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Plasma and Erythrocyte Membrane Plasmalogen Diminished in Severe Atherosclerotic Patients Undergoing Endovascular Therapy

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Plasmalogen in phospholipid class is ubiquitously found in biological membrane. Although oxidative stress is involved in human atherosclerosis and this unique phospholipid may play a role as an endogenous antioxidant, there still remains a debate concerning the role of plasmalogen in human atherosclerosis. To investigate the relationship between plasmalogen and atherosclerosis, plasmalogen in plasma and erythrocyte membrane was quantified in atherosclerotic patients (n = 20) and age–matched control subjects (n = 20) using high–performance liquid chromatography (HPLC). In the HPLC of plasma treated with phospholipase A_1, concentrations of choline plasmalogens (pl–PC) and ethanolamine plasmalogens (pl–PE) in atherosclerotic patients were significantly lower than the respective concentrations in healthy controls. In the HPLC of erythrocyte membrane phospholipid extract, the peak area of pl–PE in atherosclerotic group was reduced significantly relative to that in control group. This study demonstrated that plasmalogen in plasma and erythrocyte membrane is diminished in patients with severe atherosclerosis requiring endovascular therapy. Further studies are required to elucidate the clinical role of plasmalogen as a laboratory marker in severe atherosclerotic patients.

Keywords : atherosclerosis ／ case–control study ／ erythrocyte membrane ／ HPLC ／ plasmalogen

1. Introduction

As life expectancy prolongs, atherosclerosis is becoming a major health problem worldwide. Atherosclerotic arteries underlie aortic aneurysm, acute myocardial infarction, stroke and obstructive peripheral artery disease. Atherosclerosis is currently recognized as a sequel of oxidative stress and subsequent inflammatory process initialized at vascular endothelium, interface between the vascular smooth muscles and circulating leukocytes carrying reactive oxygen species (ROS) [1, 2]. Oxidative stress causing this common process alters the structure and impairs the function of membrane of circulating erythrocytes carrying oxygen [3, 4]. Therefore, the erythrocyte membrane phospholipid contents and profile reflect the vascular and systemic redox status [5, 6].

The phospholipid class of plasmalogen is ubiquitously found in biological membrane and involved in signaling pathway and cellular differentiation [7]. Emerging

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evidence suggests that plasmalogen acts as an endogenous antioxidant [8, 9]. Circulating erythrocytes have no intracellular organelle, and hence phospholipids are present only in the plasma membrane, indicating that plasmalogen is abundant in human erythrocyte membrane. As a matter of fact, our research group currently separated intact phosphatidylethanolamine plasmalogen (pl–PE) as well as all other phospholipids in human erythrocyte membrane by a single run of high-performance liquid chromatography (HPLC) [10], and clarified that endogenous pl–PE relative to sphingomyelin (SM) in erythrocyte membrane is reduced in patients with Alzheimer's disease [11]. Alzheimer's disease is the main cause of senile dementia, and oxidative stress in human brain plays a pivotal role which triggers β–amyloid deposition mediating neuroinflammation [12]. This line of evidence indicates that age-dependent diseases such as dementia and atherosclerosis are characterized by oxidative stress and subsequent chronic inflammation under the derangement of redox condition [13], and that plasmalogen may play a protective role against oxidative stress leading to chronic inflammation [8, 9].

Therefore, the present study was designed to investigate the plasmalogen in blood and erythrocyte membrane obtained from patients with severe atherosclerosis requiring endovascular intervention.

2. Experimental

2.1 Materials

The study population consisted of 20 Japanese atherosclerotic patients (71.7 $\pm$ 6.5 years, 6 women and 14 men) and 20 age–matched control subjects (71.5 $\pm$ 4.8 years, 10 women and 10 men) as a case–control study. Blood examination and other noninvasive routine laboratory examinations were performed in these subjects. Blood pressure was measured at sitting position by sphygmomanometer after taking a few minutes rest. Body mass index (BMI) was calculated by body weight (kg) divided by square of height (m^2) as an index of obesity. There were no current smokers in the enrolled subjects, because smoking cessation had been strongly recommended to the atherosclerotic patients.

The control group contained Japanese subjects visiting the outpatient clinic of BOOCS clinic (Fukuoka, Japan) for the purpose of health screening. They had no hypertension, dyslipidemia, diabetes or dementia, and hence they had no medication. The atherosclerotic group included patients who were referred to the Heart Center in the Kyushu University Hospital (Fukuoka, Japan) for invasive endovascular therapy. These atherosclerotic patients had coronary artery diseases such as angina pectoris (n = 10) and old myocardial infarction (n = 3), thoracic or abdominal aortic aneurysm (n = 4) and obstructive peripheral artery disease (n = 3). Endovascular therapy included percutaneous coronary angioplasty for coronary artery diseases, stent graft implantation to the aortic aneurysm or percutaneous intervention to the peripheral artery disease such as arteriosclerosis obliterans. They had at least one of the atherogenic risk factors such as hypertension (n = 15), diabetes (n = 10) and dyslipidemia (n = 11). Exclusion criteria included hemodialysis, dementia and cancer. They were treated with antihypertensive drugs including long–acting Ca antagonists (55%), angiotensin receptor blocking agents (45%) and β–blocking agents (70%), although combined antihypertensive drug prescription was common (65%). Some of them (40%) were treated with highly purified agents of eicosapentaenoic acid (EPA). EPA is rich in fish oil and one of the polyunsaturated fatty acids improving serum lipid profile and blood rheology. Anti–platelet agents (aspirin, clopidogrel, prasugrel, and the combination of these) were prescribed in most of the patients (90%). Hydroxymethylglutaryl–CoA reductase inhibitor (so–called 'statin') was prescribed in 70% of the patients to lower the serum cholesterol level and to improve the cholesterol profile. These prescriptions were under the discretion of the treating physicians.

All procedures performed in this study were in accordance with the current ethical standards of each institutional and/or national research committee and with the updated Declaration of Helsinki (2008), i.e., signed informed consent was obtained from each subject after the admission to the hospital and before the vascular intervention in the atherosclerotic group or at the enrollment into the study in the control group.

2.2 Measurements of plasma concentration of plasmalogen

Plasma concentration of plasmalogen was quantified according to the method elsewhere reported [14]. Briefly, venous blood was sampled by disposable syringe containing heparin, and plasma was separated

by centrifugation at 1,000 × g for 5 minutes. Plasma was kept at –80 ℃ until use. Phospholipase A_1 (PLA_1) purchased from Sigma–Aldrich Co. (Tokyo, Japan) and Mitsubishi Kagaku Foods Co. (Tokyo, Japan) was diluted with an equal volume of 0.1 M citrate buffer (pH 4.5), and 20 μl of the diluted PLA_1 was added to 80 μl of plasma and incubated at 45 ℃ for 60 minutes.

Lipid extraction after the treatment with PLA_1 was performed by adding 800 μl of *n*–hexane/isopropanol (3 : 2, v/v) to the PLA_1–treated plasma. After vigorous mixing, it was placed in an ultrasound bath for 5 minutes. Then, 400 μl of Na_2SO_4 solution was added and left for 5 minutes, and 400 μl of hexane layer was transferred to a new conical Eppendorf tube. Thereafter, 400 μl of hexane/isopropanol (7 : 2, v/v) was added to the lower phase and vigorously mixed, and the hexane layer (300 μl) was recovered. The combined hexane layer was dried under N_2 gas and stored at –30 ℃ until use.

Separation of phospholipid classes including ether phospholipids was performed by using HPLC–ELSD system [14], which was composed of an Agilent 1100 equipped with a four–solvent delivery system, a degasser, an automatic injector, and evaporative light scattering detector (ELSD). The column was a LiChrosphere 100 Diol (250 × 2 mm), 5 μm (Merck, Germany). Mobile phase A was *n*–hexane/2–propanol/acetic acid (82 : 17 : 1, v/v/v) with 0.08% triethylamine (TEA), and mobile phase B was 2–propanol/water/acetic acid (85 : 14 : 1, v/v/v) with 0.08% TEA. The lipid extract after PLA_1 treatment of plasma was reconstituted with 200 μl of hexane/isopropanol (3 : 2, v/v), and 20 μl was applied to the HPLC system. The column temperature was 50 ℃ and flow rate was 0.4 ml/min. The phospholipid classes were detected by ELSD (Agilent 1900 Infinity) with the following settings: evaporation temperature, 60 ℃; sensitivity gain, 6; flow rate of N_2 gas, 1 l/min. Nebulizer temperature was 30 ℃. Linear regression curves for calculation of phospholipid concentration from chromatographic peak area showed $R^2 > 0.97$ [14].

2.3 Measurements of erythrocyte membrane plasmalogen

Extraction of lipid from erythrocyte membrane was essentially performed according to the method reported previously [10]. In brief, blood was sampled using a disposable syringe containing ethylenediaminetetraacetic acid disodium salt (EDTA–2Na), cooled immediately after sampling in an ice bath, kept in a refrigerator and processed within 48 hours. Plasma and buffy coat were removed after centrifugation at 1,000 × g for 5 minutes at 4 ℃, and the erythrocytes were washed three times in cold isotonic saline at 1,000 × g for 5 minutes at 4 ℃. A small portion of the supernatant was carefully aspirated at each washing. Five hundred μl of the packed erythrocytes was hemolyzed with an equal volume of 10 mM phosphate buffer (pH 7.4). Four milliliter of methanol was added to the lysate and followed after 40 minutes by 4 ml chloroform. After an additional 30 minutes, the extract was centrifuged and the residue was re-extracted with 4 ml of methanol/chloroform (1 : 1, v/v). Methanol and chloroform used in the extraction of lipids contained butyl–hydroxytoluene (50 mg/l). Pooled extracts were washed with 10 ml of 50 mM KCl to make a biphasic mixture. One milliliter of the lower phase of the lipid extract was dried under the N_2 gas.

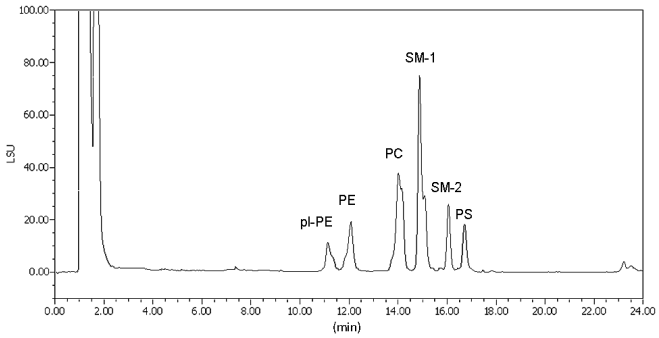

Fig. 1 Representative HPLC of sample erythrocyte membrane phospholipid extract. The system was connected to an evaporative light scattering detector (ELSD, Sedex–55, Sedere Vitry sur Seine, France). Relatively small but clear peak of phosphatidylethanolamine plasmalogen (pl–PE) is detected apart from phosphatidylethanolamine (PE) *per se* in the total phospholipids of human erythrocyte membrane. PC, phosphatidylcholine; PE, phosphatidylethanolamine; pl, plasmalogen; PS, phosphatidylserine; SM, sphingomyelin.

Separation of erythrocyte membrane phospholipid classes including plasmalogen was performed according to the previously reported HPLC [10]. The HPLC system used in this study was an Agilent HPLC system (Agilent Technologies, Tokyo, Japan) equipped with an ELSD (Sedex–55, Sedere, Vitry sur Seine, France). The system was also connected to an HPLC ChemStation (Agilent Technologies) for control and analysis

of chromatograms. The dried total lipids were dissolved in 200 μl hexane/2–propanol (1 : 1, v/v) and filtered using a filter with a pore size of 0.2 μm. Finally, 20 μl of filtered extract was injected into the HPLC system. Representative HPLC of sample phospholipid extracted from human erythrocyte membrane was demonstrated in Fig. 1. All the classes of erythrocyte membrane phospholipids including pl–PE are separated and detected by a single run of HPLC. Amount of phospholipid indicates relative value based on the peak area under the single run of chromatogram.

2.4 Statistical analyses

All data are expressed as means ± SD. For discrete data, comparison of data between the two groups was performed by chi–square (χ^2) test or the Fisher's exact test. For continuous data, the Kolmogorov–Smirnov test was first applied to investigate the normality of the data distribution. Normally distributed continuous data were compared with unpaired Student's *t* test. Comparison of continuous variables which were not normally distributed was performed by Mann-Whitney *U* test. None of the variables with missing data qualified. These analyses were performed using Predictive Analytics Software (PASW) 18.0 version for Windows (Statistical Package for Social Science; SPSS, Chicago, Ill, USA). Differences with two–sided $p < 0.05$ were considered significant.

3. Results and Discussion

3.1 Baseline characteristics of subjects

The baseline characteristics of the enrolled subjects are detailed in Table 1. There is no significant difference in body mass index (BMI), which is an index of obesity. Diastolic but not systolic blood pressure (BP) levels in the atherosclerotic group was unexpectedly lower than that in the control group ($p = 0.033$), indicating that BP control in the atherosclerotic group is strict. Although HbA1c in the atherosclerotic group was significantly greater than that in the control group ($p = 0.004$), average HbA1c in the former group (6.3 ± 0.9%) was in the upper border of the National Glycohemoglobin Standardization Program (NGSP), indicating that diabetic control in the atherosclerotic group is intensive.

Total and LDL cholesterol levels in the atherosclerotic group were lower than the respective levels in the control group, indicating that intensive treatment for dyslipidemia is conducted. The guideline–directed intensive control of atherogenic risk factors is recommended to prevent lethal event such as myocardial infarction, stroke and cardiovascular death [15]. The atherosclerotic group showed the tendency of mild anemia and hypoalbuminemia reflecting persistent inflammation. These findings indicate that intensive control of atherogenic risk factors is continued in the patients with severe atherosclerosis prior to the endovascular therapy.

3.2 Plasma concentration of plasmalogen

Plasma concentrations of plasmalogen are too low to be detected by the conventional single run of HPLC. Therefore, these concentrations were quantified by HPLC–ELSD system after treatment of plasma with PLA_1. Notably, plasma concentrations of pl–PE and pl-PC in atherosclerotic group were reduced significantly relative to the respective concentrations in control group (Table 1). Treatment of plasma with PLA_1 hydrolyzes ester (acyl) bond but not ether bond, i.e., all diacyl phospholipids are completely hydrolyzed, whereas SM and ether phospholipids remain intact. There are two types of ether bonds in ether phospholipids, i.e., alkyl bonds and alkenyl bond. Phospholipids with an alkenyl bond are termed plasmalogens [7]. Although physiological function of circulating plasmalogen is not well elucidated, plasma concentration of plasmalogen is reported to be reduced in Alzheimer disease [16], metabolic syndrome [17] and inflammatory bowel disease [18]. These reports imply that systemic or local inflammation is deeply involved in the metabolism of plasmalogens, and that plasmalogen may play a protective role against persistent inflammation. The findings of the present study is compatible to these reports [16 ~ 18] but should be interpreted carefully due to medication for dyslipidemia, 70% of the enrolled patients were prescribed with statin. The effects of statin on the plasma concentrations of plasmalogen are conflicting. In literature, pitavastatin treatment (4 mg/day) resulted in marked reduction in plasma LDL cholesterol, preferential increase in pl–PC and pl–PE and minor effects on HDL cholesterol [19], whereas rosvastatin treatment (10 or 40 mg/day) significantly reduced the plasma levels of total cholesterol, LDL cholesterol, triglyceride, SM, PC, pl–PC and pl–PE [20]. Such discrepancy may stem from the species and dose

Table 1 Baseline characteristics and laboratory data in atherosclerotic and control groups

	Atherosclerotic group (n = 20)	Control group (n = 20)	p value
age (years)	71.7 ± 6.5	71.5 ± 4.8	0.817*
gender (female/male)	6 / 14	10 / 10	0.333
BMI (kg/m^2)	23.4 ± 3.3	22.9 ± 2.1	0.587
systolic BP (mmHg)	127.8 ± 16.6	134.0 ± 16.6	0.248
diastolic BP (mmHg)	69.9 ± 9.7	78.5 ± 14.5	0.033
erythrocytes (x 10^4 /μl)	402 ± 61	436 ± 33	0.039
leukocytes (/μl)	5938 ± 1748	5105 ± 1512	0.115
platelets (x 10^4 /μl)	19.8 ± 4.9	23.1 ± 4.3	0.028
serum albumin (g/dl)	4.0 ± 0.5	4.4 ± 0.2	0.017*
AST (IU/l)	23.5 ± 9.6	23.7 ± 4.9	0.456*
ALT (IU/l)	24.6 ± 18.1	18.4 ± 7.1	0.363*
BUN (mg/dl)	19.6 ± 8.3	16.3 ± 2.9	0.379*
uric acid (mg/dl)	5.9 ± 2.0	5.5 ± 1.4	0.435
creatinine (mg/dl)	0.97 ± 0.36	0.82 ± 0.18	0.110
creatinine clearance (ml/min/kg)	63.9 ± 25.9	67.9 ± 20.0	0.317*
HbA1c (%)	6.3 ± 0.9	5.6 ± 0.3	0.004*
Total Cholesterol (mg/dL)	167.4 ± 38.0	213.2 ± 38.6	0.001
HDL Cholesterol (mg/dL)	48.0 ± 11.5	65.5 ± 12.3	< 0.001
LDL Cholesterol (mg/dL)	90.3 ± 31.6	126.5 ± 26.7	< 0.001
triglyceride (mg/dL)	139.5 ± 93.0	115.1 ± 58.4	0.425*
C-reactive protein (mg/dL)	0.23 ± 0.46	0.10 ± 0.04	0.342*
pl-PE (mg/dL)	3.61 ± 1.00	4.15 ± 0.94	0.036
pl-PC (mg/dL)	3.57 ± 0.85	4.25 ± 0.58	0.004
SM (mg/dL)	23.66 ± 5.08	27.70 ± 3.83	0.008

* Data indicate that distribution is not normal. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index calculated by body weight (kg) divided by the square of height (m^2); BP, blood pressure; BUN, blood urea nitrogen; HbA1c, hemoglobin A1c estimated according to the National Glycohemoglobin Standardization Program (NGSP); HDL, high density lipoprotein; LDL, low density lipoprotein. AST and ALT indicate liver function, whereas BUN and creatinine represent renal function. PC, phosphatidylcholine; PE, phosphatidylethanolamine; pl, plasmalogen; SM, sphingomyelin.

Table 2 Erythrocyte membrane phospholipids distribution in atherosclerotic and control groups

	Atherosclerotic group (n = 20)	Control group (n = 20)	p value
pl-PE	7.66 ± 0.91	8.46 ± 1.09	0.017
PE	10.18 ± 1.24	10.39 ± 1.14	0.575
PC	22.19 ± 3.36	25.12 ± 2.67	0.004
PS	8.93 ± 0.95	9.34 ± 1.34	0.270
SM	51.04 ± 3.97	46.71 ± 4.74	0.003

Amounts of phospholipids indicate relative values based on the chromatographic peak area(%). PC, phosphatidylcholine; PE, phosphatidylethanolamine; pl, plasmalogen; PS, phosphatidylserine; SM, sphingomyelin.

of statin, i.e., dose of statin prescribed in Japanese is lower than that in westerners [19, 20]. Although possible therapeutic effects of statin on our data remain, reduced plasma concentrations of plasmalogens in severely atherosclerotic patients are reported in literature, and our data (Table 1) are compatible to literature [21, 22].

3.3 Erythrocyte membrane plasmalogen

Erythrocyte membrane phospholipid classes were simultaneously detected by a single run of HPLC [10]. Relative composition of phospholipid classes was calculated on the basis of each chromatographic peak area. The area of pl–PE in the erythrocyte membrane of the atherosclerotic group was significantly reduced relative to the area in the control group (Table 2), which is compatible to the current studies demonstrating anti-atherogenic, anti–apoptotic actions of plasmalogen [23, 24]. It is controversial concerning whether diminished erythrocyte membrane plasmalogen is the cause or the result of severe atherosclerosis requiring endovascular therapy. Acute peroxidation of human erythrocytes exposed to *tertial* butyl–hydroperoxide (tBHP) causes formation of methemoglobin, erythrocyte membrane protein degradation, phospholipid peroxidation and selective reduction of erythrocyte membrane pl–PE (from 14.5 ± 0.5 to 11.3 ± 0.2%) in the same HPLC method as in this study [6, 10]. These results strongly support that pl–PE is consumed as an endogenous antioxidant defense for any acute or persistent oxidative damages, because lyso–ethanolamine plasmalogen, a product of acid hydrolysis of pl–PE, could not be detected by our erythrocyte lipid extraction method using hexane/isopropanol mixture [10].

It was found in this study that an increase of SM and a decrease of PC in the atherosclerotic group erythrocyte membrane relative to the corresponding phospholipids in the control group erythrocyte membrane were significant (Table 2). These findings are compatible to the results of recent studies. SM is synthetized by SM synthase. This enzyme is activated by peroxisome proliferator-activated receptor δ (PPAR–δ) signaling pathway inducing inflammation, and this enzymatic activity is reported to link to atherosclerosis [25]. Moreover, sphingolipids including SM are reported to be accumulated in human coronary plaque and are acting as inducers of atheromatous plaque inflammation and instability [26]. On the other hand, PC and PE are abundant in cellular membrane, and the altered individual phospholipid metabolism is involved deeply in various lifestyle–related diseases including dyslipidemia and atherosclerosis [27].

3.4 Atherosclerosis and plasmalogen

Long–term vascular oxidative stress is associated with atherosclerosis [28, 29]. Vascular endothelial dysfunction is a main feature of initial stage of atherosclerosis and atherogenic risk factors such as hypertension, diabetes, and dyslipidemia promote inflammatory cell infiltrations and vascular smooth muscle cell proliferations [30, 31]. The atherosclerotic group in this study included patients undergoing endovascular therapy. In this sense, these patients are in the advanced stage of systemic atherosclerosis. Increase in SM and decrease

in PC of the erythrocyte membrane support this speculation (Table 2). Moreover, these patients were under the intensive control of atherogenic risk factors to prevent lethal cardiovascular events. Accordingly, homeostasis of plasmalogen may be apart from such a conventional atherogenic risk control.

3.5 Limitations

The main findings of the present study should be interpreted with caution because of inherent study limitations. First limitation is the study design, i.e., a single–center, case–control study contains the potential bias of patients selection and treatment strategy. Second one is the lack of comparison with other techniques detecting plasmalogen such as ^{31}P–nuclear magnetic resonance (NMR) and current sophisticated mass spectrometry[9]. Third limitation is the absence of longitudinal study. Long–term follow–up of erythrocyte membrane plasmalogen may clarify the role of this unique glycerophospholipid as a putative lipophilic endogenous antioxidant in detail. At least in present, this study only supports the hypothesis that atherosclerosis is accelerated by oxidative stress and erythrocyte membrane plasmalogen is one of the biomarkers of redox condition and chronic inflammation.

4. Conclusions

The present study demonstrated that plasma concentrations of pl–PE and pl–PC in atherosclerotic patients are lower than the corresponding concentrations in healthy controls, and that pl–PE of erythrocyte membrane in atherosclerotic group is diminished relative to that in control group. Further studies are required to elucidate the physiological role of plasmalogen in severe atherosclerotic patients.

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