.8 ± 14.9, and 13.7 ± 3.67 mg/dL at the time of pentosidine administration, respectively, and 11.9 ± 1.5, 63.0 ± 30.2, and 104.7 ± 6.58 mg/dL at 24 h after administration, respectively. BUN level in bilaterally nephrectomized rats was 164.2 ± 3.88 mg/dL at 48 h after administration. Plasma free pentosidine was undetectable in all the three control groups ($N = 5$, respectively) without pentosidine administration.

sor(s) accumulating in hemodialysis patients remains unknown, it has been demonstrated that ribose and its isomers, fructose, and even ascorbate oxidation products can be precursors of pentosidine *in vitro* (22,31,33).

The origin of plasma free pentosidine remains to be discussed. The *in vivo* study presented here indicates that dietary pentosidine can be absorbed into the circulation and might be an origin of plasma free penosidine. The fact that various kinds of food contain a significant amount of pentosidine even in free-form (T. Miyata, unpublished observation), together with the chemical stability of pentosidine, which is resistant to acid hydrolysis (31,33), further support this suggestion. The dietary intake of pentosidine might thus prove of importance in the management of patients with chronic renal failure. In addition, because several matrix proteins (22–25) and albumin contain a significant amount of pentosidine, plasma free pentosidine must be released during the degradation processes of these proteins. The present finding that free pentosidine was present in uremic rats orally administered pentosidine, but not in uremic rats untreated with pentosidine, suggests that dietary pentosidine may be an important source of the free pentosidine at least in rats. However, this consideration might not apply to humans, whose longer life span might be associated with a larger incorporation of pentosidine in the matrix tissues.

Plasma free pentosidine levels in renal failure patients result, therefore, from a balance between (*1*) factors raising plasma free pentosidine, such as dietary intake of pentosidine and release of pentosidine from catabolized matrix proteins that link pentosidine, and (2) a factor decreasing plasma free pentosidine, such as GFR. Although their origin remains unclear, plasma levels of free pentosidine may provide a useful biochemical marker for the plasma levels of advanced glycoxidation in hemodialysis patients. Indeed, plasma free pentosidine can be measured more rapidly and easily than albumin-linked pentosidine

and its level is highly correlated with albumin-linked pentosidine, which almost represents total plasma pentosidine.

Our present observations have to be reconciled with those previously reported by Makita *et al.* (34–36): (*1*) these authors indicated that, among hemodialysis patients, serum AGE levels are much higher in diabetic than in nondiabetic patients, whereas, in our study, the plasma pentosidine levels were almost equivalent in both groups; and (2) they detected significant amounts of AGE in low-molecular-weight peptides (<10 kd), whereas we demonstrated that albumin (67 kd) is the major pentosidine-modified protein in the plasma. They concluded that accumulation of AGE in the plasma of hemodialysis patients, particularly in diabetic patients, resulted from the decreased removal of low-molecular-weight AGE-modified peptides (35). Because AGE constitute a heterogeneous class of structures, these discrepancies may result from the heterogeneity of AGE and an attendant difference in the measured AGE structure. Recently, it has been suggested that the epitope of polyclonal anti-AGE antibody corresponds to carboxymethyllysine (37). This is supported by our preliminary observation that the monoclonal anti-AGE antibody (38) reacts with carboxymethyllysine-modified β2-microglobulin (and BSA). In diabetic patients (24), there is a significant positive correlation between the levels of pentosidine and carboxymethyllysine in skin (0.1 pmol pentosidine per μg protein, corresponding to approximately 4 to 7 pmol carboxymethyllysine per μg protein). Although the epitope-structure recognized by the radioreceptor assay (35) or anti-AGE antibody (36) used by Makita *et al.* is yet to be identified, it is notable that their anti-AGE antibody does not crossreact with pentosidine or carboxymethyllysine (36).

Elevated levels of AGE have been recently demonstrated in β2-microglobulin amyloid deposits (14), and skin and arterial-wall collagen (30,35) of hemodialysis patients. They have, therefore, been implicated in the pathogenesis of several complications of long-term hemodialysis, such as atherosclerosis (35), hyperlipidemia (39), and dialysis-related amyloidosis (10–15). Previously, some researchers suggested that plasma AGE accumulating in uremic patients are likely to reattach matrix proteins such as collagen (34) or plasma proteins such as low-density lipoproteins (39), and result in tissue damage leading to vascular lesions and hyperlipidemia. Whether free and/or albumin-linked pentosidine share the same properties remains to be evaluated. As already mentioned, pentosidine has been demonstrated in β2-microglobulin-amyloid deposits (14) and in several collagenous structures (22–25). Furthermore, pentosidine has been linked to oxidative processes because its formation requires oxidative reaction (21,24,27). Despite the fact that neither free nor albumin-linked pentosidine are known to accelerate generation of reactive oxygen species, their accumulation in hemodialysis patients may be indicative of increased oxidative stress.

β2-Microglobulin is a major constituent of amyloid fibrils in dialysis-related amyloidosis (40,41). We recently demonstrated the possible involvement of AGE-modified β2-microglobulin in amyloid fibrils in the pathogenesis of dialysis-related amyloidosis (10–15). β2-Microglobulin-linked pentosidine levels have not yet been measured in diabetic and nondiabetic hemodialysis patients. However, the present finding that albumin-linked pentosidine levels are similar in both groups suggests that the β2-microglobulin-linked pentosidine levels are also similar because the pentosidine precursor is thought to be identical in albumin and β2-microglobulin. This agrees with the observation that diabetic subjects do not seem more prone to develop dialysis-related amyloidosis (C. van Ypersele, unpublished observation).

Further studies to elucidate the precursor(s) of albumin-linked pentosidine, the origin of plasma free pentosidine, and the pathological role of pentosidine accumulating in uremic patients will be useful to better understand the pathophysiological role of AGE, not only in renal failure but also in other diseases.

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