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The Maillard protein cross-link pentosidine in urine from diabetic patients

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Summary. The Maillard protein cross-link pentosidine is a fluorescent condensation product of lysine, arginine and ribose. It accumulates in human tissues with age, and the accumulation process is accelerated in the tissues of diabetic patients. Using SP-Sephadex C-25 in the pretreatment for HPLC, we examined levels of pentosidine in urine without hydrolysis (free form) and levels of pentosidine in urine after hydrolysis (total forms), from 23 diabetic patients and 21 control subjects. The mean percentages of the values of free form per total forms (± SD) were 89 ± 15% in diabetic patients, 88 ± 16% in control subjects and 89 ± 15% in total populations of diabetic patients and control subjects. There was a significant correlation between the values of free form and total forms in diabetic patients ($r = 0.938, p = 0.0001$), in control subjects ($r = 0.820, p < 0.02$) and in total populations of diabetic patients and control subjects ($r = 0.951, p = 0.0001$). The mean level of pentosidine per mol creatinine (± SD) was significantly elevated in urine from diabetic patients as compared to the level in control subjects (8.8 ± 4.3 μmol/mol creatinine vs 4.2 ± 1.4 μmol/mol creatinine, $p = 0.0001$ in free form; 10.1 ± 5.3 μmol/mol creatinine vs 4.7 ± 1.4 μmol/mol creatinine, $p = 0.0001$ in total forms). These results demonstrate that urinary pentosidine, especially in free form, could be a useful marker for the assessment of diabetes and diabetic complications.

Key words: Fluorometry, HPLC, human urine, laboratory medicine.

Proteins in long-lived tissues are known to be modified by reducing sugars post-transcriptionally [1, 2]. Of these compounds, Amadori products of the sugars, especially glycated haemoglobin, are popular for use in the clinical treatment of patients with diabetes mellitus [3]. These unstable compounds undergo many reactions progressively in vivo, and result in advanced Maillard or glycation end products [1, 2]. Such modifications accelerate in their formation under diabetic conditions. Some stable end products such as N^E-(carboxymethyl)hydroxylysine, N^E-(carboxymethyl)lysine and fructoselysine were studied to determine the effects of diabetic conditions on their formation in humans [2, 4–6]. However, since the detection of these end products is complicated, the method may be unsuitable for clinical use.

Pentosidine, characterized by Sell and Monnier [7] in human dura mater collagen, is a bifunctional condensation product of arginine, lysine and ribose, and is a fluorescent compound. Recent studies have revealed that its formation in human tissues is affected by the diabetic condition [1, 2, 4, 5, 8–10]. Until recently, only one study measuring the levels of pentosidine in body fluids (i. e. pentosidine levels in hydrolysate of human plasma) has been reported by Odetti et al. [8]. Since their method required the combined reverse-phase ion-exchange HPLC, a new and simple method needs to be developed to analyse levels of pentosidine in body fluids as a marker for the assessment of diabetes and diabetic complications. In a previous study, we developed a method to measure pentosidine in hydrolysates of human urine using SP-Sephadex C-25 in the pretreatment for HPLC, and discovered that urinary pentosidine increased exponentially with age in normal subjects [11]. In this study, we applied this method to examine whether pentosidine in urine could be a biomarker in the management of patients with diabetes.

Subjects and methods

Subjects

Diabetic patients ($n = 23$, 13 males and 10 females) aged 46 to 75 years (mean ± SD, 62.2 ± 8.6 years) were randomly selected following routine visits to the Division of Internal Medicine at the Hamamatsu National Hospital in Hamamatsu, Shizuoka. All the patients had Type 2 (non-insulin-dependent) diabetes currently being treated with insulin or oral hypoglycaemic agents. Any

patient who showed renal dysfunction was excluded from the study. As an age-matched control group, urine was collected from 21 healthy volunteers (9 males and 12 females), aged 49 to 73 years (58.6 ± 6.9 years) with no history of diabetes and no current known disease. Urine samples were collected in the morning between 09.00 hours and 11.00 hours and stored at -30 °C until analysis.

Reagents

The following reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan), HPLC-grade acetonitrile (MeCN), sequenation-grade n-heptafluorobutyric acid (HFBA), reagent-grade HCl. All water used in these experiments was purified using a Milli-Q, Water Purification System (Millipore Corporation, Bedford, Mass. USA).

Prefractionation of urine or its hydrolysates with SP-Sephadex C-25

We identified the pentosidine in urine without hydrolysis as the free form of pentosidine, and that in a hydrolysate of urine as the total forms of pentosidine. After thawing, a 2-ml aliquot of urine was hydrolysed with an equal volume of 12 mol/l HCl at 110 °C for 20 h in a sealed glass tube. Urine (0.125 ml) or its hydrolysate (0.25 ml) was mixed with 15 ml of water and applied to an SP-Sephadex C-25 column (H^+ form, 0.8 × 1.0 cm; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) that had been equilibrated with water. The column was washed with 20 ml of 0.1 mol/l HCl and pentosidine was eluted with 5 ml of 1.0 mol/l HCl. The eluate was evaporated under vacuum, and the residue was dissolved in 200 µl of 1% HFBA. The solutions were stored at -30 °C prior to the HPLC analysis.

High-performance liquid chromatography

The HPLC system consisted of a Model CCPM pump (Tosoh, Tokyo, Japan), a Model FS-8010 spectrofluorometer (Tosoh), a Model AS-8010 autosampler (Tosoh), and a Model SC-8010 system controller (Tosoh). A column (8 mm × 10 cm) prepacked with Radial-Pak C18, of 10-µm particle size, type 8 C1810µ (Waters Associates Inc., Milford, Mass., USA) was used. A mobile phase of MeCN/30 mmol/l HFBA (27: 73, volume/volume) was used. The flow rate was 1.0 ml/min. The volume of each sample injected was 160 µl. For the detection of pentosidine, the fluorescence at 385 nm was measured on excitation at 335 nm. The minimum amount of pentosidine detectable (signal-to-noise ratio, 2) was 1.6 pmol per injection under our experimental conditions. The levels of pentosidine content in urine samples are expressed as micromoles of pentosidine per 1 mol of urinary creatinine.

Gel filtration chromatography with Sephadex G-25

Pentosidine in urine samples, hydrolysates of urine samples from healthy control subjects and diabetic patients or standard solution was fractionated by gel filtration chromatography on a Sephadex G-25 superfine (Pharmacia LKB Biotechnology AB) column (1.2 × 12.5 cm). Two hundred microlitres of each sample which contained 25 mg of NaCl was applied on the column equilibrated with 20 mmol/l phosphate buffer, pH 7.4, containing 0.9% NaCl. Fractions of 0.92 ml were collected. Blue dextran (Pharmacia LKB Biotechnology AB) and NaCl were found to elute in fraction 5 and fractions 16–17, respectively. The content of pentosidine in each fraction was assayed by fluorometry on HPLC as described above.

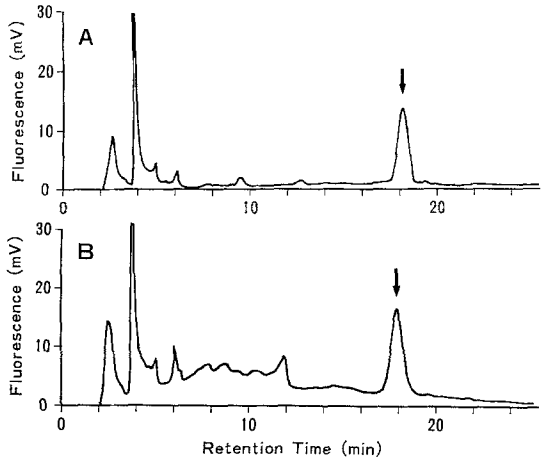

Fig. 1 A, B. Chromatograms of SP-Sephadex C-25 elute: **(A)** urine, **(B)** hydrolysate of urine. Excitation/emission wavelength was 335/385 nm. Arrow indicates elution position of authentic pentosidine

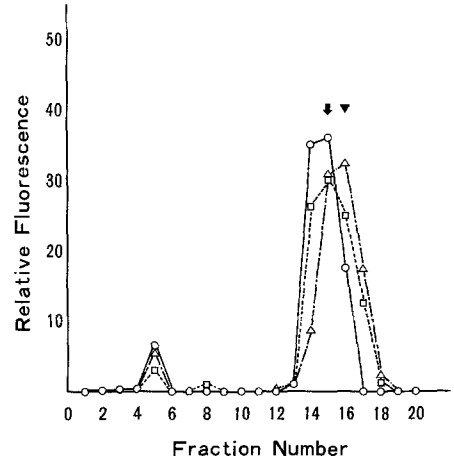

Fig. 2. Chromatograms of urine on Sephadex G-25 superfine: control subjects, —○—; diabetic patients, ----□----; hydrolysates, ---△---. Excitation/emission wavelength was 335/385 nm. Each point was calculated by the chromatography of three different samples. Arrow indicates elution position of authentic pentosidine. Arrowhead indicates elution position of NaCl

Preparation of standard solution

Pentosidine was isolated from human articular cartilage. Methods of purification and characterization were shown in our previous paper [12].

Urinary creatinine

Before hydrolysis, urinary creatinine content was determined enzymatically on an aliquot of a urine sample using a Shimadzu CL-20 clinical chemistry analyser (Shimadzu, Kyoto, Japan).

Urinary hydroxyproline

The hydroxyproline content in hydrolysed samples was measured in a Model 835–50 automated amino acid analyser system (Hitachi, Tokyo, Japan).

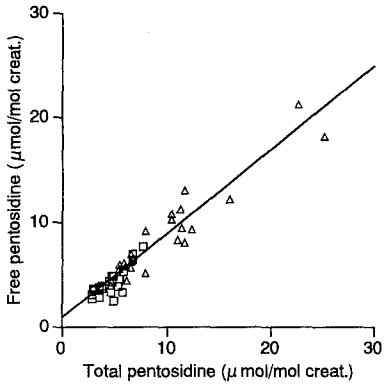

Fig. 3. Correlation between total and free pentosidine in urine from diabetic patients and control subjects. Diabetic patients, △; control subjects, □. Line indicates linear regression equation in the total of diabetic patients and control subjects: free form of pentosidine = 0.794 × (total forms of pentosidine) + 1.186, $r = 0.938$, $p = 0.0001$

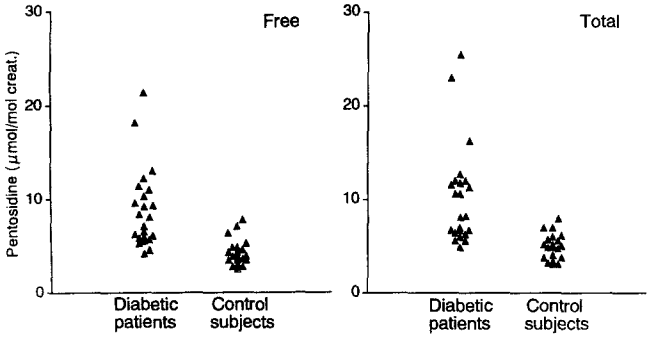

Fig. 4. Pentosidine concentration in urine samples. Free, pentosidine in urine without hydrolysis; Total, pentosidine in hydrolysate of urine

Statistical analysis

Statistical analyses were performed with a StatView II program on a Macintosh computer. The statistical significance and correlation were determined with non-parametric statistics by Mann-Whitney U tests and the Spearmann rank correlation method, respectively. *P* values below 0.05 were considered significant.

Results

Typical chromatograms of free form and total forms of pentosidine in urine from a 46-year-old diabetic male are shown in Figure 1. The chromatogram of free form (Fig. 1 A) was simpler and clearer than that of total forms (Fig. 1 B). This seems to suggest that some artifacts were produced during hydrolysis. Figure 2 shows typical gel-filtration chromatograms of pentosidine in urine samples, hydrolysates of urine samples or standard solution on a Sephadex G-25 superfine. Each point was obtained by the chromatography of three different samples. Since the arrow in the figure indicates the elution position of standard pentosidine, we conclude that most of the free form pentosidine has almost the same molecular weight as that of standard pentosidine. The recovery of standard pentosidine ($n = 6$, mean ± SD) and the coefficient of variation in the hydrolysate were 95.4 ± 2.6 and 2.9 %, respectively. In addition, since the level of free form pentosidine seemed to be the same as for the total forms of pentosidine, we compared values of two forms in each population of diabetic patients and control subjects and total populations of diabetic patients and control subjects. There was significant correlation between the values: free form pentosidine (μmol/ mol creatinine) = 0.753 × (total forms of pentosidine) + 1.186, $r = 0.938$, $p = 0.0001$ in diabetic patients; free form pentosidine = 0.829 × (total forms of pentosidine) + 0.2, $r = 0.820$, $p < 0.02$ in control subjects; free form pentosidine = 0.794 × (total forms of pentosidine) + 0.583, $r = 0.951$, $p = 0.0001$ in the combined study populations (Fig. 3). The mean percentages (± SD) of the free form per total forms were 89 ± 15 % (range = 64 to 112 %) in diabetic patients; 88 ± 16 % (range = 48 to 112 %) in control subjects; 89 ± 15 % in the total population.

Furthermore, we compared the values of the two forms per 1 mol of urinary creatinine between diabetic patients and control subjects. The mean value (± SD) in diabetic patients was significantly higher than it in control subjects: 8.8 ± 4.3 μmol/mol creatinine vs 4.2 ± 1.4 μmol/mol creatinine, $p = 0.0001$ for free form; 10.1 ± 5.3 μmol/mol creatinine vs 4.7 ± 1.4 μmol/mol creatinine, $p = 0.0001$ for total forms (Fig. 4).

Discussion

During the first stage of the discovery of pentosidine, there was uncertainty as to whether it was formed artificially during acid hydrolysis [7]. Our results showed that more than 80 % of pentosidine was present in free form and most of it remained intact after hydrolysis. These results support the idea that pentosidine is an endogenous component in humans.

Pentosidine is known to accumulate in human tissues with age and its accumulation accelerates in patients with diabetes and uraemia [1, 2, 4, 8–10]. Although we first detected pentosidine in urine, the origin of pentosidine is unclear. Currently, there are two hypotheses: one is that pentosidine is formed with free forms of reducing sugars, arginine and lysine, but since it takes a long time for pentosidine formation reactions to occur under physiological conditions [1, 2, 7], it seems unlikely that this mechanism would cause the production of pentosidine in vivo. The other hypothesis is that pentosidine is produced by degradation reactions of proteins in which pentosidine has accumulated. Hydroxyproline concentrations (mean ± SD) of hydrolysates of the urine samples were 5.93 ± 2.22 mmol/mol creatinine in diabetic patients and 6.98 ± 2.02 mmol/mol creatinine in control subjects. From these values, the concentrations of pentosidine in human urine were about 1.63 nmol/mg collagen in diabetic patients and about 0.64 nmol/mg collagen in control subjects, and the urinary excretion of pentosidine was approximately 75 μg per day in diabetic patients and 29 μg per day in the control subjects. The concentrations of pentosidine per mg collagen shown in this report are the

highest of all results previously reported [4, 5, 7–9], which suggests that there are some mechanisms in tissues which prevent pentosidine from accumulating. We currently have no data which identifies the cause of increased concentration of pentosidine in the urine of diabetic patients; however, it is possible that oxidative stress may play an important role [6].

In this study, using cation exchange chromatography (SP-Sephadex C-25), we have shown that more than 80 % of urinary pentosidine is present in its free form. We also provide the evidence that the levels of urinary pentosidine in patients with diabetes were significantly higher than those in control subjects. Pentosidine is a fluorescent compound, and using fluorometry with HPLC, we could measure pentosidine in urine at a concentration as low as 1.6 pmol per injection. Therefore, these results suggest that the free form of pentosidine in human urine could be a biomarker for diagnostic use and will be useful in the management of diabetic patients. Our results also reveal that it may be possible to develop a new method for detection of the levels of urinary pentosidine using immunological techniques.

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