Association of circulating levels of total and protein-bound sphingosine 1-phosphate with osteoporotic fracture

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ABSTRACT

The biological activity and effects of circulating sphingosine 1-phosphate (S1P) might be dependent on the carrier protein. Although S1P is known to be a biomarker for osteoporotic fracture (OF), its role according to its carrier protein (high-density lipoprotein (HDL), low-density lipoprotein (LDL), or albumin) has not yet been studied. We measured the protein-bound S1P levels and bone mineral density (BMD) in 58 postmenopausal women with OF and 58 age-matched and body mass index–matched postmenopausal women without OF. Albumin-bound S1P was the most abundant. Before adjustment, women with OF had higher total S1P (p=0.046) and albumin-bound S1P (p=0.026) levels than those without OF, but there was no difference in the levels of HDL-bound or LDL-bound S1P. After adjustment for confounders including BMD, women with OF had only higher levels of total S1P than those without OF (p=0.047). Before adjustment, the OR for OF was higher in subjects in the highest quartile for total S1P (OR 5.36, 95% CI 1.22 to 23.63) or albumin-bound S1P (OR 4.48, 95% CI 1.22 to 16.42). After adjustment for confounders including BMD, statistical significance persisted only for total S1P (OR 2.23, 95% CI 1.12 to 4.81). These findings suggest that the positive association of S1P with OF is mainly due to level of total plasma S1P and not due to the differing contributions from specific carrier protein-bound fractions.

INTRODUCTION

Several studies showed sphingosine 1-phosphate (S1P) as an important lipid mediator with various roles in bone metabolism, that is, increased bone formation[1–3] and increased bone resorption.[4–6] S1P increased bone resorption by stimulation of osteoclast differentiation[6] and of the migration of osteoclast precursor cells from the blood to the bone marrow (BM) due to S1P concentration gradient between the blood with a high S1P level and BM with a low S1P level.[4 5] High circulating S1P levels in human had association with increased bone resorption markers and lower bone mineral density (BMD), but not with bone formation markers,[7–10] so effects of S1P on bone metabolism in human seem to be dominant to bone resorption rather than bone formation. Furthermore, increased plasma S1P levels were associated with higher risks of osteoporotic fracture (OF), independent of the BMD or other clinical risk factors (CRFs).[7–9] Collectively, these data suggest that circulating S1P levels could be a new biological marker of fracture risk, independent of CRFs and BMD.[11]

Circulating S1P is carried by lipoproteins (high-density lipoprotein (HDL) and low-density lipoprotein (LDL)) and albumin. In general, approximately 50% of S1P binds to HDL, and the remaining S1P binds to albumin,[12] although more than 50% of S1P was found to be albumin bound in some studies.[13 14] The biological activity of circulating S1P might be dependent on the carrier protein. For example, in contrast to the potentially beneficial effects of HDL-bound S1P as an anti-inflammatory or anti-atherogenic mediator,[15–18] albumin-bound S1P appears to promote inflammatory responses, resulting in a pro-atherogenic mediator.[17] However, the functional roles of S1P according to the carrier protein (HDL, LDL, or albumin) have not been studied in the context of bone biology. In the present study, we investigated the association of protein-bound S1P with OF in humans.

MATERIALS AND METHODS

Study participants and protocol

We selected study subjects from postmenopausal Korean women who either visited the clinic for concerns regarding the possibility of diagnosis with osteoporosis or were referred to the clinic for osteoporosis that had been detected during a routine examination at the Asan Medical Center (Seoul, Korea) between January 2010 and October 2017. Exclusion criteria was premature menopause (<40 years of age), history of medication, and diseases that could affect bone metabolism. To rule out any systemic illness, subjects with a fever and abnormality in complete blood counts, liver, kidney, or thyroid function, or concentrations of calcium, phosphorus, or alkaline phosphatase were excluded. After excluding subjects, 540 women were eligible for participation in the study. Among these women, 58 cases of OF were identified. For a case–control study,

controls were randomly selected from the remaining 482 subjects and matched 1:1 to cases by both age (within 1.0 year) and body mass index (BMI; within 1.0 kg/m^2). Written informed consent was obtained from all study subjects.

Measurement of BMD and fracture assessment

Areal BMD values at the femur neck (FN-BMD) (g/cm^2) were measured using dual-energy X-ray absorptiometry (Lunar system V.9.30.044; Prodigy, Madison, WI). The precision of the equipment, the coefficient of variation, was 1.25%.

For detection of morphological vertebral fracture (VF), lateral thoracolumbar radiographs were measured from all participants. VF was assessed in accordance with the recommendations of the Working Group on Vertebral Fractures.[19] Definition of VF was a >20% reduction in any vertebral height (ie, anterior, middle, or posterior).[20] Non-vertebral fractures at osteoporosis-related locations (the hip, distal radius, proximal humerus, and pelvis) were evaluated using a self-administered questionnaire. Non-osteoporotic fractures (ie, fractures by major trauma or falls from higher than standing height) were excluded, so low-trauma fractures after menopause were only included.

Measurement of different forms of protein-bound S1P

Lipoproteins and the albumin fraction were separated using sequential density gradient ultracentrifugation.[21] Samples were electrophoresed on agarose gels to confirm the separation of lipoproteins and the albumin fraction. S1P was extracted from each protein fraction separated by ultracentrifugation using liquid–liquid extraction.[22] The quantities of S1P extracted from the isolated protein fractions were measured using liquid chromatography–tandem mass spectrometry (LC-MS/MS).[12 23] S1P and C17-S1P were purchased from Avanti Polar Lipids (USA). Human HDL and serum albumin were from Sigma-Aldrich (USA). Solvents and other chemicals were purchased from Sigma-Aldrich, JT Baker, or Merck (USA), unless otherwise stated.

Statistical analysis

The baseline characteristics of the cases and controls were compared using Student's t-tests or Mann-Whitney U tests for continuous variables and χ^2 tests for categorical variables. Multivariate-adjusted least-square means and 95% CIs for S1P levels according to fracture status were estimated by analysis of covariance before and after adjustment for CRFs that might have effects on bone metabolism and FN-BMD. CRFs included age, BMI, smoking status, alcohol intake (≥3 units/day), regular outdoor exercise (≥30 min/day), and parental history of hip fracture. For both total S1P levels and protein-bound S1P levels, conditional logistic regression analyses were performed to generate ORs that compared the association with OF for subjects in each of the highest three quartiles with the association with OF for subjects in the lowest quartile after adjustment for confounders. All statistical analyses were performed with SPSS statistical software, and p value <0.05 was considered statistically significant.

RESULTS

Characteristics of the study subjects are listed in table 1. Subjects with OF had higher levels of total S1P (p=0.046) and albumin-bound S1P (p=0.026), but not HDL-bound S1P (p=0.171) or LDL-bound S1P (p=0.104), than those without OF. Albumin-bound S1P was the most abundant (57.4% in subjects without OF and 58.2% in those with OF), and HDL-bound S1P (36.6% and 35.8%, respectively) was more abundant than LDL-bound S1P (6.0% and 6.0%,

Table 1 Baseline characteristics in 1:1 matched pairs of patients with fracture and without fracture

	Subjects without fracture (n=58)	Subjects with fracture (n=58)	P value
Age (years), mean±SD	63.7±6.8	64.3±6.6	0.660
Weight (kg), mean±SD	58.5±6.9	57.5±7.3	0.460
Height (cm), mean±SD	155.3±4.9	154.4±5.7	0.374
BMI (kg/m^2), mean±SD	24.3±2.8	24.1±2.9	0.820
Exercise ≥30 min/day, n (%)	22 (37.9%)	21 (36.2%)	>0.999
Current smoker, n (%)	1 (1.7%)	0 (0.0%)	>0.999
Alcohol intake ≥3 U/day, n (%)	1 (1.7%)	1 (1.7%)	>0.999
Parental history of hip fracture, n (%)	5 (8.6%)	6 (10.3%)	>0.999
FN-BMD (g/cm^2), mean±SD	**0.787±0.112**	**0.736±0.109**	**0.013**
Corrected calcium (mg/dL), mean±SD	8.8±0.5	9.0±0.4	0.185
Phosphorus (mg/dL), mean±SD	3.8±0.4	3.7±0.37	0.096
S1P levels (pmol/mL), mean (95% CI)			
Total S1P	**1030.3 (965.5–1095.2)**	**1123.9 (1059.1–1188.8)**	**0.046**
Albumin-bound S1P	**584.8 (542.8–626.4)**	**651.9 (610.1–693.8)**	**0.026**
HDL-bound S1P	377.5 (345.1–409.9)	409.3 (377.0–441.7)	0.171
LDL-bound S1P	59.4 b(52.3–66.4)	67.6 (60.5–74.6)	0.104

Patients with fracture and without fracture were matched for age (±1.0 years) and BMI (±1.0 kg/m^2).
All values are the mean±SD unless otherwise specified.
Boldface values are statistically significant.
*Corrected calcium concentration (mg/dL)=total calcium concentration (mg/dL)+0.8×[4.0 g/dL−serum albumin concentration (g/dL)].
BMD, bone mineral density; BMI, body mass index; FN-BMD, BMD at femur neck; HDL, high-density lipoprotein; LDL, low-density lipoprotein; S1P, sphingosine 1-phosphate.

Table 2 ORs (95% CIs) for fracture according to levels of total and protein-bound S1P by quartile

	Model 1			Model 2			Model 3		
	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
Total S1P (pmol/mL)									
Q1 (535.46–919.50)	Ref			Ref			Ref		
Q2 (919.51–1063.61)	**4.75**	**1.16 to 19.37**	**0.030**	1.99	0.89 to 4.49	0.083	1.96	0.84 to 4.54	0.117
Q3 (1063.62–1245.48)	**5.13**	**1.34 to 19.71**	**0.017**	2.09	0.96 to 4.71	0.063	**2.14**	**1.03 to 4.76**	**0.044**
Q4 (1245.49–1664.76)	**5.36**	**1.22 to 23.63**	**0.026**	**2.38**	**1.16 to 5.25**	**0.031**	**2.23**	**1.12 to 4.81**	**0.033**
Albumin-bound form (pmol/mL)									
Q1 (301.15–514.30)	Ref			Ref			Ref		
Q2 (514.31–617.72)	2.70	0.83 to 8.77	0.099	1.80	0.87 to 3.94	0.110	1.79	0.75 to 4.25	0.186
Q3 (617.73–709.74)	3.10	0.99 to 9.74	0.053	1.90	0.88 to 4.22	0.085	1.93	0.82 to 4.55	0.134
Q4 (709.75–999.19)	**4.48**	**1.22 to 16.42**	**0.024**	**2.08**	**1.02 to 4.45**	**0.048**	1.98	0.91 to 4.36	0.090
HDL-bound form (pmol/mL)									
Q1 (164.86–306.18)	Ref			Ref			Ref		
Q2 (306.19–375.11)	1.38	0.47 to 4.02	0.553	1.20	0.54 to 2.69	0.655	1.24	0.55 to 2.77	0.603
Q3 (375.12–479.63)	1.84	0.58 to 5.86	0.302	1.35	0.63 to 2.88	0.442	1.31	0.61 to 2.80	0.485
Q4 (479.64–709.68)	2.92	0.84 to 10.14	0.091	1.58	0.73 to 3.41	0.242	1.63	0.76 to 3.50	0.211
LDL-bound form (pmol/mL)									
Q1 (16.29–42.74)	Ref			Ref			Ref		
Q2 (42.76–60.21)	1.00	0.37 to 2.71	0.996	0.98	0.45 to 2.12	0.956	0.99	0.46 to 2.15	0.990
Q3 (60.22–79.38)	1.11	0.41 to 2.98	0.842	1.07	0.50 to 2.29	0.852	1.10	0.52 to 2.34	0.801
Q4 (79.39–131.63)	1.66	0.56 to 4.88	0.358	1.28	0.62 to 2.63	0.505	1.31	0.64 to 2.69	0.457

Boldface values are statistically significant.
Model 1: unadjusted model. Model 2: adjusted for age, BMI, current smoking status, alcohol intake (≥3 units/day), parental history of hip fracture, and secondary osteoporosis. Model 3: adjusted for FN-BMD, in addition to the risk factors included in model 2.
BMD, bone mineral density; BMI, body mass index; FN-BMD, BMD at femur neck; HDL, high-density lipoprotein; LDL, low-density lipoprotein; S1P, sphingosine 1-phosphate.

respectively). There was no significant difference in the proportion of protein-bound S1P between subjects with or without OF.

After adjustment for CRFs and FN-BMD, there was a statistically significant difference between subjects with and without OF in total S1P (mean (95% CI) pmol/mL: 1137.7 (1064.3–1211.2) vs 1029.9 (956.8–1102.2), p=0.047), but not in albumin-bound S1P (652.7 (602.7–702.7) vs 600.7 (551.2–650.1), p=0.159), HDL-bound S1P (407.4 (374.7–440.1) vs 379.4 (346.7–412.1), p=0.239), or LDL-bound S1P (67.9 (60.7–75.1) vs 60.0 (51.8–66.20), p=0.091).

The association with OF was higher in subjects in the highest total S1P quartile (Q4) than in those in the lowest quartile (Q1), both before and after adjustment for CRFs (OR 5.36, 95% CI 1.22 to 23.63 and OR 2.38, 95% CI 1.16 to 5.25, respectively) (table 2). Even after adjustment for FN-BMD, the association remained statistically significant (OR 2.23, 95% CI 1.12 to 4.81). The association with OF was higher in subjects in the highest quartile for albumin-bound S1P (Q4) than in those in the lowest quartile (Q1), before and after adjustment for CRFs (OR 4.48, 95% CI 1.22 to 16.42 and OR 2.08, 95% CI 1.02 to 4.45, respectively). However, the statistical significance was lost after further adjustment for FN-BMD (OR 1.98, 95% CI 0.91 to 4.36). There was no statistically significant difference in the association with OF according to HDL-bound or LDL-bound S1P levels.

DISCUSSION

In this age-matched and BMI-matched case–control study of postmenopausal women, subjects with OF had markedly higher total S1P levels than those without OF, both before and after adjusting confounders. Furthermore, the association with OF was increased by 2.23-fold in subjects with the highest S1P levels compared with those with the lowest S1P levels. Albumin-bound S1P, the most abundant form of protein-bound S1P in postmenopausal women, was also higher in subjects with OF than in those without OF before adjustment for confounders. However, the statistical significance of the difference in albumin-bound S1P levels and of the association of albumin-bound S1P levels with OF were weakened after adjustment for confounders.

Consistent with the results of previous studies,[7–9 24] total S1P levels were associated with OF. Circulating S1P may exert different, even contradictory, effects on the cardiovascular system depending on whether it exists in a HDL-bound form or in an albumin-bound form.[17] In general, biologically active HDL-bound S1P has been shown to contribute to several beneficial effects of HDL-mediated anti-inflammatory, antioxidant, and antithrombotic processes.[15–18] By contrast, albumin-bound S1P levels have been shown to promote inflammatory responses.[17] Therefore, we investigated the association of different types of protein-bound S1P with OF. One of the interesting findings of this study was that albumin-bound S1P levels, but not HDL-bound S1P levels, tended to be associated with OF. Furthermore, OF was more strongly associated with total S1P levels than albumin-bound S1P levels. We were not able to elucidate the reason for these findings in this study, but the data suggest that fracture is mainly associated with the high level of circulating total S1P and is not associated with the

any variation in biological activity according to the carrier protein. The hypothesis regarding the beneficial effects of biologically active HDL-bound S1P was that HDL might neutralize or buffer the potentially deleterious effects of free S1P by scavenging plasma S1P.[17] Thus, the higher levels of albumin-bound S1P in patients with OF in the present study might reflect higher circulating S1P levels due to increased bone resorption, which might be associated with OF despite the scavenging of plasma S1P by HDL. Furthermore, the stronger correlation between total S1P and albumin-bound S1P (γ=0.856) than between total S1P and HDL-bound S1P (γ=0.738, p=0.013) might also explain the association of albumin-bound S1P, but not HDL-bound S1P, with OF. Collectively, these data suggest that high levels of total S1P itself, not differing effects of S1P according to the carrier protein, negatively affect bone metabolism.

Another interesting finding was that albumin-bound S1P was more abundant than HDL-bound S1P (57.4%–58.2% vs 35.8%–36.5%) in the study subjects. A previous study showed that circulating S1P exists in both HDL-bound (approximately 50% of total S1P) and albumin-bound (40%–50% of total S1P) forms,[12] although more than 50% of S1P was albumin-bound in some subjects.[13 14] This difference in the distribution of protein-bound S1P is probably related to differences in gender, age, and race, as well as differences in the method used to detect the S1P. This study examined 116 postmenopausal Korean women with a mean age of 64 years, compared with a previous study, which examined only four Japanese individuals of unknown sex and age.[12] We used direct LC-MS/MS measurements, in contrast to the in vitro [^{3}H]S1P radioreceptor assay used in the previous study.[12] Thus, we believe that this study provides reliable information on the distribution of plasma S1P levels in a large number of individuals.

Several potential limitations should be considered when interpreting these results. First, platelets when activated by thrombin and mast cells when activated by immunoglobulin E-bound antigen can secrete S1P. Therefore, platelets and mast cells affect S1P levels. We intentionally applied strict inclusion criteria to minimize the possibility of including subjects who may have had infectious or immune disorders. In addition, it has been reported that neither platelets nor mast cells have a role in regulating the homeostatic levels of S1P in the blood.[25] Second, the study population comprised postmenopausal Korean women. Further prospective studies that include large numbers of patients and other ethnic groups are therefore needed.

Levels of both total S1P and albumin-bound S1P, the most abundant protein-bound form of S1P, were significantly associated with OF before adjustment. After adjustment for confounding factors, only total S1P was still significantly associated with OF. These results suggest that the deleterious effects of S1P on bone metabolism are mainly due to the high level of total S1P itself, and not due to differences in the biological activity of S1P bound to different carrier proteins.

Correction notice This article has been corrected since it was first published. Hyun Ju Yoo has been added as a joint corresponding author. Additionally, the author's institution has been added to the second affiliation: 'University of Ulsan College of Medicine'.

Contributors HJY and J-MK contributed to the study conception and design. Acquisition and analysis of data were performed by HES, SHL, SJK, and B-JK. Interpretation of data was performed by SHL, HJY, and J-MK.The first draft of the manuscript was written by HES and SHL. All authors read and approved the final manuscript.

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