

Original Article

Serum sulfatide abnormality is associated with increased oxidative stress in hemodialysis patients

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Running head: Sulfatide abnormality in HD patients

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Abstract

Background: Sulfatides are major glycosphingolipids of lipoproteins that influence atherosclerosis and blood coagulation. Our previous cross-sectional study of hemodialysis patients showed that serum sulfatide levels decreased markedly with increasing duration of hemodialysis treatment, which may contribute to the development of cardiovascular disease. However, this past study could not demonstrate the time-dependent change in serum sulfatide levels in each patient, and the underlying mechanism is unknown.

Methods: To confirm the time-dependent aggravation of serum sulfatide abnormality, 95 stable hemodialysis outpatients were followed for 3 years. To show the underlying mechanisms, we statistically analyzed correlations between serum sulfatide levels and clinical factors including an oxidative stress marker, malondialdehyde. Serum sulfatides were quantified by mass spectrometry after conversion to lysosulfatides.

Malondialdehyde was measured using a colorimetric assay.

Results: The results showed a time-dependent decrease in serum sulfatide levels associated with increased malondialdehyde levels, although the absolute level of serum malondialdehyde does not determine the baseline level of serum sulfatides. Multiple linear regression analysis showed a significant correlation only between the

time-dependent change in serum sulfatide levels and the time-dependent change in serum malondialdehyde levels.

Conclusions: This study demonstrated, for the first time, a time-dependent aggravation of serum sulfatide abnormality in hemodialysis patients, as well as the potential relationship between serum sulfatide abnormality and increasing oxidative stress. These findings suggest that oxidative stress might be an aggravating factor in serum sulfatide abnormality. As continuation of hemodialysis treatment hardly improves abnormal serum sulfatide levels or increased oxidative stress, development of novel therapeutic strategies may be important.

Keywords: cardiovascular disease, hemodialysis, malondialdehyde, oxidative stress, serum sulfatide level.

INTRODUCTION

Sulfatides are amphiphilic molecules that are synthesized from galactosylceramides by sulfation at C3 of the galactosyl residue.¹ Sulfatides are widely distributed in mammalian tissues such as the brain, kidney, liver, and digestive tract, and also exist in the sera of various mammals as major glycosphingolipids of lipoproteins.¹⁻⁴ The physiological roles of serum sulfatides are still unclear. Our previous study found obvious accumulation of sulfatides in the sclerotic aorta of Watanabe hereditary hyperlipidemic rabbits, which provide an animal model for human familial hypercholesterolemia, suggesting that sulfatides play a significant role in the progression of atherosclerosis.^{2,5} Other experimental studies reported that intravenous administration of exogenous sulfatides had significant effects on blood coagulation.^{6,7} Most studies reported that serum sulfatides had anticoagulant and antiplatelet effects, but some reported that sulfatides accelerated blood coagulation.⁷ The results of these studies suggest that abnormal serum sulfatide levels may play a role in the pathogenesis of cardiovascular disease (CVD).

Chronic kidney disease patients undergoing hemodialysis (HD) therapy have a high incidence of CVD, which is the major cause of death in this population.^{8,9} Interestingly, our recent cross-sectional study found that serum sulfatide levels were

remarkably low in HD patients compared with normal subjects, and that serum sulfatide levels were significantly lower in HD patients with CVD than in HD patients without CVD.¹⁰ These findings suggest that decreased serum sulfatide levels may contribute to the development of CVD in HD patients because of abnormal sulfatide function.

The mechanisms underlying the decrease in serum sulfatide levels in HD patients are currently unknown. Our previous cross-sectional clinical study found an inverse correlation between the serum sulfatide level and the duration of HD, suggesting that alteration of the internal environment caused by long-term kidney failure gradually decreases the serum sulfatide level.¹⁰ However, this past study could not demonstrate the time-dependent change in serum sulfatide levels in each patient. To confirm the time-dependent change in serum sulfatide levels in each HD patients and to elucidate the underlying mechanisms, we conducted a 3-year observational study and investigated the clinical factors correlated with serum sulfatide levels.

Serum sulfatides are produced and secreted mainly by the liver as the major component of lipoproteins.² Our previous experimental studies using an acute kidney injury model and an acute hepatic injury model found that increased systemic oxidative stress reduced the hepatic expression of an essential synthetic enzyme of sulfatides, cerebroside sulfotransferase (CST), followed by a decrease in serum sulfatide levels.^{11,}

¹² Furthermore, we reported that kidney transplantation normalized the serum sulfatide abnormality and oxidative stress synchronously in kidney transplant recipients.^{13, 14}

These findings suggest a close relationship between serum sulfatide abnormality and oxidative stress. Many past studies reported that HD patients are exposed to high levels of oxidative stress, caused by increased production of reactive oxygen species because of their uremia and HD procedures, as well as by decreased antioxidant activity and decreased uptake of dietary antioxidants.^{15, 16} This unhealthy internal condition in HD patients may suppress hepatic sulfatide synthesis, leading to a decrease in serum sulfatide levels.

In the current study, we investigated whether there is a relationship between serum sulfatide abnormality and oxidative stress in HD patients. Because malondialdehyde (MDA) is one of the most well-known classical oxidative stress markers and the analytical method of MDA is well established, we selected and utilized the serum MDA levels as a representative oxidative stress marker, which is common with all of our studies.

MATERIALS AND METHODS

HD patients and study protocol

To examine the general characteristics in HD patients, we sought to survey all outpatients undergoing maintenance HD therapy at affiliated hospitals of Shinshu University. Among them, 201 patients agreed to participate in the study and were enrolled. From this population, unstable patients with cancer (n=3) and/or serum positivity of chronic infection (hepatitis C virus, n=26; hepatitis B virus, n=12; syphilis, n=1), one patient who planned to shift to the kidney transplantation unit, and two patients who withdrew their consent were excluded at the beginning of the observation period (baseline number = 156). Additionally, to confirm the time-dependent change in serum sulfatide levels in HD patients over 3 years, 34 patients who died during the study period and 27 patients without clinical data and/or serum samples were excluded. Finally, this study investigated 95 patients. Serum samples were collected at the beginning of the observation period (baseline) and after 18 and 36 months, and were stored in a deep freezer. All serum samples were obtained just before the first HD session of the week. Serum sulfatide levels and serum MDA levels in the frozen samples were analyzed together with many other laboratory data. The study protocol complied with the guidelines of the 2004 revision of the Declaration of Helsinki, and

was approved by the ethics committee of Shinshu University (approval number: 750).

Written informed consent was obtained from all patients before inclusion in the study.

Quantification of serum sulfatide levels

Sulfatides were extracted from the serum samples using the hexane/isopropanol method, and were converted to lysosulfatides (LSs; sulfatides without fatty acids) by saponification with sodium hydroxide.¹⁷⁻¹⁹ The LS samples were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry using a Voyager Elite XL Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA, USA) as described previously.¹⁸ Seven molecular species of LS were detected, based on differences in sphingoid base structures: LS-sphingadienine (LS-d18:2), LS-(4E)-sphingenine (LS-d18:1), LS-sphinganine (LS-d18:0), LS-4D-hydroxysphinganine (LS-t18:0), LS-(4E)-eicosasphingenine (LS-d20:1), LS-eicosasphinganine (LS-d20:0), and LS-4D-hydroxyeicosasphinganine (LS-t20:0). The total serum sulfatide level was calculated as the sum of the levels of these seven LS molecular species. When sulfatide levels were measured for new samples, the same standard serum sample and the chemically prepared internal standard, hydrogenated N-acetyl LS (LS-d18:0 NAc), were always used in order to verify the consistency of the data.

Analysis of serum oxidative stress marker

The serum level of the oxidative stress marker MDA was measured using a lipid peroxidation colorimetric assay kit purchased from Oxis International (Beverly Hills, CA, USA).

Statistical analysis

Continuous variables were handled as non-parametric variables and are shown as the median (minimum–maximum). Time-dependent changes in paired continuous variables were analyzed using the Wilcoxon matched-pairs signed-rank test. Two independent groups of continuous variables were compared using the Mann–Whitney *U* test. Correlations between clinical factors and serum sulfatide levels were determined using Spearman’s correlation coefficient. The clinical parameters that were most strongly correlated with the time-dependent changes in serum sulfatide levels were analyzed by multivariate linear regression analysis. The multivariate analysis included all clinical follow-up parameters that changed significantly during the follow-up period. All statistical analyses were performed using SPSS version 18.0J (SPSS, Inc., Chicago, IL, USA). The level of statistical significance was set at $P < 0.05$.

RESULTS

Baseline characteristics

The baseline characteristics of the HD patients included in the study are shown in Table 1. Compared with the latest overview of regular dialysis treatment in Japan by the Japanese Society for Dialysis Therapy,²⁰ HD patients in the current study had a lower median age (63 vs. 67 years), higher proportion of males (67% vs. 63%), and lower prevalence of diabetes mellitus (31.6% vs. 37%) at study entry (Table 1). The median duration of maintenance HD treatment was 8.4 years. This study included only HD patients who could be followed up for 3 years, and excluded unstable patients and the dead during the follow-up, and therefore may have enrolled patients with relatively good physical condition compared with the general population of Japanese HD patients. Nevertheless, 30.5% of enrolled patients had a history of CVD at study entry. These HD patients were treated with various medicines; however, no patient used any antioxidant medicine, such as alpha-tocopherol, vitamin C, and N-acetylcysteine.

Cross-sectional analysis of correlations between clinical factors and serum sulfatide levels

The mean serum sulfatide level of the 95 HD patients at study entry was 3.66 ± 1.00 nmol/mL. When compared with the normal serum sulfatide level in healthy control participants (8.21 ± 1.50 nmol/mL) reported in our previous study,¹⁰ the serum sulfatide level in HD patients appeared to be less than 50%. Consistent with our previous study,¹⁰ the mean serum sulfatide level was markedly lower in HD patients with a history of CVD than in HD patients without a history of CVD (3.14 ± 0.62 vs. 3.90 ± 1.05 nmol/mL, $P < 0.01$). To show the mechanisms underlying the decrease in serum sulfatide levels in HD patients, we analyzed correlations between baseline clinical parameters and serum sulfatide levels. Spearman's rank correlation analysis and scatter diagrams showed an inverse correlation between serum sulfatide levels and the duration of HD, but no significant correlations between serum sulfatide levels and other clinical parameters including serum MDA levels (Table 2, Figure 1A and B).

Analysis of clinical factors correlated with time-dependent changes in serum sulfatide levels

Follow-up of the enrolled HD patients for 3 years confirmed a time-dependent decrease in serum sulfatide levels (Figure 2A). Interestingly, this follow-up analysis also showed a marked time-dependent increase in serum MDA levels (Figure 2B). The

composition of the different molecular species of serum sulfatides in the 95 HD patients did not differ at each follow-up point, suggesting that all molecular species similarly decreased in a time-dependent manner (Figure 2C). Therefore, we used sum data of the seven sulfatides as representative data of sulfatides in the statistical analyses. The time-dependent decrease in serum sulfatide levels was not affected by sex, CVD history, smoking history, diabetes mellitus, type of vascular access, dialysis method (conventional HD or on-line hemodialysis filtration), or various oral medications (Figure 3). To identify other factors potentially correlated with time-dependent changes in serum sulfatide levels, we examined the changes in serum levels of various parameters during the follow-up period (Table 3). Linear regression analysis was performed using all observational point data of candidate parameters including serum MDA levels, and found that the MDA level was the only factor significantly correlated with the time-dependent change in serum sulfatide level (Table 4). The inverse correlation between serum sulfatide level and serum MDA level was confirmed by a scatter diagram (Figure 4), suggesting that serum sulfatide levels may decrease as oxidative stress increases in a time-dependent manner.

DISCUSSION

The current follow-up study of maintenance HD patients found that serum sulfatide levels decreased and serum MDA levels increased from the baseline values in each patient, in a time-dependent manner. It is thought that the HD procedure cannot directly remove serum sulfatides, because sulfatides are incorporated in lipoproteins with high molecular weight.² Indeed, our preliminary study measuring serum sulfatide levels before and after a single HD session did not show a reduction in sulfatide levels by HD therapy (data not shown). This finding suggests that the time-dependent reduction in serum sulfatide levels in HD patients might be induced by alteration of the internal environment. Our multivariate statistical analysis showed a significant correlation between the time-dependent changes in serum sulfatide levels and serum MDA levels. This finding suggests that time-dependent aggravation of abnormal serum sulfatide levels might be associated with increased oxidative stress, and that the internal environment of HD patients continues to gradually deteriorate. This progressively unhealthy situation may contribute to the poor prognosis in HD patients.

Interestingly, the inverse correlation between serum sulfatide levels and oxidative stress could only be detected by follow-up study, and not by cross-sectional analysis. To exclude the factor of HD duration, we also examined the correlation

between serum sulfatide levels and serum MDA levels in 64 chronic kidney disease patients (stage 5) just before the start of HD. This preliminary cross-sectional analysis also could not detect a significant correlation between these two parameters. These findings suggest that serum sulfatide levels are different in each patient even if the absolute level of serum MDA is the same, and that other unknown factors may also play a role in regulating the baseline level of serum sulfatides. Oxidative stress should be considered as a time-dependent aggravating factor that reduces serum sulfatide levels, rather than an essential factor regulating the baseline level of serum sulfatides. The factors regulating baseline level of serum sulfatides are still unclear, but the proteins influencing sulfatide synthesis and/or metabolism should be considered. Sulfatides are biosynthesized by only two enzymes, CST and ceramide galactosyltransferase, and are degraded by galactosylceramidase and arylsulfatase A.⁴ Our recent experimental study found that expression of CST was regulated by a transcriptional factor, peroxisome proliferator-activated receptor α (PPAR α).²¹ It is reported that PPAR α becomes degraded with conditions such as kidney dysfunction.²² PPAR α ability in each patients and its degradation by kidney dysfunction might influence in CST expression and serum sulfatide levels. Furthermore, it is possible that alterations of other key enzymes such as arylsulfatase A, ceramide galactosyltransferase, and galactosylceramidase affect serum

sulfatide levels in HD patients. We currently have no information regarding alterations of the function of PPAR α and sulfatide metabolizing enzymes in HD patients or in experimental models of chronic kidney disease, and further study is required in this area.

The time-dependent increases in oxidative stress in HD patients have been controversial. As baseline levels of oxidative stress appear to differ among HD patients depending on their background characteristics, it seems that cross-sectional studies hardly detect the time-dependent increase in oxidative stress. The current follow-up study found that oxidative stress increased progressively in HD patients, regardless of the baseline level, suggesting that continuation of HD treatment hardly suppress the increase in oxidative stress. Intervention to treat this unhealthy internal condition is therefore important. Many studies reported that the oxidative stress of HD patients could be ameliorated by administration of alpha-tocopherol, vitamin C, and N-acetylcysteine.¹⁶ However, neither normalization nor sufficient reduction of oxidative stress in HD patients have yet been established. Our previous clinical studies found that the serum MDA levels and serum sulfatide levels could be normalized by kidney transplantation after a few years.^{13, 14} As continuation of hemodialysis treatment hardly improve oxidative stress and serum sulfatide abnormality, development of novel

therapeutic strategies, including promotion of kidney transplantation, may be important.

This study has some limitations that should be considered. First, as organ biopsy is not performed in HD patients without clinical need, we could not measure the levels of sulfatide synthesis enzymes. Our proposed molecular mechanism for the decrease in serum sulfatide levels is therefore speculative. Second, the observed inverse correlation between serum sulfatide levels and serum MDA levels does not prove direct causality. Third, we did not measure the other pro-oxidant and antioxidant markers; therefore, further investigation regarding time-dependent alterations of these markers is necessary. Fourth, our results may have been influenced by confounding factors that were not accounted for. Fifth, this study included only HD patients who were followed up for 3 years, and patients who died during the study period were excluded. Sixth, this study did not determine whether decreased serum sulfatide levels affect prognosis, and this should be further investigated in future studies. Finally, this study seems to have a relatively small population size when compared with other clinical studies investigating common risk factors in HD patients; therefore, assessment of whether this was an adequate sample size is necessary. We examined a published study investigating serum sulfatide abnormality in kidney transplantation recipients in which there was a total of 81 samples from 17 recipients. In that study, nevertheless, we were able to detect a

relationship between serum sulfatide abnormality and oxidative stress.¹⁴ In the current study, we investigated 285 samples from 95 HD patients and were able to detect a potential relationship between time-dependent serum sulfatide abnormality and oxidative stress, a finding consistent with our past studies. Therefore, we believe that the sample size of the current study meets the level necessary to evaluate serum sulfatide abnormality in HD patients.

CONCLUSION

Results of the current 3-year follow-up study demonstrated, for the first time to the best of the authors' knowledge, the time-dependent aggravation of serum sulfatide abnormality in HD patients, accompanied by increased MDA levels. Although the absolute level of serum MDA does not determine the baseline level of serum sulfatides, time-dependent deterioration of the internal environment, such as increasing oxidative stress, might be an aggravating factor that results in decreased serum sulfatide levels. As continuation of HD treatment hardly correct abnormal serum sulfatide levels or the increased oxidative stress, development of novel therapeutic strategies may be important.

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FIGURE LEGENDS

Figure 1

Serum sulfatide levels in hemodialysis (HD) patients. (A) Scatter plot showing serum sulfatide levels according to duration of HD. (B) Scatter plot showing cross-sectional analysis of serum sulfatide levels and serum malondialdehyde (MDA) levels.

Figure 2

Time-dependent alterations in serum levels of sulfatide and an oxidative marker. (A) Serum sulfatide levels, and (B) serum MDA levels during 3 years of follow-up. $***P < 0.001$ compared with baseline, $###P < 0.001$ compared with 18 months. (C) The composition (means) of the different molecular species of serum sulfatides in 95 HD patients at baseline, 18 months and 36 months.

Figure 3

Time-dependent alterations in serum sulfatide levels in various subgroups. Blue, green, and blown box plot distributions indicate values at baseline, 18 months, and 36 months, respectively. $**P < 0.01$, $***P < 0.001$ compared with baseline. $##P < 0.01$, $###P < 0.001$ compared with 18 months. $¶P < 0.05$, $¶¶P < 0.01$ compared with the other group.

AVF, arteriovenous fistula; AVG, arteriovenous graft; HDF, hemodialysis filtration;

RAS, renin-angiotensin system.

Figure 4

Scatter plots of serum sulfatide levels and serum MDA levels, showing all the data from all patients. Blue diamonds, red squares, and green triangles indicate values at baseline, 18 months, and 36 months, respectively.