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SHORT COMMUNICATION

Kidney dysfunction induced by protein overload nephropathy reduces serum sulphatide levels in mice

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Running title: Low serum sulphatide levels in nephropathy mice

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ABSTRACT

Aim: We recently proposed serum sulphatides as a novel biomarker for cardiovascular disease in patients with end-stage renal failure (ESRF), based on the possible anti-thrombotic properties of this molecule. In this earlier study, the level of serum sulphatides was gradually decreased in parallel with kidney dysfunction; however the precise mechanism underlying this decrease was unknown. The aim of the present study was to investigate the mechanism underlying the decrease in serum sulphatide levels caused by kidney dysfunction in an experimental animal model.

Methods: To produce a kidney dysfunction animal model, we prepared a mouse model of protein overload nephropathy. Using high-throughput analysis combined with matrix-assisted laser desorption ionisation time-of-flight mass spectrometry, we measured the levels of sulphatides in the sera, livers, small intestines and kidneys of protein overload nephropathy mice.

Results: As the disease progressed, the levels of sulphatides in sera decreased. Also, the levels in livers and small intestines decreased in a similar manner to those in sera, to approximately 60% of the original levels. On the contrary, those in kidneys increased by approximately 1.4-fold.

***Conclusion*:** Our results indicate that kidney dysfunction affects the levels of

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sulphatides in lipoprotein-producing organs, such as livers and small intestines, and lowers the levels of sulphatides in sera.

Key words: lipoprotein-producing organs, protein overload nephropathy, kidney dysfunction, serum sulphatides,

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INTRODUCTION

Sulphatides (I^3SO_3-GalCer) are a type of acidic glycosphingolipid, composed of sphingoid, fatty acid, galactose and sulphate, and are thought to be present on the outer leaflets of biological membranes in various tissues, such as brain, kidney, liver, and gastrointestinal tract.[1] They are also present in serum lipoproteins.[1,3] An immunofluorescence study showed that sulphatides in rat kidneys were distributed predominantly on the luminal (apical) membrane of the thick ascending limb.[2] We have been investigating the possible involvement of sulphatides in thrombosis/atherosclerosis since the late 1980s. We have found that sulphatide levels are increased in the sera of animal models for familial hypercholesterolemia, and that they prolong blood-coagulation time and bleeding time.[3, 4, 5, 6] These results led us to the hypothesis that sulphatides might be natural anticoagulant/antithrombotic agents in blood.[7] Cardiovascular disease (CVD) is a serious problem for patients with kidney dysfunction. Such patients are more likely to die of CVD than to develop kidney failure. Mortality due to CVD is 10 to 30 times higher in these patients than in the general population. Thus, CVD in patients with end-stage renal failure (ESRF) is a world-wide social and health problem, and many risk factors have been proposed.[8, 9, 10] Previously, we established a high-throughput method for the analysis of lysosulphatides (LSs) as a

measure of the level of sulphatides in a small amount of sera, using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS).[11] Subsequently, using this method, we demonstrated that the level of serum sulphatides was gradually decreased in parallel with kidney dysfunction and found a close correlation between low serum sulphatides levels and the high incidence of CVD in patients with ESRF.[12] However, the precise mechanism underlying this decrease in serum sulphatide levels with kidney dysfunction remains unknown. As a first step to elucidate this mechanism, we investigated the amounts and compositions of sulphatides in major serum sulphatide-producing organs in the kidney dysfunction state. For this purpose, we employed mouse protein overload nephropathy as a kidney dysfunction model, because this animal model develops reproducible kidney dysfunction owing to proximal tubular injuries in short time.[13, 14]

MATERIALS AND METHODS

Animal Studies

Male mice on an Sv/129 genetic background were used as described elsewhere.[15]

Mice (18 weeks) were treated in a specific pathogen-free facility according to the Shinshu University and National Institutes of Health animal care guidelines and the Accreditation of Laboratory Animal Care guidelines. Protein overload nephropathy was

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chosen as a kidney dysfunction animal model[14]. The mice were given consecutive daily intraperitoneal bolus injections of 0.4 g of bovine serum albumin (BSA) for 10 days. Protein overload did not result in glomerular injuries as well as diffuse interstitial fibrosis, while the treatment obviously caused proximal tubular injuries, such as tubular vacuolation, tubular dilatation, tubular hyaline cast formation, and detachment of tubular cells from the tubular basement membrane, resulting in kidney dysfunction. Under general anaesthesia, mice were killed by exsanguinations for analysis on days 4 and 10. The numbers of mice subjected to analysis were as follows: day 0 group, normal mice, n = 4; day 4 group, n = 6; day 10 group, n = 8. Sera, livers, small intestines, and kidneys were removed and stored at -80°C until analysis.

Quantitation and qualitation of sulphatides

Sulphatides were extracted from specimens using a hexane-isopropanol mixture.[18] After chemically converting sulphatides to LSs (sulphatides without fatty acids), samples were desalted by Mono-tip C18 tips (GL Sciences Inc. Tokyo, Japan) and analysed by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry using a Voyager Elite XL Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA) in the negative ion mode, as described previously.[11, 12, 16, 17] This method allowed quantitation of serum sulphatides from 100 serum specimens within one working day.

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Tissues were fixed in formaldehyde and embedded in paraffin. Thin sections (3 microns) were cut and stained with haematoxylin and eosin.

Statistics

Kruskal-Wallis tests were used to compare values. A P value of <0.05 was considered to show statistical significance.

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RESULTS AND DISCUSSION

Figure 1 (left half) shows typical mass spectra of LSs converted from sulphatides in the sera, livers, small intestines, and kidneys of normal mice. There are seven molecular species of lysosulphatides, namely, LS-d18:2, (LS containing sphingadienine as a sphingoid), LS-d18:1, (LS containing sphingosine), LS-d18:0 (LS containing sphinganine), LS-t18:0 (LS containing phytosphingosine), LS-d20:1 (LS containing icosasphingosine d20:1), LS-d20:0 (LS containing icosasphinganine), and LS-t20:0 (LS containing icosaphytosphingosine) (Fig. 1). The LS compositions of sulphatides in sera, livers, small intestines, and kidneys were determined by MALDI TOF-MS analysis, as shown in the right half of Fig. 1. The major LS species were LS-d18:1 and LS-t20:0,

which represented 32.6±0.7% and 24.9±0.3%, respectively, of the LSs in sera, 28.9±1.0% and 19.7±0.9%, respectively, of the LSs in livers, and 15.6±0.3% and 36.1±0.6%, respectively, of the LSs in small intestines. In addition, significant proportions of LS-d18:2, LS-d18:0, LS-t18:0 and LS-d20:1 were commonly observed in these three sample types. The LS composition of sulphatides in sera was very similar to that in livers and partly similar to that in small intestines. Serum sulphatides are present as a major glycosphingolipid in serum lipoproteins.[3] It is well known that most serum lipoproteins originate from livers (approximately 90%), with the rest being derived from the small intestines (approximately 10%), as confirmed by sphingoid analysis of sulphatides.[3] Our results indicate that the compositions of molecular species of LSs converted from sulphatides reflect the locations where serum sulphatides are probably produced. LSs in kidneys were predominantly composed of LS-d18:1 (60.0±0.7%). The composition of LSs in kidneys is thought to be specific to this organ. Judging from the obvious differences in the levels of different molecular species of sulphatides between kidneys and sera, livers or small intestines, there seemed to be no metabolic correlation between the sulphatides in the kidneys and those in lipoprotein-related organs. The levels of sulphatides in normal mice were determined to be 1598.5±60.3 pmol/ml in sera, 31.3±1.1 pmol/mg in livers, 60.1±1.1 pmol/mg in small intestines, and

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1160.9±130.8 pmol/mg in kidneys. It is well known that the kidneys contain a high level of sulphatides, second only to nervous tissues.[1] Protein overload led to the development of acute tubular injury and produced kidney dysfunction, as reported elsewhere[14]. The serum urea nitrogen concentrations (mg/dl) on days 0, 4 and 10 were 21.0±3.9, 38.7±5.8 and 39.6±4.2, respectively. The serum creatinine concentrations (mg/dl) on the same days were 0.28±0.06, 0.46±0.05 and 0.72±0.18, respectively. The values for total urine protein (mg/day) were 2.4±0.1, 156.3±5.6 and 101.6±4.7, respectively. As the disease advanced, the levels of sulphatides in livers and small intestines were significantly decreased in a similar manner to those in sera (Fig. 2). No compositional differences in LSs were observed among the control group (day 0), the day 4 group and the day 10 group, for all three specimen types (data not shown). These results suggest that the reduction in the levels of sulphatides in sera is caused by renal dysfunction. Furthermore, this reduction is related to the reduction in the levels of sulphatides in the lipoprotein-producing organs, namely livers and small intestines, because both the LS species (Fig. 1) and the rates of decreases are similar among these three specimens (Fig. 2). On the other hand, the levels of sulphatides in kidneys were moderately increased without remarkable differences in the composition of LSs with progression of the disease. Using mouse and rat models of tubulointerstitial injury, Shikata's group

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reported that sulphatides were L-selectin ligands in kidneys, and relocated from the distal tubules to the peritubular capillaries with possible new synthesis of sulphatides in kidneys, where many infiltrating mononuclear cells, which contain sulphatides, were observed.[19,20] In the present study, a small but significant number of infiltrating monocytes was also observed by pathological examination of the kidneys of mice with renal dysfunction (data not shown). However, the numbers of monocytes seemed to be too small to explain the increase in the levels of sulphatides. More detailed examinations concerning the regulation of renal sulphatides will be needed in the future. Various studies have suggested that sulphatides are involved in renal functions, such as urine production/urine concentration[1]. The renal dysfunction caused by injection of BSA might somehow lead to an increment in the level of sulphatides in kidneys to compensate for the loss of kidney function. Recently, Nagai et al reported that the addition of gluconeogenic renal nutritional substrate (glutamine) to medium containing isolated renal tubules from rats accelerated the turnover rate of sulphatides to twice that of controls.[21] In our experiment, daily injection of BSA might supply enough amino acids to affect the synthesis of sulphatides, and thus, might increase the amounts in kidneys.

It is suggested that the reduction in the levels of serum sulphatides found in

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patients with ESRF is not due to long-term haemodialysis, but probably due to a reduction in the levels of sulphatides in livers and small intestines where serum sulphatides are supposed to be produced and supplied to the blood stream as components of serum lipoproteins. The present experiment, using mice with protein overload nephropathy, indicated that kidney dysfunction affects the levels of sulphatides in lipoprotein-producing organs, such as livers and small intestines, and lowers the level of sulphatides in sera. It remains to be studied how kidney dysfunction affects the levels of sulphatides in livers and small intestines. A current working hypothesis is that kidney dysfunction might influence the expression levels of sulphatides-metabolic enzymes in livers and small intestines. Studies concerning the expression levels of these enzymes are currently underway.

The present study suggests that the reduction in the levels of serum sulphatides found in patients with kidney dysfunction might be due to a reduction in the levels of sulphatides in livers and small intestines, where serum sulphatides are probably produced and supplied to the blood stream as components of serum lipoproteins. Since serum sulphatides are thought to be a natural anti-thrombotic agent in blood [3,4,5,6], low levels of sulphatides may lead to the development of CVD in patients with kidney dysfunction. We believe that elucidation of the mechanism underlying the decrease in

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serum sulphatide levels associated with kidney dysfunction might contribute to an understanding of the relationship between kidney dysfunction and the high incidence of CVD in such patients in the future.

ACKNOWLEDGEMENT

This work was supported in part by GL Science Inc. Japan.

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Legends

Figure 1

Typical mass spectra (left) and compositions (right) of the seven molecular species of LSs in specimens from normal mice; A, LS-d18:2, B, LS-d18:1, C, LS-d18:0, D, t18:0, E, LS-d20:1, F, LS-d20:1 G, LS-d20:0. The peaks represent lysosulphatide molecular species. ISTD is an internal standard, *N*-acetyl lyso-d18:0. The second isotopic peak of LS-d18:1 (m/z 542) and the second peak of the ITSD (m/z 586) had the same molecular masses 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 calculated from the peak areas, and were used to determine the actual amounts of LS-d18:0 and LS-t20:0.

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Figure. 2

The total levels of sulphatides in specimens from the control group (normal mice), the day 4 group and the day 10 group. The significances of differences among these three groups in each fluid or organ were examined with Kruskal-Wallis tests and are shown in the upper right of each drawing. A *P* value of less than 0.05 was considered to indicate statistical significance.

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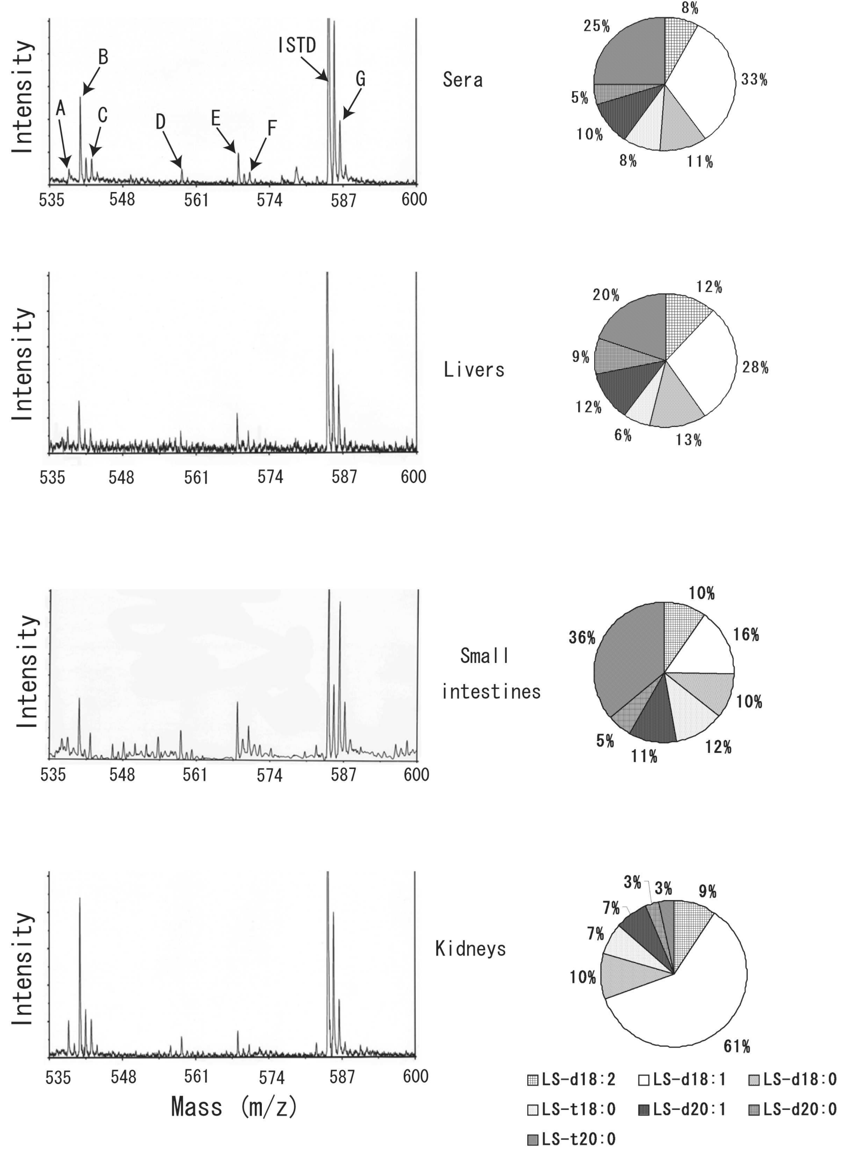

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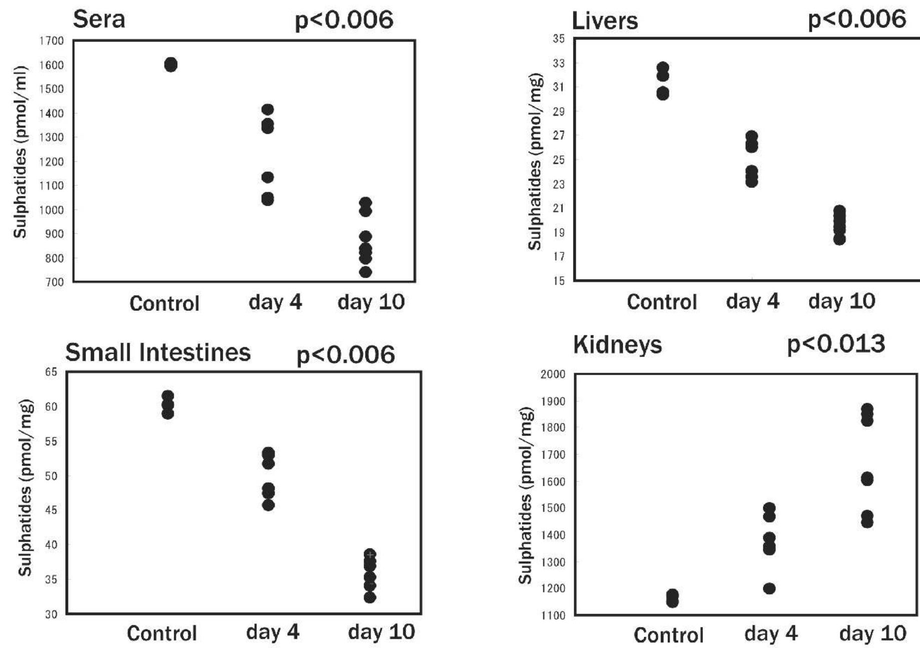
Figure 2



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