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Serum sulfatides as a novel biomarker for cardiovascular disease in patients with end-stage renal failure

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Abstract Sulfatides, normal components of serum lipoproteins, may play an important role in cardiovascular disease due to their various modulatory functions in haemostasis. The incidence of cardiovascular disease in patients with end-stage renal failure undergoing maintenance hemodialysis has been reported to be approximately 10 to 30 times higher than that in the general population. To elucidate the possible roles of serum sulfatides in this high incidence, we measured the level of sulfatides in 59 such patients, by converting them to lysosulfatides according to a recently developed quantitative, qualitative, high-throughput technique using matrix-assisted laser desorption ionization-time of flight mass spectrometry. The mean level of sulfatides in patients 3.58±1.18 nmol/ml was significantly lower than that in age-matched normal subjects (8.21±1.50 nmol/ml; $P<0.001$). Patients receiving maintenance hemodialysis over a longer period had lower levels of sulfatides. When the mean levels of sulfatides were compared between patients with cardiovascular disease ($N=22$) and those without the disease ($N=37$), the level in the former group 2.85±0.67 nmol/ml was found to be significantly lower than that in the latter group 4.01±1.22 nmol/ml ($P<0.001$). These findings reveal a close correlation between low levels of serum sulfatides and a high risk of cardiovascular disease in these patients. Determination of the level of serum sulfatides can contribute to predictions of the incidence of cardiovascular disease in patients with end-stage renal failure undergoing maintenance hemodialysis.

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Keywords Serum sulfatides · Cardiovascular disease · End-stage renal failure · Hemodialysis

Abbreviations

WHHL	Watanabe hereditable hyperlipidemic
CVD	cardiovascular disease
ESRF	end-stage renal failure
VLDL	very low-density lipoprotein
LDL	low-density lipoprotein
HDL	high-density lipoprotein.

Sphingoids are abbreviated such as (4E)-sphingenine d18:1 and t18:0, 4D-hydroxysphinganine (or phytosphingosine) where the first number indicates the number of carbon atoms and the second indicates the number of double bonds and d and t mean di- and tri-hydroxyl groups respectively

LS	lysosulfatide
LS-d18:1	LS possessing d18:1 as a sphingoid

Introduction

Sulfatides are esters of sulfuric acid with galactosylceramides at C3 of the galactosyl residue [1].

Over the last two decades, we have been continuously reporting the possible involvements of sulfatides in haemostasis/thrombosis. First through a series of experiments comparing normal and WHHL (Watanabe hereditable hyperlipidemic) rabbits, an animal model for human familial hypercholesterolemia, we revealed that sulfatides, the major glycosphingolipids in serum lipoproteins, are markedly elevated in WHHL rabbits [2] and that they are accumulated in atheromatous plaques in the aortae of WHHL rabbits [3]. We also subsequently identified sulfatides as substantial constituents of the sera from various normal animals including humans [4]. When exogenous sulfatides were simply injected into animals, they prolonged the bleeding time, probably due to both their binding activity to fibrinogen, disturbing the fibrin formation [5, 6] and their direct inhibitory activity on thrombin [7]. On the other hand, exogenous sulfatides enhanced thrombosis when injected into mice with blood vessel walls that were heavily damaged [7, 8] or when continuously infused into mice through plastic cannulae [9]. The possible participation of blood coagulation factor XII was suggested to underlie these *in vivo* thrombotic activities [7–9]. In addition to our observations, there have been many reports of sulfatides, some of which have reported thrombogenic activities, and others of which have reported anti-thrombotic activities; many different mechanisms for these activities, such as the involvement of sulfatides in coagulation [10, 11], platelet functions [12–15] and/or intimal hyperplasia [16] have been suggested. However, most of these were of experiments using exogenous sulfatides. Although the reports were contradictory, it is likely that endogenous sulfatides affect the pathogenesis of cardiovascular disease (CVD) as seen daily in a clinical setting [17]. Therefore, it has long been desired to determine the level of sulfatides in many clinical specimens with high accuracy. Very recently, we established a method for the quantitative, qualitative and high-throughput analysis of serum sulfatides, which represent a type of endogenous sulfatides, using matrix assisted-laser desorption ionization time-of flight mass-spectrometry [18].

Since patients with end-stage renal failure (ESRF) undergoing maintenance hemodialysis frequently present dyslipoproteinaemia [19–24] and suffer from an accelerated atherosclerosis, this abnormality is believed to increase the risk of CVD, which is responsible for the majority of morbidity and mortality in ESRF patients on hemodialysis [22–24]. In fact, the incidence of death caused by CVD in these patients is approximately 10 to 30 times higher than that in the general population [22, 23]. Although many possible risk factors are suggested, no crucial factor has yet been determined. In this paper, we aimed to quantitatively and qualitatively compare sulfatides in the sera of ESRF patients undergoing maintenance hemodialysis with those in the sera of age-matched normal controls, in order to determine whether sulfatides are involved in the pathogenesis of CVD in ESRF patients.

Materials and methods

Materials

Lysosulfatide possessing (4*E*)-sphingenine as a sphingoid (LS-d18:1), and hydrogenated *N*-acetyl LS (LS-d18:0 NAc), were chemically prepared from sulfatides using a deacylation procedure as described in our previous study [25]. MonoTip C18 cartridges were obtained from GL Sciences Inc (Tokyo, Japan). All other reagents used in the experiments were of the highest grade, and were commercially available in Japan.

Subject profiles

Fifty-nine patients with ESRF on maintenance hemodialysis, being treated at the Shinshu University Hospital, were examined. Their ages were between 40 and 86 years old (mean ± SD, 68.32±11.40). The average duration of maintenance hemodialysis treatment (12 h/week) was 8.60± 7.08 years. The patients were separated into two groups. One group was made up of ESRF patients with a complication of CVD, such as myocardial infarction and angina pectoris (22 patients, male 13 and female 9). The other group was made up of ESRF patients without any CVD (37 patients, male 18 and female 19). There was no significant difference in age or hemodialysis duration between these groups. A further 43 age-matched normal persons, from 40 to 80 years old (mean ± SD, 65.56±10.66), were used as controls. This study was conducted in accordance with the Declaration of Helsinki. Signed informed consent was obtained from all of the subjects, and the Medical Ethics Committee of the Shinshu University School of Medicine approved the study protocols. Blood samples were obtained from all subjects early in the morning prior to a hemodialysis session. Clinical data such as

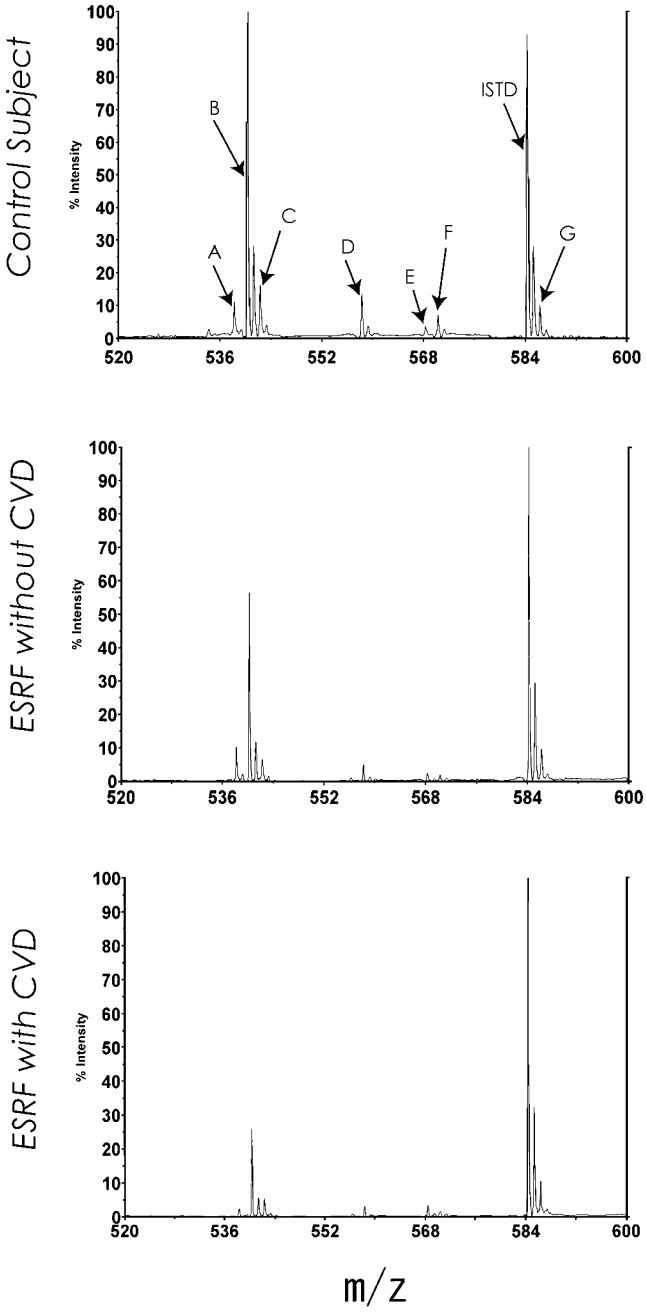

Fig. 1 Typical mass spectra of lysosulfatides in serum obtained from control subjects, ESRF patients without cardiovascular disease and ESRF patients with cardiovascular disease. The peaks representing the different lysosulfatide molecular specie, LS-d18:2 (*A*), LS-d18:1 (*B*), LS-d18:0 (*C*), LS-t18:0 (*D*), LS-d20:1 (*E*), LS-d20:0 (*F*) and LS-t20:0 (*G*) are shown in the mass spectra of the age-matched normal control subject. ISTD is the internal standard, N-acetyl LS-d18:0. The second isotopic peak of LS-d18:1 (m/z 542) and the second peak of the ISTD (m/z 586) had the same mass numbers as the monoisotopic peaks of LS-d18:0 and LS-t20:0, respectively. The ratios of the second isotopic peaks to the monoisotopic peaks were estimated from the areas, and were used to determined the actual amounts of LS-d18:0 and LS-t20:0, respectively

total cholesterol, HDL-cholesterol, triacylglycerol, serum calcium, serum inorganic phosphorus and number of platelets, in blood samples, were measured by an autobiochemical analysis system (AU2700, Olympus, Japan). For the measurement of the sulfatides, the serum was stored at −80°C until analyzed.

Quantitation of sulfatide in serum

Sulfatides were extracted from serum using a *n*-hexane-isopropanol mixture [26] and analyzed as lyso-forms using a high-throughput method developed in our laboratory [18]. Briefly, the total lipids extracted from 50 μl of serum with *n*-hexane: isopropanol (3:2, *v*/*v*) were dried and partially hydrolyzed with 0.1 N NaOH in 90% methanol at 150°C for 30 min to convert sulfatides to LSs. After neutralizing, *n*-hexane was added with vigorous shaking, and the upper layer was discarded to remove non-polar lipids dissolved in the *n*-hexane. The lower layer containing LSs was evaporated, and desalted by Monotip C18. After adding

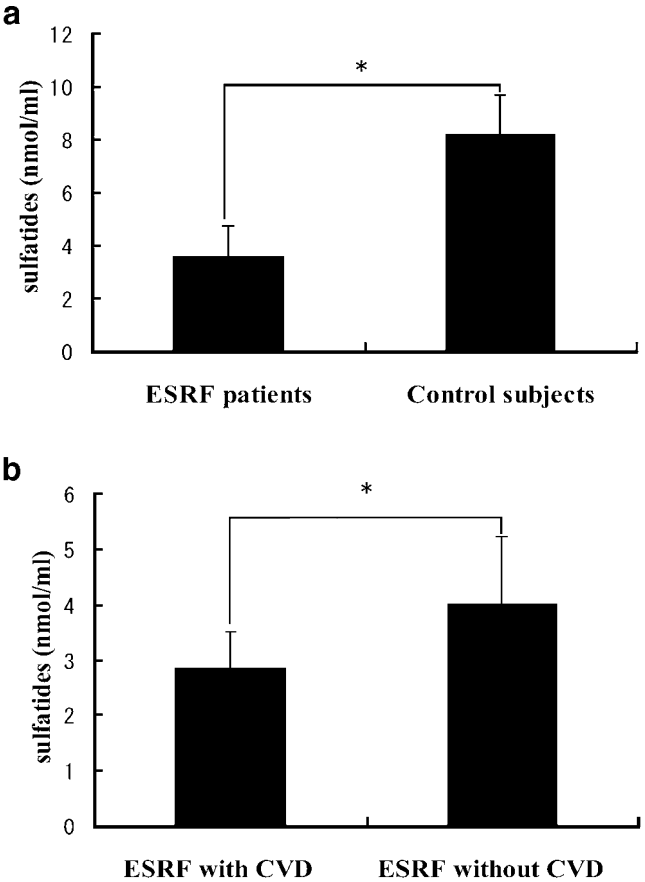

Fig. 2 **a** The concentrations of sulfatides in the sera of ESRF patients on maintenance hemodialysis (*N*=59) and control subjects (*N*=43). Values are means ± SD. Significant differences are shown: **p*<0.001. **b** The concentrations of sulfatides in the sera of ESRF patients with (*N*=22) and without cardiovascular disease (*N*=37). Values are means ± SD. Significant differences are shown: **p*<0.001

LS-d18:0 NAc as an internal standard, the sample was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry with delayed ion extraction using a Voyager Elite XL (6.5 m flight length in the reflector mode) Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA). A nitrogen laser (337 nm) was used for ionization and negative ion mode detection was employed. A two-point external calibration was performed in all experiments with the use of LS-d18:1 ($[M–H]^-$, 540.2843) and LS-d18:0 NAc ($[M–H]^-$, 584.3105).

Statistics

Student t-tests were used to compare the values obtained between the two groups. A P value of <0.05 was considered to be statistically significant. Student t tests and regression analysis were performed using Microsoft Excel version 2003 SP2.

Results

Figure 1 shows typical mass spectra of serum sulfatides determined as LSs from a normal control subject, an ESRF patient undergoing maintenance hemodialysis without CVD, and an ESRF patient with CVD. While in the normal control individual, the major molecular species of LS-d18:1 was dominantly observed together with some LS-d18:2, LS-d18:0, LS-t18:0, LS-d20:1, LS-d20:0 and LS-t20:0, in the patients, every peak was decreased in magnitude, and these decreases were especially remarkable for LS-d18:2 and LS-d18:1.

Figures 2 and 3 present statistical analyses of the serum sulfatide concentration and the composition of sphingoid molecular species in patients with and without CVD and age-matched control subjects, respectively. There was a significant decrease in the total level of serum sulfatide in the patients; the average value in patients 3.58±1.18 nmol/ml was about 56% lower than that in age-matched normal control subjects (8.21±1.50 nmol/ml; Fig. 2a). Regarding the molecular species of LSs, marked decreases in the level of LS-d18:2 (corresponding to 66% of LS-d18:2 in age-matched control subject, and significant differences between in patients and normal control subjects; $P<0.001$) and LS-d18:1 (63%, $P<0.001$) were observed in ESRF patients. Moderate decreases in the level of LS-d18:0 (38%, $P<0.001$), LS-t18:0 (49%, $P<0.001$) and LS-d20:0 (36%, $P<0.001$) were observed in ESRF patients. Smaller decreases were observed in LS-d20:1(12%, no significance) and LS-t20:0 (10%, no significance). These results are clearly reflected in the compositional comparison of molecular

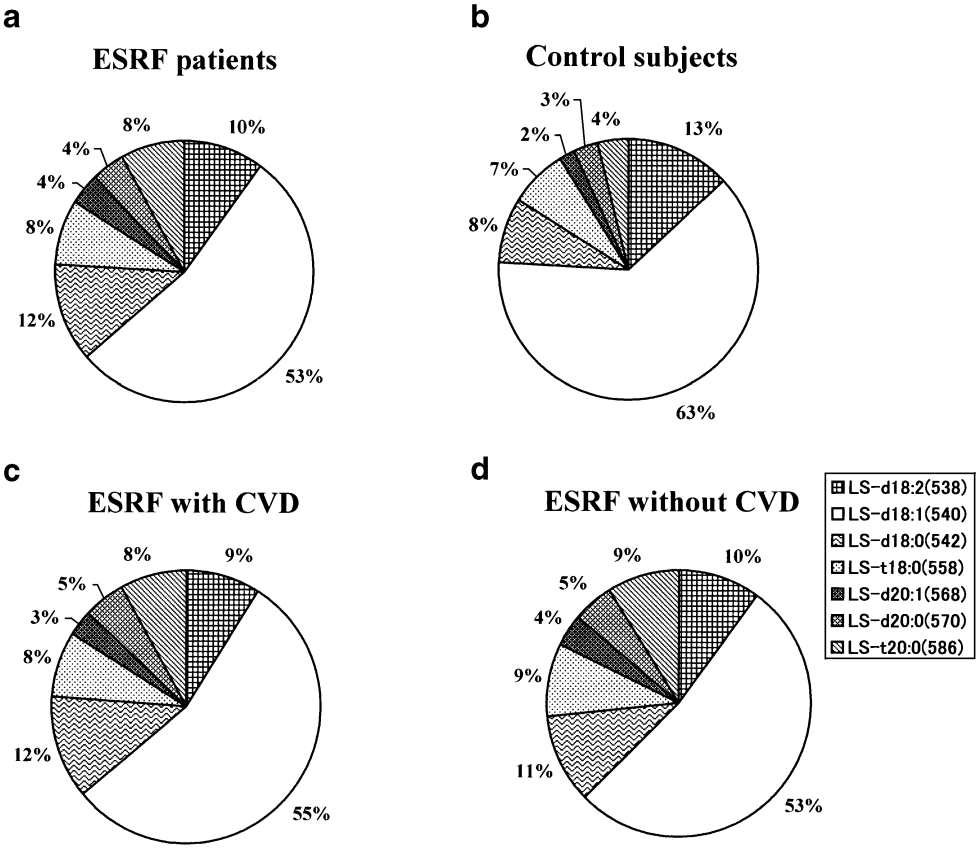

Fig. 3 The composition (means) of the different molecular species of sulfatides in sera from ESRF patients on hemodialysis (**a**) ($N=59$), control subjects (**b**) ($N=43$), ESRF patients with cardiovascular disease (**c**) ($N=22$) and ESRF patients without cardiovascular disease (**d**) ($N=37$)

species of LSs between ESRF patients and age-matched control subjects. Namely, in comparison to the control subjects, the compositional percentages of LS-d18:1 and LS-d18:2 in ESRF patients are decreased while those of LS-d18:0, LS-d20:1 and LS-t20:0 are increased (Fig. 3a,b). Although the total levels of serum sulfatides were significantly different between in the patients with (2.85± 0.67 nmol/ml) and without (4.01±1.22 nmol/ml) CVD (Fig. 2b), the compositions of LSs between these groups are similar (Fig. 3c,d).

An inverse relationship between sulfatide levels in the serum and the duration of hemodialysis is observed in both ESRF patients with CVD and without CVD (Fig. 4). Because the slopes of both regression lines are similar, sulfatide levels were decreased at similar constant rates (about 1 nmol/ml serum for 10 years) in both groups. When sulfatide levels were compared between patients that had received the same duration of hemodialysis, the patients with CVD showed a sulfatide level of about 1 nmol/ml lower than patients without CVD. The maximum level of serum sulfatide in the patients with CVD was 3.92 nmol/ml of serum as shown in Fig. 4a. These results indicate that a low level of serum sulfatides (of less than 3.92 nmol/ml serum) warns of the onset of CVD.

In order to clarify whether the serum sulfatide levels are correlated with other clinical factors underlying CVD, we measured total cholesterol, high-density lipoprotein (HDL) cholesterol, triacylglycerol, serum calcium concentration, serum inorganic phosphorus concentration and the number of platelets, but no significant correlation was found among them (Table 1).

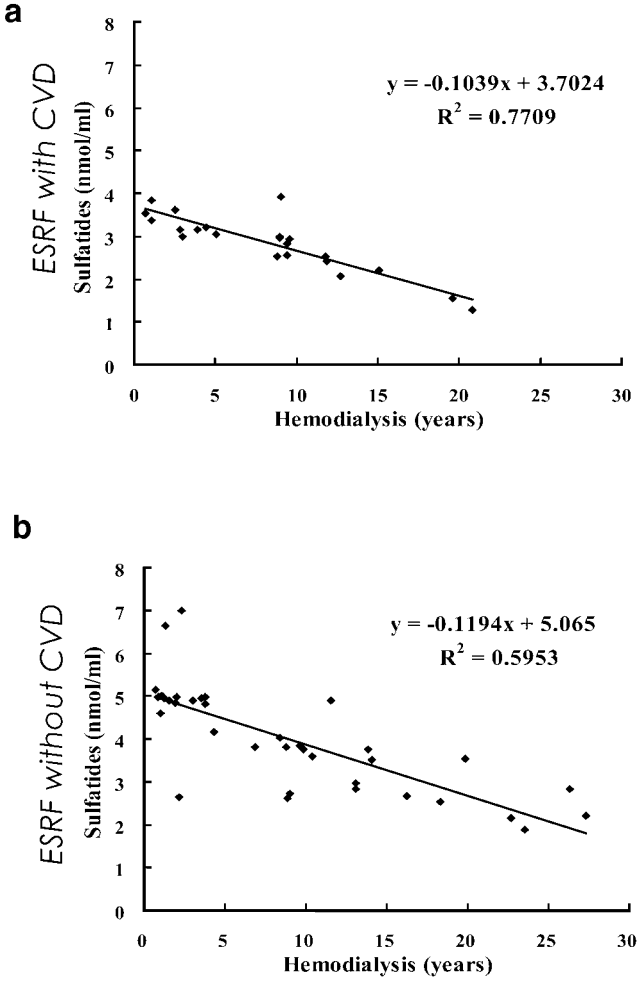

Fig. 4 **a** The concentration of sulfatides in the sera of ESRF patients with cardiovascular disease undergoing maintenance hemodialysis. The regression coefficient for the correlation between concentration of sulfatides in serum and the duration of maintenance hemodialysis is 0.7709. **b** The concentration of sulfatides in sera of ESRF patients without cardiovascular disease undergoing maintenance hemodialysis. The regression coefficient for the correlation between concentration of sulfatides in serum and the duration of maintenance hemodialysis is 0.5953

Discussion

There were two aims for the present study. First, to determine whether the levels of serum sulfatides were altered in ESRF patients on maintenance hemodialysis compared with normal controls. Second, if this is the case, to determine whether the levels of serum sulfatides in patients are correlated with the incidence of CVD in these patients.

It is well known that CVD is closely associated with ESRF. Patients with ESRF are more likely to die of CVD than kidney failure [22–24]. Although many clinical factors such as hypertension, left ventricular hypertrophy, a higher

Table 1 Clinical variables in ESRF patients with and without CVD (means ± SD values)

	ESRF patients with CVD	ESRF patients without CVD	*P*
Sulfatides (nmol/ml)	2.85±0.67	4.01±1.22	<0.001
Age (years)	68.59±10.33	68.16±12.13	n.s.
Duration of hemodialysis (years)	8.20±5.68	8.84±7.85	n.s.
HDL-cholesterol (mg/dl)	41.77±11.94	42.46±10.66	n.s.
Total cholesterol (mg/dl)	152.50±26.55	153.27±31.37	n.s.
Triacylglycerol (mg/dl)	94.59±41.28	92.24±31.30	n.s.
Ca (mg/dl)	9.10±1.02	9.22±0.94	n.s.
P (mg/dl)	4.82±0.82	4.98±1.42	n.s.
Platelets (×10,000/μl)	17.70±5.41	16.74±6.31	n.s.

n.s. Not significant

level of HDL cholesterol, lower level of cholesterol, and the balance of serum calcium and phosphorus are suggested to be the risk factors for CVD, the most crucial factors remain unknown [22–24]. Indeed we could not find clear differences in the levels of total cholesterol, HDL cholesterol, triacylglycerol, serum calcium and phosphorus, and the number of platelets in blood, between patients with CVD and those without CVD; only the level of serum sulfatides clearly enabled two groups to be discriminated.

Although a low level of serum sulfatides is closely correlated to a high incidence of CVD in the ESRF patients, the mechanism underlying this correlation is still unclear. We previously reported anti-coagulant activities of exogenous sulfatides to prolong bleeding time [5–9], probably due to both their binding to fibrinogen [6], and their direct inhibition of thrombin [7]. The anti-platelet activities of exogenous sulfatides were also reported to inhibit the adhesion of platelets to von Willebrand factor [14], to which sulfatides can bind [27], and to inhibit the aggregations induced by thrombin, collagen and ristocetin [12]. Low levels of serum sulfatides may contribute to a decrease in these anti-thrombotic functions in blood, although contradictorily, exogenous sulfatides exhibit strong thrombotic activities in certain experimental conditions [7–9, 15, 16]. Also, prolongation of the bleeding time paradoxically occurs in ESRF patients, even though they are prone to develop CVD [22]. It remains to be elucidated whether the sulfatides incorporated into serum lipoproteins really enable them to exhibit anti-thrombotic activities *in vivo*. In addition, the roles of other endogenous sulfatides such as those on the surfaces of platelets should also be examined in this disease.

The molecular species of sphingoids differ, depending upon the organs in which sulfatides are synthesized [28]. It is well known that serum lipoproteins are synthesized in liver and small intestine. In WHHL rabbits, the major sphingoid molecular species of sulfatides in liver and small intestine were d18:1 and t18:0, respectively, and we previously concluded that a large proportion of all serum sulfatides were derived from liver and that the rest were derived from the small intestine [2]. Since a marked decrease in the total level of serum sulfatides was observed in ESRF patients, especially in the level of LS-d18:1, it is suggested that the proportion of serum sulfatides from liver may be significantly reduced in ESRF patients, although the molecular species of sphingoids in sulfatides from the human liver are currently unknown. On the other hand, no marked compositional difference was observed between patients with CVD and those without CVD. These observations may present a clue to the mechanism involved. Since abnormalities in apolipoprotein profile and lipoprotein composition have been reported in ESRF patients [19], the states of sulfatides in the lipoprotein might be influenced, consequently affecting the functions of the sulfatides in haemostasis. Although, we reported sulfatides as major glycosphingolipids in all kinds of serum lipoproteins, such as chylomicrons, VLDL, LDL, and HDL from WHHL rabbits [2], detail analysis of those sulfatides from humans has not been performed yet. A comparative analysis of sulfatides from each serum lipoprotein from normal volunteers as well as the patients remains to be investigated.

Interestingly, the rate of decrease in the level of total serum sulfatides with duration of hemodialysis seemed to be similar between the patients with and without CVD, because the slopes for the two groups are similar (−0.1039 and −0.1194, respectively; Fig. 4a,b). It may be possible not only to predict the risk of CVD but also to predict the time of onset of CVD in ESRF patients. In addition, an initial low level of serum sulfatides of less than 3.92 nmol/ml at the induction of hemodialysis should be considered as indicating a poor prognosis of these patients.

In conclusion, we reveal: (1) low levels of serum sulfatides in ESRF patients on maintenance hemodialysis as compared to age-matched normal subjects; (2) a reduction in the total level of serum sulfatides with constant rate, along with the prolongation of hemodialysis maintenance duration; (3) a significantly lower level of serum sulfatides in ESRF patients with CVD compared with ESRF patients without CVD.

Although it is still premature to conclude, we believe that the determination of the level of serum sulfatides could provide a new means for the diagnosis and prevention of CVD in ESRF patients undergoing maintenance hemodialysis, which would contribute to an improvement in patients' quality of life and to an increase in their life span. Large-scale clinical studies to confirm and expand on our findings are currently ongoing.

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