Free *p*-Cresol Sulfate Is Associated with Survival and Function of Vascular Access in Chronic Hemodialysis Patients

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Key Words

Vascular access • Hemodialysis • Uremic toxins

Abstract

Background: Protein-bound uremic toxins indoxyl sulfate (IS) and *p*-cresol sulfate (*p*-CS) have been implicated as an important factor in uremic syndrome. Recent evidence indicates that both IS and *p*-CS are predictors of cardiovascular as well as all-cause mortality among chronic dialysis patients. We conducted a study to analyze the relationship between IS and *p*-CS and vascular access (VA) outcome in chronic hemodialysis (HD) patients. **Methods:** A total of 91 chronic stable HD patients were divided into groups according to survival of VA and frequency of VA dysfunction. Demographic and biochemical data were reviewed and recorded. Serum levels of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1, and the total and free forms of IS and *p*-CS were determined. **Results:** Patients with a history of frequent VA failure and dysfunction had lower albumin and higher levels of ICAM-1, free IS, free and total *p*-CS. Diabetes was associated with higher IS and *p*-CS. Logistic regression revealed that diabetes and free *p*-CS were independent factors associated with poor outcome of VA.

Conclusion: Endothelial dysfunction and uremic toxins were associated with survival and function of VA. Diabetes and free *p*-CS were significantly related to the outcome of VA among chronic HD patients.

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Background

Patent and functional vascular accesses are crucial for uremic patients who need chronic hemodialysis (HD). They are anastomosed with an artery and a vein directly or by a grafted Gortex against normal physiology and hemodynamics. A variety of factors such as mechanical stress (e.g. surgery, needle punctures and angioplasty), physical alteration (e.g. hemodynamic change) and chemical stress (e.g. dialysate, artificial kidney, arterio-venous graft and tubing) affect survival and function of the vascular accesses [1]. In general, vascular access is subject to repeated punctures with resultant vascular in-

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jury and subsequent repair. Strategies to prevent vascular injury and prolong the survival of vascular access are of utmost importance for chronic HD patients. Those modifiable factors should thus be identified and treated appropriately.

As chronic kidney diseases progress, molecules that are metabolized or secreted via the kidney will accumulate in the body and elicit deleterious effects. Although HD therapy can effectively remove excessive fluid and small solutes, it increases our concern that protein-bound uremic toxins such as indoxyl sulfate (IS) and *p*-cresol sulfate (*p*-CS) are not eliminated by traditional HD efficiently [2]. A previous study [3] revealed that IS and *p*-CS inhibit endothelial proliferation and wound repair. Furthermore, IS causes endothelial and vascular dysfunction by promoting vascular smooth muscle cell proliferation [4]. Levels of IS were also associated with increased aortic calcification and vascular stiffness in patients with chronic kidney disease [5]. On the other hand, endothelial dysfunction is a well-recognized complication of uremia [6]. Nevertheless, the role of endothelial dysfunction in function and survival of vascular access in chronic HD has not been illuminated clearly.

We hypothesize that the protein-bound uremic toxins can cause vasculopathy and thus are detrimental to the function and survival of vascular access in HD patients. A cross-sectional study was conducted to analyze the association between serum levels of total and free IS and *p*-CS, and outcome of vascular access in chronic HD patients.

Methods

Patients

Stable adult uremic patients that have been on regular HD therapy in our hospital for at least 6 months were recruited. The HD therapy proceeds with 4.0 h per session and 3 sessions per week. We retrospectively reviewed their history of vascular access surgery or angioplasty during the period from January to December 2010. Patients who experienced at least three episodes of vascular access dysfunction that needed angioplasty or re-creation of a new vascular access were enrolled as the high-risk group. Patients who did not experience any vascular access dysfunction, no history of angioplasty or re-creation of a new vascular access either with native arteriovenous fistula (AVF) maintained for more than 5 years were identified as low-risk group.

Patients were excluded if there was a chronic inflammatory disease such as rheumatoid arthritis, systemic lupus erythematosus, malignancy, or active infectious disease. Patients with a recent infection (occurring within 3 months prior to entry into this study) were not eligible. Informed consent was obtained from each enrolled patient. This study was approved by the Institu-

tional Review Board of Chang-Gung Memorial Hospital (IRB No. 99-1304C). All patients received three sessions HD every week. Demographic data, including age, gender, types of shunt, duration of HD therapy and body mass index (BMI) were reviewed and recorded. Diabetes was defined based on the guidelines of the American Diabetes Association (ADA). Hypertension was identified as those with predialysis blood pressure >140/90 mm Hg or already on antihypertensive agents. Medication with statins and antiplatelets or anticoagulants continuously for at least 6 months was reviewed and recorded. Blood samples were collected on the midweek dialysis day prior to the start of HD. To present biochemical data, results of monthly laboratory tests of 12 months before the study were collected and averaged. The adequacy of HD was estimated by calculating Kt/V (Daugirdas).

Measurements of Inflammatory Cytokines and Adhesion Molecules

Blood levels of interleukin (IL)-18 were determined using the commercial kits (Quantikine Human IL-18; Medical and Biological Laboratory, Nagoya, Japan). Levels of intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) were determined using commercial kits (Quantikine® Human sICAM-1/CD54 and Quantikine® Human sVCAM-1; R&D Systems, Inc., USA). Levels of high-sensitivity C-reactive protein (hs-CRP) were detected by nephelometry (Behring Diagnostics, Marburg, Germany).

Measurement of Blood Concentration of Total and Free IS and p-CS

Serum samples were deproteinized by the addition of 3 parts methanol to 1 part serum for determination of IS. Total *p*-CS (i.e. combined free and protein-bound fraction) was analyzed after deproteinization (acid and heat) and extraction (ethyl acetate) of serum samples. Free concentrations of IS and *p*-CS were measured in serum ultrafiltrates obtained using Microcon YM-30 separators (Millipore, Billerica, Mass., USA), followed by the same sample preparation and analysis. A UPLC assay was performed at room temperature on an Acquity UPLC BEH phenyl column of 2.1 × 100 mm (Waters, Milford, Mass., USA). Buffer flow was 0.4 ml/min using 10 mmol/l NH₄H₂PO₄ (pH 4.0) (A) and 100% acetonitrile (B) with a gradient from 82.5% A/17.5% B to 55% A/45% B over 9 min. Under these conditions, IS and *p*-CS appeared at 1.4 and 2.75 min, respectively. There were standard curves for IS and *p*-CS at 0.5, 1, 2.5, 5, and 10 mg/l; processed samples had average *r*² values of 0.999 ± 0.001. Quantitative results were obtained and calculated as concentrations in milligrams per liter. Quantitative results were obtained and calculated as concentrations in milligrams per liter. The sensitivity of this assay was 0.225 mg/l for IS and 0.425 mg/l for *p*-CS [7].

Statistical Analysis

All data are presented as mean ± SD. Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, Ill., USA). Student's *t* test was used for comparison between the two groups. For comparison of category variables, χ^2 test was utilized. To examine the relationship between IS/*p*-CS and other variables, Pearson's correlation test was used. Lastly, logistic regress analysis was utilized to identify independent associates of outcome of vascular access. A value of *p* < 0.05 was interpreted as statistically significant.

Table 1. Demographic characteristics and biochemical data of all patients

	Total (n = 91)	High risk (n = 37)	Low risk (n = 54)	p value
Male:female	39:52	16:21	23:31	0.952
Age, years	57.6 ± 1.2	60.8 ± 1.6	55.5 ± 1.6	0.028
Dialysis duration, months	139.3 ± 7.1	100.0 ± 11.7	166.4 ± 6.9	<0.001
Diabetes, %	20.9	43.2	5.9	<0.001
Hypertension, %	31.9	40.5	25.9	0.155
Statins used, %	14.3	18.9	11.1	0.322
Antiplatelet or anticoagulants, %	18.7	24.3	14.8	0.276
BMI	22.0 ± 0.4	22.8 ± 0.6	21.4 ± 0.4	0.057
Vascular access type (AVF), %	71.4	29.7	100	<0.001
Kt/V	1.7 ± 0.05	1.67 ± 0.1	1.73 ± 0.04	0.559
Hemoglobin, g/dl	10.9 ± 0.3	10.7 ± 0.2	10.9 ± 0.1	0.415
Platelets, 10 ³ /μl	187.5 ± 5.6	188.2 ± 9.0	187.1 ± 7.2	0.924
Albumin, g/dl	3.9 ± 0.3	3.8 ± 0.1	3.93 ± 0.03	0.031
BUN, mg/dl	69.0 ± 1.3	65.9 ± 2.0	71.2 ± 1.7	0.380
Creatinine, mg/dl	11.3 ± 0.2	10.8 ± 0.4	11.6 ± 0.3	0.079
Calcium, mg/dl	9.4 ± 0.2	9.3 ± 0.1	9.4 ± 0.1	0.739
Phosphorous, mg/dl	4.9 ± 0.12	4.9 ± 0.2	4.9 ± 0.1	0.930
hs-CRP, mg/l	4.5 ± 0.5	5.0 ± 0.7	4.2 ± 0.6	0.374
TIS, mg/l	36.2 ± 1.7	37.2 ± 17.0	35.6 ± 15.8	0.649
FIS, mg/l	2.9 ± 0.2	3.3 ± 2.1	2.6 ± 1.5	0.072
TPCS, mg/l	25.4 ± 1.9	33.1 ± 19.9	20.1 ± 14.9	0.001
FPCS, mg/l	2.1 ± 0.2	2.9 ± 2.3	1.5 ± 1.2	0.001
IL-18, pg/ml	437.4 ± 166.6	444.6 ± 157.2	432.1 ± 156.5	0.712
ICAM-1, ng/ml	193.8 ± 30.6	218.6 ± 43.4	175.9 ± 60.7	0.046
VCAM-1, ng/ml	1,919.6 ± 830.1	2,056.9 ± 948.9	1,820.0 ± 618.9	0.160

BUN = Blood urea nitrogen; TIS and FIS = total and free indoxyl sulfate; TPCS and FPCS = total and free *p*-cresol sulfate.

Results

Table 1 displays the demographic characteristics and biochemical data of the enrolled patients. Initially 118 patients (58 and 60 subjects in the high- and low-risk groups, respectively) were recruited. After exclusion of non-eligible patients because of recent infection, chronic inflammation, malignancy or those who did not sign informed consent, 37 patients in the high-risk group and 54 patients in the low-risk group were enrolled for further investigation. The gender distribution was even between the two groups. Patients of the high-risk group were older and had shorter HD duration. More than 40% patients were diabetics in the high-risk group while it was much lower in the low-risk group. 15 patients of the high-risk group were hypertensive (40.5%), and there were 14 patients in the low-risk group (25.9%). Patients using an arteriovenous graft accounted for 70.3% in the high-risk group, while all patients were using primary AVF in the low-risk group ($p < 0.001$). Medication review revealed

that in the high-risk group, 9 patients were on antithrombotic therapy (24.3%; 7 patients on aspirin and 2 patients on clopidogrel, and 1 patient on both agents); in the low-risk group, there were 8 patients (14.8%; 6 patients on aspirin and 2 patients on clopidogrel). The usage of statins was similar between the two groups.

The indicator of dialysis adequacy measured as Kt/V was similar between the two groups. Patients of the high-risk group had a lower serum albumin level. Their serum levels of hs-CRP were comparable between the two groups. No significant difference in concentrations of total and free IS was noted between the two groups. Both total and free *p*-CS levels were significantly higher in the high-risk group. The levels of IL-18 were similar between the two groups. Patients of the high-risk group had higher levels of ICAM-1 than that of the low-risk group.

Patients with and without diabetics were also compared. We found that diabetics were associated with a higher total and free *p*-CS levels than non-diabetics (total: 34.1 ± 20.3 vs. 23.1 ± 17.0 mg/l; free: 3.2 ± 2.5 vs.

Table 2. Association analysis of selected variables in chronic HD patients

	Age	BMI	Duration	TIS	FIS	TPCS	FPCS	IL-18	ICAM-1	VCAM-1
Age	–	–0.001	0.619	0.098	0.213 ^a	–0.027	0.067	0.211 ^a	0.095	0.058
BMI	–	–	–0.204	–0.01	0.064	–0.133	–0.068	0.065	0.072	–0.017
Duration	–	–	–	0.066	–0.07	–0.281 ^b	–0.292 ^b	0.202	–0.094	0.1
TIS	–	–	–	–	0.833 ^c	0.281 ^b	0.335 ^b	–0.107	–0.018	0.032
FIS	–	–	–	–	–	–0.436 ^b	0.611 ^b	0	0.199 ^a	0.195 ^a
TPCS	–	–	–	–	–	–	0.930 ^c	–0.230 ^a	0.148	–0.016
FPCS	–	–	–	–	–	–	–	–0.136	0.308 ^b	0.077
IL-18	–	–	–	–	–	–	–	–	0.353 ^b	0.349 ^b
ICAM-1	–	–	–	–	–	–	–	–	–	0.443 ^b
VCAM-1	–	–	–	–	–	–	–	–	–	–

TIS and FIS = Total and free indoxyl sulfate; TPCS and FPCS = total and free *p*-cresol sulfate.

^a *p* < 0.05; ^b *p* < 0.01; ^c *p* < 0.001.

Table 3. Logistic regression analysis of factors associated with survival and patency of vascular access

Variables	Correlation coefficient (SE)	OR	95% CI		p value
			lower	upper	
Diabetes	2.257 (0.713)	9.553	2.361	38.652	0.002
FPCS	0.485 (0.171)	1.624	1.162	2.270	0.005

The forward stepwise logistic regression method utilizing the likelihood ratio test was applied with the following variables initially entered: age, diabetes, albumin. FPCS = Free *p*-cresol sulfate; TPCS = total *p*-cresol sulfate; SE = standard error; OR = odd ratio; CI = confidence interval.

1.8 ± 1.5 mg/l, both *p* < 0.05). The levels of free IS were also significantly higher in diabetics (3.8 ± 2.4 vs. 2.7 ± 1.5 mg/l, *p* < 0.05). The levels of total IS were similar between diabetics and non-diabetics (39.4 ± 18.8 vs. 35.4 ± 15.4 mg/l, *p* > 0.05).

Relationship between Uremic Toxins and Selected Variables (table 2)

The associations among several selected variables are shown in table 2. Patient age was positively correlated with levels of free IS and IL-18. No association between BMI and uremic toxins was noted. Dialysis duration was negatively correlated with levels of total and free *p*-CS. There was a significant association between IS and *p*-CS, both in the total and free forms. Levels of ICAM-1 were directly related with VCAM-1. IL-18 levels positively cor-

related with ICAM-1 and VCAM-1. A positive correlation between free IS and ICAM-1, VCAM-1 was noted. Levels of free *p*-CS correlated positively with ICAM-1. We further analyzed the relationship in the high- and low-risk patients, respectively (data not shown). In the high-risk group, age and dialysis duration did not have a significant association with other variables. The association between IS and *p*-CS remained significant. No relationship between free IS and adhesion molecules was found. In the low-risk group, age had a positive association with HD duration, free IS and IL-18 levels. Dialysis duration was inversely related to levels of total and free *p*-CS. The relationship between IS and *p*-CS was inconsistent. Only the free form of IS and *p*-CS was interrelated. No relationship between IL-18, ICAM-1 and VCAM-1 was observed.

Logistic Regression Analysis of Survival and Function of Vascular Access

As shown in table 3, significant factors were identified in univariate analysis: age, diabetes, albumin, total and free *p*-CS, ICAM-1 were selected for logistic regression analysis. The result revealed that diabetes and levels of free *p*-CS were independent associates of outcome of vascular access.

Discussion

In the present study, we demonstrated for the first time that in addition to the traditional risk factors, protein-bound uremic toxin such as *p*-CS was associated with

survival and function of vascular access in chronic HD patients. Furthermore, our study also revealed a significant association between uremic toxin and circulating markers of activation of endothelial cells. This finding indicates that the protein-bound uremic toxin may exert a deleterious effect on endothelium. However, how elevated protein-bound uremic toxins affect vascular access in terms of function and chronic use remains unclear.

There are numerous factors that can affect the outcome of vascular access. In our subjects, patients with frequent dysfunction or failure of vascular access were older and had a shorter dialysis duration. Comorbidity such as diabetes accounted for almost half of the patients in the high-risk group. The dialysis adequacy of both groups was similar and achieved the current recommended guideline. Thus, these results indicate that clinical characters play an important role in determining the outcome of vascular access. All patients in the low-risk group were using native AVF. This selection bias is partially due to the retrospective nature of our study but also represents the fact that patients with a history of multiple failures eventually need the creation of a graft for chronic use as their vascular access.

Leukocyte migration and activation of adhesion molecules occur during endothelium stimulation due to direct injury or inflammation. The overwhelming vascular incompetence, especially the endothelial dysfunction, has been implicated in dialysis patients [8]. It has been noted that local expressions of VCAM-1, IL-6, and tumor necrosis factor- α were significantly higher in HD patients with thrombosed AVF [9]. Elevated circulating levels of vascular endothelial growth factor and sVCAM-1 have also been reported in HD patients with thrombosed AVF when comparing non-thrombosed fistulas [10]. Our study confirmed the higher levels of circulating adhesion molecules such as ICAM-1 and VCAM-1 in patients in the high- and low-risk groups. Moreover, a significant association between levels of adhesion molecules and protein-bound uremic toxins was documented.

IS and *p*-CS are protein-bound uremic toxin molecules which share similar characteristics including their production and strong albumin binding [11, 12]. Of note, these toxins may exert deleterious effects on a variety of cells [13–15]. Tumor et al. [13] reported that IS upregulated the expression of ICAM-1 and monocyte chemoattractant protein-1 by oxidative stress-induced NF- κ B activation in cultured endothelial cells. Levels of both *p*-CS and IS have been shown to correlate with the long-term outcome in dialysis and chronic kidney disease patients [16–18]. The underlying mechanism responsible for the prob-

able toxic effects remains unclear. Increased oxidative stress, enhanced inflammation and even a defect in repair might mediate uremia-associated vasculopathy, thus leading to increased morbidity and mortality [6, 8]. The impact of protein-bound uremic toxin on survival of vascular access has not been investigated previously. Compared with HD, patients on peritoneal dialysis had lower levels of protein-bound uremic toxins [19, 20]. Despite the fact that the removal of these toxins is more effective by HD, reason to explain the difference is currently unknown. Efforts have been made to improve the clearance of protein-bound uremic toxins [2]. Whether a higher level of uremic toxins observed in HD leads to any clinical events is not clear. The present study explores the association between free *p*-CS and outcome of vascular access in chronic HD patients. Although direct evidence supporting an active role of *p*-CS is lacking, our result suggests a probable causal relationship. A previous study revealed hypoalbuminemia increased the free fraction of *p*-CS [21]. Patients of the high-risk group had a lower albumin level, it is thus reasonable to speculate that a lower albumin level may contribute to the elevation of free *p*-CS.

In the present study, we found diabetes affects the outcome of vascular access in chronic HD patients. Because diabetes is associated with generalized vascular disease, it is conceivable to identify diabetes as an underlying factor that influences survival and function of vascular access. Our result also demonstrated that diabetic patients had higher levels of uremic toxins. Comparing with non-diabetics, there was a 41% increase in free IS and 78% increase in free *p*-CS in diabetics. A previous study had also found higher free *p*-CS levels in HD patients with diabetes [17]. Because logistic regression analysis revealed both diabetes and free *p*-CS independently correlated with outcome of vascular access, it is likely that another mechanism is involved linking *p*-CS and disorders of vascular access.

IL-18 is a pro-inflammatory cytokine produced mainly by macrophages and has multiple biological effects such as immunoregulation, atherosclerosis, and insulin resistance [22]. Several studies demonstrate IL-18 is a prognostic marker of dialysis-associated morbidity and hospitalization [23, 24]. However, the role of IL-18 in vascular access has not been clarified. The relationship between IL-18 and inflammatory marker was not evident [25]. In our patients, level of IL-18 correlated with circulating adhesion molecules but not with either IS or *p*-CS. This result suggests IL-18 might not be involved in uremic toxin-associated vascular injury.

There are limitations in the present study. First, the retrospective study design allows association analysis only and further study is needed to prove the prognostic or pathogenic role *p*-CS. Secondary, a large-scale study with more clinical and laboratory data would definitely provide more comprehensive understanding of the mechanism of how protein-bound uremic toxin affects outcome of vascular access. Thirdly, as we mention, there is selection bias in the present study. A more precise recruitment of study subjects can help to determine the role of protein-bound uremic toxins and their influence on different types of vascular access.

In conclusion, our study reveals that function and survival of vascular access in HD patients was associated

with activation of endothelial cells and protein-bound uremic retention compounds such as IS or *p*-CS. Levels of IS or *p*-CS correlated significantly with circulating adhesion molecules. Diabetes and free *p*-CS were independent factors relevant to the outcome of vascular access of chronic HD patients.

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