The Involvement of Pentosidine in Aortic Calcification Associated with Hemodialysis Patients

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Abstract

BACKGROUND/AIM: We measured serum level of pentosidine in 39 dialysis patients, whose atherosclerotic change was evaluated by means of aortic calcification index (ACI) and tried to confirm a significant correlation between them.

METHOD: Thirty-nine dialysis patients (22 men, 17 women) aged 20-79 years (52.4 ± 10.8 years) were studied. The mean duration of HD was 141.8 ± 52.4 months. ACI was assessed using abdominal CT scans obtained from 1993 to 1997, and the annual change (Δ ACI) was calculated. Determination of pentosidine was performed by a competitive ELISA method.

RESULTS: ACI was increased progressively and significantly as follows: 21.1 ± 18.7 in 1993, 32.4 ± 21.2 in 1995, and 38.4 ± 21.8 in 1997 ($p < 0.0001$). Δ ACI (1993-1997) was 4.0 ± 2.16 on average. Serum concentration of pentosidine was remarkably increased to as high as 2425 ± 1161 pmol/mL on average. Serum value of pentosidine/mgTP correlated weakly, but significantly with ACI ($p = 0.353$ $p = 0.0297$), but not Δ ACI in univariable analysis. Multiple regression analysis showed that pentosidine/mg total protein (TP) together with calendar age were significant explanatory variables for ACI.

CONCLUSION: Pentosidine is probably involved in some process common to an ubiquitous mechanism of maturation in atherosclerosis.

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Introduction

Pentosidine is an advanced glycation endproduct (AGE) molecule firstly identified in 1989 by Sell and Monnier¹⁾. Soon after, pentosidine was reported to be detected in various human tissues including the aorta²⁾ and amyloid tissue in dialysis patient, too³⁾. Vlassara et al.⁴⁾ showed AGE substance to be involved in atherosclerotic vasculopathy. Scleicher et al.⁵⁾ similarly, detected carboxymethyl lysine, another chemically identified AGE molecule, in human aortic tissue.

As for dialysis patients, premature atherosclerosis has been the most vital problem for their survival since as early as 1974⁶⁾. Kabaya et al.⁷⁾ documented an augmented and expanded arterial calcification in a dialysis patient. Current research has indicated growing evidence of OX-LDL, Lp(a) and homocystein contributing to the development and progression of atherosclerosis in dialysis patients⁸⁻¹⁰⁾. To date, however, we can find no report that describes an implication of pentosidine in cardiovascular pathology in dialysis patients.

We measured the serum level of pentosidine in 39 dialysis patients, whose atherosclerotic change was evaluated by means of aortic calcification index (ACI), and could confirm a significant correlation between them.

Patients and Methods

Patients

Thirty-nine dialysis patients (22 men, 17 women) aged 20-79 years (52.4 ± 10.8 years) were studied after informed consent. They underwent regular hemodialysis (HD) using bicarbonate dialysate under heparin sodium anticoagulation 2 or 3 times a week.

The primary disease of chronic renal failure was glomerulonephritis in 38 patients, and polycystic kidney disease in one patient. The mean duration of HD was 141.8 ± 52.4 months, varying from 41 to 258, in which 29 long-term hemodialysis patients of more than 100 months were included.

All patients do not have lipid rich meals. Fifteen patients use antihypertensive drugs. Thirty patients use active vitamin D supplements. Twenty-nine patients use precipitated calcium carbonate.

Assessment of ACI

ACI was assessed according to the method reported originally by Yukawa et al.¹¹⁾ with some modification using abdominal CT scans obtained from 1993 to 1997.

In brief, an abdominal aorta was scanned from the diaphragm to the aortic bifurcation in consecutive sequential 10 mm slices by CT9200 (Yokogawa Co. Ltd, Tokyo). The circumference of the aorta in each slice was divided into 12 segments and the segments with calcification were counted in each slice. Then, the accumulative number of segments with calcification was divided by the total number of slices and ACI was calculated by means of a percentage according to the following formula:

$$\text{ACI} = (\text{number of segments with calcification in each slice}) \times 1/12 \times 100 / (\text{total number of slices}) \times 100$$

In addition, the annual change (ΔACI) was calculated according to the following formula:

$$\Delta\text{ACI}(\text{1993-1997}) = [\text{ACI}(\text{1997}) - \text{ACI}(\text{1993})] / \text{interval time (M)} \times 12$$

ACI was measured by one author (J.N) and the intra-assay CV value was 7.5% when 5 measurements were done in 5 patients who were selected at random.

Determination of pentosidine by a competitive ELISA

Pentosidine standard and specific anti-pentosidine polyclonal antibody were kindly provided by Dr. Miyata (Tokai University of School of Medicine). Determination of pentosidine was performed by a competitive ELISA method as described by Miyata¹²⁾ with some modifications. Each sample (200 μL) was hydrolyzed in 12 M HCL overnight at 107°C , neutralized with 6M Na_2CO_3 , and diluted with 0.5M phosphate buffer (pH 7.4). Sample (serum or standard) and anti-pentosidine rabbit polyclonal antibody were placed in a microplate well coated with pentosidine, and the plate was incubated overnight at 4°C . Each well was washed, followed by incubation with peroxidase-labeled anti-rabbit IgG (Immunotech, France) for 60 min at room temperature. After washing any unbound anti-pentosidine antibody, the bound pentosidine was measured using tetramethylbenzidine as the chromogen. Absorbance at 450nm was measured with a microplate reader system (model EAR340AT, SLT-LAB INSTRUMENTS, Austria).

Laboratory tests

Lp(a) was measured by ELISA. Other parameters were determined using standard laboratory tests.

Statistical analysis

Data are expressed as the mean $\pm$ SD. The significance of changes in ACI over time was analysed by One-Way Repeated ANOVA, and ΔACI was assessed using the paired Student's t-test. Simple regression analysis of pentosidine relative to other parameters was done with the Pearson's correlation coefficient, while ACI and ΔACI , were assessed using Spearman's correlation coefficients. Multivariate analysis was performed by multiple regression analysis. For all methods, $p < 0.05$ was regarded as significant.

Results

1. ACI and ΔACI

ACI was increased progressively and significantly as follows: 21.1 ± 18.7 in 1993, 32.4 ± 21.2 in 1995, and 38.4 ± 21.8 in 1997 ($p < 0.0001$) (Fig. 1).

ΔACI decreased significantly from 4.8 ± 3.1 during the first period from 1993 to 1995 compared to 3.0 ± 3.2 during second period from 1995 to 1997. $\Delta\text{ACI}(\text{1993-1997})$ was 4.0 ± 2.2 on average.

A significant correlation between ACI (1997) and age ($\rho = 0.428$ $p = 0.0083$), and between the duration of HD and ΔACI (1993-1997) ($\rho = -0.388$ $p = 0.0169$) could be found.

2. Laboratory data

Routine laboratory data are listed in Table 1. Serum Ca and Pi were determined twice a month. The mean values throughout the year of 1997 were 2.47 ± 0.15 mmol/L for Ca, 2.16 ± 0.32 mmol/mL for Pi, and 5.32 ± 0.80 for [Ca $\times$ P]. Twenty (51.3%) patients showed a serum Pi of 2.26 mmol/L or more and 11 (28.2%) patients showed a high value of Ca $\times$ Pi ≥ 5.64 .

Dyslipidemia was found in 28 patients in all, hypercholesterolemia was more than 5.69 mmol/L in 3 (7.7%), hypertriglycemia was more than 1.69 mmol/mL in 13 (33.3%), and hypo-high density lipoproteinemia was less than 1.03 mmol/L in 24 (61.5%) patients. As for atherogenic lipid, the serum value of LDL was 2.40 ± 0.85 mmol/L varying from 1.24 to 4.76 mmol/mL. In addition, the serum value of Lp(a) was 187 ± 122 mg/L varying from 10 to 540 mg/L in which a high level of more than 300 mg/L could be found in 8 (20.5%) patients.

3. Serum pentosidine

The serum concentration of pentosidine was remarkably increased to as high as 2425 ± 1161 pmol/mL on average (range: 1021 to 6669 pmol/mL) compared with 189.0 ± 45.2 pmol/mL in healthy subjects.

We calculated pentosidine/mg TP, because 95% of the serum pentosidine we measured was linked to protein.

Similarly, the serum level of pentosidine/mgTP was remarkably increased to as high as 37.7 ± 19.0 nmol/mgTP on average (range: 16.2 to 98.1 nmol/mgTP).

There was no significant correlation of pentosidine/mgTP with age, or duration of HD.

4. Correlations between ACI or Δ ACI and pentosidine/mgTP

The serum value of pentosidine/mgTP correlated weakly, but significantly with ACI ($\rho=0.353$ $p=0.0297$) (Fig. 2), but not Δ ACI in univariable analysis.

On the other hand, multiple regression analysis (dependent variable: ACI, explanatory variables: age, duration of HD, serum Ca $\times$ Pi, HDL, pentosidine/mgTP) showed that pentosidine/mgTP together with calendar age are significant explanatory variables for ACI (Table 2).

However, multiple analysis with the same explanatory variables showed no significant relationships between serum pentosidine and Δ ACI.

Discussion

In dialysis patients, a persistent excess of oxidative stress has been well documented¹³⁾. As clearly stated in Ross's hypothesis¹⁴⁾, oxidative stress is assumed to be a very important factor in the pathomechanism of atherosclerosis. Current research¹³⁾ concerning AGEs has proven that pentosidine, as well as carboxymethyl lysine, is an integral marker of persistent oxidative stress.

Pentosidine and carboxymethyl lysine have both been reported to be highly elevated in the circulation of uremic and dialysis patients¹³⁾. Yet, we can find no data regarding the direct participation of oxidative stress in

Fig. 1 The change with the passage of time of ACI: ACI was increased progressively and significantly as follows: 21.1 ± 18.7 in 1993, 32.4 ± 21.2 in 1995, 38.4 ± 21.8 in 1997 ($p < 0.0001$).

Fig. 2 Correlation between pentosidine/mgTP and ACI (1997): Serum value of pentosidine/mgTP correlated weakly, but significantly with ACI ($\rho=0.353$ $p=0.0297$).

Table 1 Clinical data

Hb (g/L)	89 ± 13	(60–120)
BUN (mmol/L)	28.7 ± 4.96	(17.2–39.4)
CRE (μ mol/L)	1175 ± 230	(663–1688)
TP (g/L)	63 ± 5.0	(54–78)
Ca (mmol/L)	2.47 ± 0.15	(2.12–2.72)
Pi (mmol/L)	2.16 ± 0.32	(1.29–2.78)
Ca $\times$ P	5.32 ± 0.80	(3.25–6.90)
TC (mmol/L)	4.12 ± 1.11	(2.59–7.42)
TG (mmol/L)	1.43 ± 0.75	(0.35–2.98)
HDL (mmol/L)	1.04 ± 0.34	(0.52–1.84)
Lp(a) (mg/L)	187 ± 122	(10–540)

Table 2 Multiple regression analysis of ACI ($R^2=0.369$, $p=0.0073$)

Explanatory variable	Regression coefficient	Standard error	Standard regression coefficient	t	p
Age	.921	.295	.456	3.122	.0037
Duration of HD	-.029	.064	-.068	-.443	.6608
Ca $\times$ P	-4.08×10^{-4}	4.027×10^{-4}	-.149	-1.014	.3180
HDL	-19.174	9.940	-.295	-1.929	.0624
*pento./mgTP	.402	.164	.350	2.450	.0197

*pento.: pentosidine

aortic calcification. This process is presumed likely to develop in the subendothelial space under a damaged endothelium and unlikely to develop in an intact layer of vasculature in a patient.

Aortic calcification (AC) is one of the clinical features commonly found in dialysis patients who are inevitably compelled to survive in a nonphysiological state such as a dialysis setting. However, AC is not a clinical feature common to all dialysis patients. AC was generally found along with the aging process and was associated with progeria in limited cases¹⁵. Very recently, Kuro-o et al.¹⁶ identified a key aging gene, the Klotho gene, in mice in which severe AC could be recognized.

Originally, AGE had been reported to be a deranged protein, part of the aging process¹⁷. As for pentosidine, Sell and Monnier reported a progressive increase of pentosidine in skin collagen together with increasing age¹⁸. Based upon those considerations, our data of a significant correlation between AC and pentosidine (Table 2) could be interpreted to indicate not only an involvement of pentosidine in AC, but with an accelerated aging process which develops in the clinical setting of dialysis.

The background of AC was multifactorial and included several factors already recognized; abnormal parathyroid function, high product value of Ca $\times$ P, dyslipidemia and hyper homocysteinemia. Furthermore, it is anticipated that predisposing factors for AC might vary not only from patient to patient, but also from time to time.

As a consequence, we cannot recognize a significant correlation between a probable predisposing factor and AC from clinical sample in dialysis patients. Nevertheless, we did obtain a significant correlation between the serum level of pentosidine/mgTP and AC in this study. It could be interpreted that pentosidine is probably involved in some process common to the mechanism of maturation in atherosclerosis. Currently, we are lacking data about that mechanism and need more information based on evidence.

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References

- 1) Sell DR, Monnier VM: Structure elucidation of senescence cross-link from human extracellular matrix. *J Biol Chem* 264: 21597-21602, 1989
- 2) Hoshino H, Takahashi M, Kushida K, Ohishi T, Kawanaka K, Inoue T: Quantitation of the crosslinks, pyridinoline, deoxypyridinoline and pentosidine, in human aorta with dystrophic calcification. *Atherosclerosis* 112: 39-46, 1995
- 3) Miyata T, Ueda Y, Shinzato T, Iida Y, Tanaka S, Kurokawa K, Strihow CY, Maeda K: 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. *J Am Soc Nephrol* 7: 1198-1206, 1996
- 4) Vlassara H, Li YM, Imani F, Wojciechowicz W, Yang Z, Liu F, Cerami A: Identification of galectin-3 as a high-affinity binding protein for advanced glycation end products (AGE): A new member of the AGE-receptor complex. *Molecular Medicine* 1: 634-646, 1995
- 5) Scleicher ED, Wagner E, Nerlich AG: Increased accumulation of the glycoxylation product N ϵ -(carboxymethyl) lysine in human tissues in diabetes and aging. *J Clin Invest* 99: 457-468, 1997
- 6) Linder A, Charra B, Sherrard DJ, Scribner BH: Accelerated atherosclerosis in prolonged maintenance hemodialysis. *N Engl J Med* 290: 697-701, 1974
- 7) Kabaya T, Nitta K, Kimura H, Kawashima A, Narusawa K, Nihei H: Increased aortic calcification index in hemodialysis patients. *Nephron* 81: 354-355, 1999
- 8) Holvoet P, Donck J, Landeloos M, Brouwers E, Luijckens K, Arnout J, Lesaffre E, Vanrenterghem Y, Collen D: Correlation between oxidized low lipoproteins and von Willebrand factor in chronic renal failure. *Thromb Haemost* 76: 663-669, 1996
- 9) Kronenberg F, Katherein H, Konig P, Neyer U, Sturm W, Lhotta K, Grochenig E, Utermann G, Dieplinger H: Apolipoprotein(a) phenotypes predict the risk for carotid atherosclerosis in patients with end-stage renal disease. *Arterioscler Thromb* 14: 1405-1411, 1994
- 10) McCully KS: Homocysteine and vascular disease. *Nature Med* 2: 386-389, 1996
- 11) Yukawa S, Mori K, Nishikawa O, Nomoto H, Nishikawa N, Okuda R, Takuda H, Nishide I: Aortic calcification index and pulse wave velocity in chronic hemodialysis patients. *J Atheroscler Thromb* 13: 1237-1243, 1985
- 12) Miyata T, Tanada S, Kawai R, Ueda Y, Horiuchi S, Hara M, Maeda K, Monnier VM: Identification of pentosidine as a native structure for advanced glycation end products in β 2-microglobulin-containing amyloid fibrils in patients with dialysis-related amyloidosis. *Proc Natl Acad Sci USA* 93: 2353-2358, 1996
- 13) Miyata T, Wada Y, Cai Z, Iida Y, Horie K, Yasuda Y, Meda K, Kurokawa K, Charles YS: Implication of an increased oxidative stress in the formation of advanced glycation end products in patients with end-stage renal failure. *Kidney Int* 51: 1170-1181, 1997
- 14) Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362: 801-809, 1993
- 15) Baker PB, Baba N, Boesel CP: Cardiovascular abnormalities in progeria. Case report and review of the literature. *Arch Pathol Lab Med* 105:384-386, 1981
- 16) Kuro-o M, Matsumura Y, Aizawa H, et al.: Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 390: 45-51, 1997
- 17) Araki N, Ueno N, Chakrabarti B, Morino Y, Horiuchi S: Immuno-chemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. *J Biol Chem* 267: 10211-10214, 1992
- 18) Sell DR, Lane MA, Johnson WA, Masoro EJ, Mock OB, Reiser KM, Fogarty JF, Cutler RG, Ingram DK, Roth GS, Monnier VM: Longevity and the genetic determination of collagen glycoxylation kinetics in senescence. *Proc Natl Acad Sci USA* 93: 485-490, 1996