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Original Article

Relationships of Leg Ischemia Symptoms and Carotid Artery Atherosclerosis with Hypertensive-Disorders-of-Pregnancy-Associated Peptides in Patients with Lower Extremity Arterial Disease

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Objectives: We have proposed seven peptides with low molecular weights in blood as biomarkers for the diagnosis of hypertensive disorders of pregnancy (HDP). The purpose of this cross-sectional study was to investigate the relationships of the HDP-associated peptides with symptoms of leg ischemia and degree of atherosclerosis in patients with lower extremity arterial disease (LEAD).

Methods: The subjects were 165 outpatients with LEAD (145 men and 20 women aged 74.3 ± 8.1 years [47–93 years]). Their symptoms of leg ischemia, leg arterial flow, and degree of atherosclerosis were evaluated using the Rutherford classification of Clinical Ischemia Category, ankle-brachial index (ABI) and the intima-media thickness (IMT) of carotid arteries, respectively. Serum concentrations of the HDP-related peptides were measured by mass spectrometry.

Results: The grade of the Rutherford classification was positively associated with levels of the peptides with m/z 2091 and 2378 and was inversely associated with levels of the peptide with m/z 2081. The category of the Rutherford classification was inversely associated with ABI. There were no HDP-associated peptides that showed significant relationships with IMT.

Conclusions: The peptides with m/z 2081, 2091, and 2378 are possible biomarkers of leg ischemia but are not associated with carotid atherosclerosis in LEAD patients.

Keywords: carotid atherosclerosis, hypertensive disorders of pregnancy, lower extremity arterial disease, peptide biomarker, Rutherford classification

Introduction

Hypertensive disorders of pregnancy (HDP) are common complications of pregnancy and are worldwide leading causes of maternal morbidity and mortality.[1,2] Women with HDP have a long-term higher risk than women without HDP for the development of cardiovascular diseases including ischemic heart disease, stroke, and heart failure.[3–5] We proposed seven peptides with low molecular weights in blood as biomarkers for diagnosis of HDP.[6] These peptides were named based on their mass-to-charge ratios (m/z) as P-2081 (m/z 2081), P-2091 (m/z 2091), P-2127 (m/z 2127), P-2209 (m/z 2209), P-2378 (m/z 2378), P-2858 (m/z 2858), and P-3156 (m/z 3156) and were identified to be fragments of parent proteins including kininogen (P-2081, P-2127, P-2209), fibrinogen-α (P-2091), complement C4 (P-2378), α-2-HS glycoprotein (P-2858), and inter-α-trypsin inhibitor heavy chain H4 (P-3156).[6] However, the significance of these HDP-associated peptides in cardiovascular health in the general population remains to be elucidated.

Hypertension is a risk factor for lower extremity arterial disease (LEAD),[7–9] and patients with LEAD have higher risks of ischemic heart disease and stroke, which determine the prognosis of the patients.[10,11] Moreover, hypertension in pregnancy was reported to be an independent risk factor for LEAD decades after pregnancy.[12] In a recent study, we demonstrated that serum levels of P-2081,

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P-2091, P-2127, P-2209, P-2378, and P-2858 were associated with leg arterial flow evaluated by ankle-brachial index (ABI) and a change in ABI after leg exercise in patients with LEAD.[13] However, it remains to be determined whether the HDP-associated peptides are related to symptoms of leg ischemia in patients with LEAD. Progression of atherosclerosis is deeply involved in the pathogenesis of LEAD.[14,15] However, it is also unknown whether the HDP-associated peptides are related to the degree of atherosclerosis.

The purpose of this study was therefore to investigate the relationships of the HDP-associated peptides with symptoms of leg ischemia and degree of atherosclerosis. Leg ischemia symptoms were assessed using the Rutherford classification,[16] and intima-media thickness (IMT) of the carotid arteries was measured for evaluation of the degree of atherosclerosis. IMT was reported to be associated with the incidence and prevalence of LEAD.[17,18]

Materials and Methods

Subjects

The subjects were 165 outpatients of the Department of Cardiovascular Surgery of Yamagata Saisei Hospital who had been diagnosed as having LEAD defined as a low ABI (≤0.9)[19] and had already received medication therapy for LEAD. All of the subjects gave informed consent to participate in the present study. Histories of subjects regarding illness, medication, cigarette smoking, and alcohol consumption were surveyed by questionnaires. A history of smoking was categorized by daily average cigarette consumption as nonsmokers (never), light smokers (20 cigarettes or less), and heavy smokers (21 cigarettes or more). The frequency of habitual alcohol drinking was also asked in the questionnaires and was categorized as nondrinkers (never), occasional drinkers (4 days or less per week), and regular drinkers (5 days or more per week). The degree of symptoms of leg ischemia was evaluated using the Rutherford classification.[16] Patients with advanced LEAD (categories 3–6 of the Rutherford classification) were not included in the subjects.

Evaluation of leg arterial blood flow

After each subject had rested quietly in a supine position, ABI was measured by an oscillometric method using an automatic ABI device (VaSera VS-1500, Fukuda Denshi, Tokyo, Japan). The lower ABI value of the left and right legs of each individual was used for analysis.

Evaluation of the degree of atherosclerosis in carotid arteries

IMT was measured by ultrasonography in the supine position as described previously.[20] Briefly, high-resolution B-mode ultrasound images were scanned with an L12-3 MHz transducer (Philips CX50, PHILIPS Electronics Japan, Tokyo, Japan). Three arterial wall segments in each common carotid artery were imaged from a fixed lateral transducer angle at the far wall, and the far wall IMT of both common carotid arteries was measured at three determinations (greatest thickness point and 1 cm upstream and 1 cm downstream points from the greatest thickness point). The averages of each of the mean IMT and maximum IMT over the six segments of the left and right common carotid arteries were designated as IMT_{mean} and IMT_{max}.

Measurements of cardiovascular risk factors

Height and body weight were measured with light clothes at the health checkup. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Fasted blood was collected from each subject in the morning. Hemoglobin A_{1c} was measured using an automatic glycohemoglobin analyzer based on high-performance liquid chromatography (ADAMSTM A_{1c} HA-8170, Sekisui Medical Co., Ltd, Tokyo, Japan). Hemoglobin A_{1c} values were calibrated using the formula proposed by the Japan Diabetes Society.[21] Serum low-density lipoprotein (LDL) cholesterol concentrations were measured by an enzymatic method using a commercial kit, Metabolead LDL-C (Kyowa Medex Co., Ltd, Tokyo, Japan). Plasma fibrinogen concentrations were measured by the thrombin time method using a commercial kit, Thrombocheck-Fib (Sysmex, Kobe, Japan). Subjects with diabetes were defined as those receiving drug therapy for diabetes and/or those showing high hemoglobin A_{1c} levels (≥6.5%), according to the criteria for diagnosis of diabetes by the American Diabetes Association.[22] Systolic and diastolic blood pressure of the right brachial artery was also recorded using VaSera VS-1500. The mean arterial pressure was defined as a diastolic blood pressure level plus one-third of the difference between systolic and diastolic blood pressure levels.

Determination of peptide concentrations in serum

The concentration of each peptide in serum was measured according to the method described previously.[23,24] Briefly, each serum sample was spiked with stable isotope-labeled (SI) internal standard peptides, and the peptide fraction was prepared with a graphite carbon tip device. The seven target peptides were quantified with liquid chromatography with a tandem mass spectrometry (LC-MS/MS) system using the multiple reaction monitoring mode. The serum concentration of each peptide was calculated by the ratio of the peak areas of the natural and internal standard SI peptides. Each ratio of the area of a stable

isotope-labeled peptide to the area of an isotope-unlabeled peptide was reproducible relatively well (coefficient of variation: 0.008–0.147).

Statistical analysis

Statistical analyses were performed using a computer software program (IBM SPSS Statistics for Windows, Version 25.0., IBM Corp., Armonk, NY, USA). Continuous variables showing normal distributions are summarized as means with standard deviations or means with 95% confidence intervals. In univariable correlation analyses, Spearman's rank correlation coefficients were calculated for variables not showing normal distributions, and Pearson's correlation coefficients were calculated for variables showing normal distributions. Since the levels of each peptide did not show a normal distribution, they were used after base-10 logarithmic transformation for normalization in linear analyses as described below. In multivariable linear regression analysis, standardized partial regression coefficients (β) were calculated for variables showing normal distributions. Comparison of variables between the two groups of category 1 and category 2 of the Rutherford classification was performed using Student's t-test in univariable analysis and using analysis of covariance followed by Student's t-test after Bonferroni correction in multivariable analysis. The concentrations of each peptide in the subjects were arranged in ascending order and then the subjects were divided into three tertile groups of an equal size (55 subjects per tertile). Mean levels of each variable were compared among three tertile groups of each peptide using analysis of variance followed by Scheffé's F-test as a post hoc test in univariable analysis and by analysis of covariance followed by Student's t-test after Bonferroni correction in multivariable analysis. In logistic regression analysis, crude and adjusted odds ratios for category 2 of the Rutherford classification (versus category 1) in the 2nd and 3rd tertile groups of each peptide versus the 1st tertile group were calculated. Bonferroni's multiple comparison test was used in all analyses for relationships between each variable and levels of the seven peptides. In the multivariable analyses, age, gender, BMI, mean arterial pressure, LDL cholesterol, habits of smoking and alcohol drinking, and histories of diabetes and therapy using anticoagulants were used as variables for adjustment. Receiver operating characteristic (ROC) analysis was performed to examine an optimal cutoff point of each peptide concentration for leg ischemia evaluated by the Rutherford classification (category 2 versus category 1) as an outcome. The area under the ROC curve (AUC) and 95% confidence interval were estimated empirically. The optimal cutoff point was selected by maximizing Youden's index, which is the difference between the true-positive rate (sensitivity) and the false-positive rate (1-specificity) in the ROC curve. Probability (p) values less than 0.05 were defined as significant.

Table 1 Characteristics of subjects with LEAD

Variables	Values
Gender	145 men and 20 women
Age (years)	74.3 ± 8.1
Smokers (%)	24.2 (light, 21.2; heavy, 3.0)
Alcohol drinkers (%)	56.4 (occasional, 19.4; regular, 37.0)
Rutherford classification	Category 1, n = 78; Category 2, n = 87
History of diabetes (%)	41.2
History of anticoagulation therapy (%)	79.4
Height (cm)	161.6. ± 7.9
Body weight (kg)	59.8 ± 9.8
BMI (kg/m^2)	22.9 ± 3.0
Systolic blood pressure (mmHg)	134.6 ± 14.3
Diastolic blood pressure (mmHg)	71.1 ± 11.0
Mean arterial pressure (mmHg)	92.3 ± 10.6
Hemoglobin A_{1c} (%)	6.30 ± 0.97
LDL cholesterol (mg/dl)	111.0 ± 31.5
Fibrinogen (mg/dl)	292.0 ± 69.9
IMT_{max} (mm)	2.74 ± 0.92
IMT_{mean} (mm)	1.08 ± 0.37
ABI	0.812 ± 0.196
P-2081 (ng/ml)	0.75 (0.39, 1.44)
P-2091 (ng/ml)	2.55 (0.72, 9.56)
P-2127 (ng/ml)	0.94 (0.11, 2.24)
P-2209 (ng/ml)	2.47 (0.74, 7.15)
P-2378 (ng/ml)	5.60 (1.19, 41.37)
P-2858 (ng/ml)	986.8 (553.2, 1720.8)
P-3156 (ng/ml)	6.44 (3.86, 10.63)

Shown are numbers, proportions, means with standard deviations, and medians with interquartile ranges in parentheses.

ABI: ankle-brachial index; BMI: body mass index; IMT: intima-media thickness; LEAD: lower extremity arterial disease; LDL: low-density lipoprotein

Results

Characteristics of the subjects

Table 1 shows the characteristics of the subjects. The subjects were 145 male and 20 female outpatients (mean age: 74.3 [47–93] years). About one-fourth of the subjects were smokers, and the proportions of subjects with diabetes and subjects with a history of anticoagulation therapy were 41.2% and 79.4%, respectively. The mean ABI was 0.812, and 59.4% of the subjects (n = 98) showed low ABI (0.9 or lower). Mean IMT_{max} and IMT_{mean} were 2.74 mm and 1.08 mm, respectively, and 50.3% of the subjects (n = 83) showed high IMT_{mean} (≥1.0 mm). There was a large range of concentrations of the seven HDP-associated

Table 2 Comparisons of mean levels of each peptide between the subject groups with category 1 and category 2 of the Rutherford classification

	Univariable		Multivariable	
	Rutherford-category 1	Rutherford-category 2	Rutherford-category 1	Rutherford-category 2
P-2081	0.022 (–0.071 to 0.114)	–0.265 (–0.360 to –0.170)**	–0.005 (–0.104 to 0.094)	–0.241 (–0.334 to –0.148)**
P-2091	0.222 (0.092–0.353)	0.625 (0.488–0.762)**	0.251 (0.110–0.391)	0.600 (0.467–0.733)**
P-2127	–0.013 (–0.149 to 0.123)	–0.468 (–0.627 to –0.309)**	–0.081 (–0.231 to 0.069)	–0.407 (–0.548 to –0.265)*
P-2209	0.578 (0.448–0.708)	0.249 (0.112–0.385)**	0.538 (0.398–0.679)	0.284 (0.151–0.417)
P-2378	0.538 (0.370–0.706)	1.037 (0.866–1.207)**	0.596 (0.425–0.766)	0.985 (0.824–1.146)*
P-2858	2.914 (2.840–2.987)	3.047 (2.980–3.114)	2.932 (2.863–3.002)	3.030 (2.965–3.096)
P-3156	0.842 (0.760–0.925)	0.787 (0.704–0.869)	0.843 (0.757–0.929)	0.786 (0.705–0.868)

Shown are means with 95% confidence intervals of levels of each peptide after log-10 transformation. In multivariable analysis, age, gender, habits of smoking and alcohol drinking, histories of diabetes and medication therapy using anticoagulants, body mass index, mean arterial pressure, and low-density lipoprotein cholesterol were used as other explanatory variables. Symbols denote significant differences from the group with category 1 of the Rutherford classification (*p <0.05; **p <0.01).

peptides in serum: The medians of concentrations of the peptides were from 0.75 ng/ml (P-2081) to about 1 μg/ml (P-2858).

Relationships between the HDP-related peptides and symptoms of leg ischemia

Symptoms of leg ischemia in patients with LEAD were evaluated using the Rutherford classification. Because the subjects had already received medication therapy for LEAD, they were categorized into only two groups, category 1 and category 2 of the Rutherford classification (category 1, n = 78 [47.3%]; category 2, n = 87 [52.7%]), and subjects having more severe symptoms of leg ischemia (categories 3 and higher of the Rutherford classification) were not included in the subjects of the present study. Levels of each of the seven HDP-related peptides were compared in the subject groups with Rutherford category 1 and category 2 (**Table 2**). Since values of the seven peptide levels did not show normal distributions, they were analyzed after log-10 transformation. Both in univariable and multivariable analyses, levels of P-2081 and P-2127 were significantly lower in the group with category 2 of the Rutherford classification than in the category 1 group, while levels of P-2091 and P-2378 were significantly higher in the category 2 group than in the category 1 group. Levels of P-2209, P-2858, and P-3156 were not significantly different in the category 1 and category 2 groups in multivariable analysis.

Odds ratios for category 2 (versus category 1) of the Rutherford classification of the 2nd and 3rd tertile groups versus the 1st tertile group of each peptide are shown in **Table 3**. Both in univariable and multivariable logistic regression analyses, the odds ratios of the tertiles for P-2081, P-2127, and P-2209 tended to be lower with an increase in the tertile for each peptide, while the odds ratios of the tertiles for P-2091 and P-2378 tended to be higher with an increase in the tertile for each peptide. The odds ratios of the 3rd versus 1st tertiles for P-2081 and P-2209 were significantly (in univariable analysis) or marginally significantly (in multivariable analysis) lower than the reference level of 1.00, while the odds ratios of the 3rd versus 1st tertiles for P-2091 and P-2378 were significantly higher than the reference level in univariable analysis and multivariable analysis. The odds ratios of the 2nd and 3rd versus 1st tertiles for P-2127, P-2858, and P-3156 were not significantly different from the reference level in multivariable analysis. Thus, from the above results of analysis of covariance and multivariable logistic regression analysis, P-2081, P-2091, and P-2378 were associated with the symptoms of leg ischemia evaluated by the Rutherford classification in patients with LEAD.

ROC analysis for the relationship between each peptide level and symptoms of leg ischemia

The results of ROC analysis for the relationship between each peptide level and symptoms of leg ischemia are shown in **Supplementary Table 1**. AUCs for the peptides except for P-3156 were significantly higher than the reference level of 0.5. The cutoff values (ng/ml) for P-2081, P-2091, P-2127, P-2209, P-2378, and P-2858 were 0.758, 3.99, 0.501, 2.02, 19.28, and 1020.8, respectively.

Relationships of IMT and ABI with symptoms of leg ischemia

IMT_{max}, IMT_{mean}, and ABI were compared in the subject groups with category 1 and category 2 of the Rutherford classification (**Table 4**). ABI was significantly lower in the category 2 group than in the category 1 group, while IMT_{max} and IMT_{mean} were not significantly different in the category 1 and category 2 groups.

Table 3 Odds ratios for the higher (2nd) category of the Rutherford classification in the 2nd and 3rd tertile groups versus the 1st tertile group of each peptide level

	Odds ratio		
	1st tertile	2nd tertile	3rd tertile
P-2081			
Univariable	1.00	0.50 (0.23–1.09)	0.26 (0.12–0.57)**
Multivariable	1.00	0.63 (0.27–1.45)	0.34 (0.14–0.83)
P-2091			
Univariable	1.00	1.82 (0.85–3.89)	4.27 (1.92–9.50)**
Multivariable	1.00	1.74 (0.76–3.98)	3.86 (1.55–9.64)*
P-2127			
Univariable	1.00	0.40 (0.18–0.87)	0.25 (0.11–0.56)**
Multivariable	1.00	0.46 (0.19–1.13)	0.37 (0.15–0.93)
P-2209			
Univariable	1.00	0.40 (0.18–0.87)	0.25 (0.11–0.56)**
Multivariable	1.00	0.43 (0.18–1.04)	0.33 (0.14–0.76)
P-2378			
Univariable	1.00	1.16 (0.54–2.48)	4.39 (1.95–9.89)**
Multivariable	1.00	1.03 (0.46–2.31)	3.87 (1.51–9.90)*
P-2858			
Univariable	1.00	1.16 (0.55–2.45)	2.66 (1.22–5.77)
Multivariable	1.00	1.16 (0.52–2.60)	2.19 (0.86–5.61)
P-3156			
Univariable	1.00	0.38 (0.18–0.82)	0.86 (0.40–1.84)
Multivariable	1.00	0.22 (0.08–0.58)*	0.69 (0.29–1.64)

Shown are odds ratios with 95% confidence intervals. In multivariable analysis, age, gender, habits of smoking and alcohol drinking, histories of diabetes and medication therapy using anticoagulants, body mass index, mean arterial pressure, and low-density lipoprotein cholesterol were used as other explanatory variables. Symbols denote significant differences from the reference level of 1.00 (*, $p < 0.05$; **, $p < 0.01$).

Table 4 Comparisons of mean levels of IMT and ABI between the subject groups with category 1 and category 2 of the Rutherford classification

	Univariable		Multivariable	
	Rutherford-category 1	Rutherford-category 2	Rutherford-category 1	Rutherford-category 2
IMT_{max} (mm)	2.764 (2.540–2.988)	2.721 (2.537–2.905)	2.797 (2.587–3.007)	2.691 (2.493–2.890)
IMT_{mean} (mm)	1.050 (0.970–1.130)	1.114 (1.031–1.197)	1.065 (0.980–1.149)	1.101 (1.021–1.181)
ABI	0.884 (0.846–0.922)	0.747 (0.705–0.789)**	0.883 (0.841–0.926)	0.747 (0.707–0.787)**

Shown are means with 95% confidence intervals of each variable. In multivariable analysis, age, gender, habits of smoking and alcohol drinking, histories of diabetes and medication therapy using anticoagulants, body mass index, mean arterial pressure, and low-density lipoprotein cholesterol were used as the covariates. Symbols denote significant differences from the group of category 1 of the Rutherford classification (**p <0.01).

Relationships between the HDP-related peptides and the degree of atherosclerosis in the carotid arteries

The correlation coefficients of each peptide level with IMT_{max} and IMT_{mean} are shown in **Supplementary Table 2.** Among the seven HDP-related peptides, there was no peptide that showed a significant correlation with IMT_{max} or IMT_{mean} in univariable analysis and multivariable analysis. Next, IMT_{max} and IMT_{mean} were compared in the three tertile groups of each peptide (**Table 5**). Both in univariable analysis and multivariable analysis, there were no significant differences in IMT_{max} or IMT_{mean} among the three tertile groups of each peptide. Thus, none of the seven HDP-related peptides were associated with the degree of atherosclerosis in carotid arteries.

Discussion

In this study, levels of P-2081, 2091, and 2378 were shown to be associated with symptoms of leg ischemia evaluated using the Rutherford classification (**Tables 2 and 3**), which agrees with the results of our recent study

Table 5 Comparisons of mean levels of IMT_{max} and IMT_{mean} in the tertile groups of each peptide level

	IMT_{max}		IMT_{mean}	
	Univariable	Multivariable	Univariable	Multivariable
P-2081				
1st tertile	2.838 (2.606–3.070)	2.813 (2.563–3.063)	1.163 (1 053–1.273)	1.145 (1.047–1.244)
2nd tertile	2.702 (2.469–2.935)	2.745 (2.494–2.996)	1.036 (0.955–1.116)	1.044 (0.941–1.147)
3rd tertile	2.684 (2.400–2.968)	2.666 (2.413–2.918)	1.053 (0.945–1.162)	1.047 (0.939–1.155)
P-2091				
1st tertile	2.847 (2.546–3.148)	2.795 (2.543–3.047)	1.103 (0.997–1.209)	1.096 (0.994–1.198)
2nd tertile	2.545 (2.327–2.763)	2.600 (2.350–2.849)	1.031 (0.930–1.132)	1.054 (0.953–1.155)
3rd tertile	2.832 (2.616–3.048)	2.829 (2.578–3.080)	1.118 (1.021–1.215)	1.101 (1.000–1.203)
P-2127				
1st tertile	2.851 (2.630–3.071)	2.831 (2.571–3.090)	1.140 (1.031–1.248)	1.115 (1.011–1.220)
2nd tertile	2.715 (2.472–2.957)	2.767 (2.515–3.018)	1.048 (0.961–1.136)	1.070 (0.969–1.172)
3rd tertile	2.659 (2.375–2.943)	2.627 (2.375–2.878)	1.064 (0.957–1.170)	1.066 (0.964–1.167)
P-2209				
1st tertile	2.737 (2.520–2.954)	2.714 (2.462–2.966)	1.075 (0.981–1.168)	1.050 (0.950–1.151)
2nd tertile	2.766 (2.543–2.989)	2.777 (2.528–3.026)	1.127 (1.023–1.231)	1.142 (1.042–1.241)
3rd tertile	2.721 (2.417–3.024)	2.733 (2.483–2.982)	1.050 (0.944–1.156)	1.059 (0.960–1.159)
P-2378				
1st tertile	2.862 (2.560–3.164)	2.836 (2.584–3.087)	1.096 (0.988–1.204)	1.100 (0.999–1.201)
2nd tertile	2.540 (2.330–2.750)	2.587 (2.340–2.833)	1.011 (0.930–1.093)	1.033 (0.933–1.132)
3rd tertile	2.822 (2.600–3.045)	2.801 (2.546–3.057)	1.144 (1.034–1.254)	1.119 (1.016–1.222)
P-2858				
1st tertile	2.566 (2.308–2.825)	2.547 (2.292–2.803)	1.030 (0.929–1.131)	1.037 (0.933–1.140)
2nd tertile	2.850 (2.591–3.108)	2.841 (2.593–3.089)	1.110 (1.007–1.214)	1.116 (1.015–1.216)
3rd tertile	2.808 (2.579–3.037)	2.835 (2.572–3.098)	1.112 (1.012–1.211)	1.099 (0.993–1.206)
P-3156				
1st tertile	2.730 (2.517–2.942)	2.696 (2.445–2.948)	1.072 (0.974–1.171)	1.049 (0.940–1.159)
2nd tertile	2.844 (2.554–3.133)	2.797 (2.547–3.047)	1.176 (1.058–1.295)	1.135 (1.030–1.240)
3rd tertile	2.651 (2.408–2.893)	2.730 (2.478–2.982)	1.003 (0.924–1.082)	1.021 (0.914–1.128)

Shown are means with 95% confidence intervals of IMT_{max} and IMT_{mean}. In multivariable analysis, age, gender, habits of smoking and alcohol drinking, histories of diabetes and medication therapy using anticoagulants, body mass index, mean arterial pressure, and low-density lipoprotein cholesterol were used as the covariates.

showing associations of these peptides with leg arterial flow evaluated by ABI.[13] Patients with LEAD are prone to suffer from other atherosclerotic diseases including ischemic heart disease and stroke.[10,11] An association between ABI and IMT was shown in a general population.[25] However, none of the seven HDP-related peptides showed an association with the degree of carotid atherosclerosis evaluated by IMT (**Table 5 and Supplementary Table 2**), which agrees with no associations between IMT and leg ischemia symptoms (**Table 4**). Therefore, P-2081, P-2091, and P-2378 are thought to be related to leg ischemia but not to the degree of atherosclerosis. This is the first study that showed relationships of HDP-related peptides with symptoms of leg ischemia and the progression of carotid atherosclerosis.

The subjects of this study were outpatients of LEAD after receiving medication therapy for LEAD, and the grades of leg ischemia in the subjects were low (categories 1 and 2 of the Rutherford classification). This agrees with the fact that about 40% of the subjects showed normal levels of ABI (>0.9). Therefore, the HDP-related peptides (P-2081, P-2091, and P-2378) were suggested to be useful for the evaluation of leg ischemia after intervention therapy for LEAD. Further studies using a database of patients before medication therapy and patients showing more severe leg ischemia are needed to confirm whether the HDP-related peptides are useful as biomarkers for the diagnosis of LEAD. In addition to P-2081, P-2091, and P-2378, other peptides, P-2127 and P-2209, were shown to be associated with leg arterial flow evaluated by ABI in our previous study[13] but not with symptoms of leg ischemia in the multivariable analysis in the present study. These dissociations of the results for P-2127 and P-2209 may be due to a difference in the methods for evaluation of leg ischemia: ABI may be more sensitive for detecting leg ischemia than its symptoms evaluated by the Rutherford

classification. The above dissociations may also imply that P-2081, P-2091, and P-2378 are more sensitive biomarkers than P-2127 and P-2209 for leg ischemia in patients with LEAD. On the other hand, P-3156 was not associated with symptoms of LEAD as well as ABI.[13] Thus, P-3156 is not a biomarker of leg ischemia in patients with LEAD. Interestingly, P-3156 was reported to show inverse associations with BMI and triglycerides, which are variables of risk factors for atherosclerotic diseases, in healthy men.[24] However, P-3156, as well as the other six peptides, was not associated with carotid atherosclerosis in LEAD patients. Therefore, it is thought that the HDP-related peptides are not biomarkers of atherosclerotic progression in patients with LEAD.

The results for values of HDP-related peptides in healthy individuals in our previous study using a database of men receiving annual health checkup examinations[24] were compared with the results of the present study using a database of patients with LEAD (**Supplementary Table 3**). As shown in the table, the mean age was much older in the patient group than in the healthy group, and levels of P-2081, P-2127, and P-2209 were much higher and levels of P-2091, P-2378, and P-2858 were much lower in the patient group than in the healthy group, while P-3156 levels were not remarkably different between the two groups. These tendencies of the differences in levels of all of the six peptides (except for P-3156) between healthy individuals and patients with LEAD were completely opposite to the tendencies of the differences in the peptide levels between the high and low ABI groups in patients with LEAD: levels of P-2081, P-2127, and P-2209 were lower and levels of P-2091, P-2378, and P-2858 were higher in the group with prominent leg ischemia than in the group without prominent leg ischemia in patients with LEAD.[13] Compared with healthy individuals, atherosclerotic progression is more prominent in patients with LEAD, and the healthy individuals were much younger than the patients with LEAD (46.4 vs. 74.3 years old). Since atherosclerosis progresses with an increase in age, changes in the peptide levels due to atherosclerotic progression and leg ischemia due to LEAD are speculated to be directed oppositely. This may be the reason for the finding in the present study that the degree of atherosclerosis evaluated by IMT was not associated with the peptide levels: the effects of atherosclerosis on the peptide levels were possibly canceled by the effects of leg ischemia on the peptide levels in patients with LEAD. Interestingly, P-3156 levels were reported to be associated with major cardiovascular risk factors including obesity and blood lipids[24]; however, P-3156 was not associated with leg ischemia in LEAD patients in the present study and there was no significant difference between P-3156 levels in healthy individuals and patients with LEAD (**Supplementary Table 3**). Therefore, P-3156 levels, which were associated with atherosclerotic risk factors, were speculated to be oppositely affected by leg ischemia, resulting in no difference between the groups with and without prominent leg ischemia in patients with LEAD.

P-2081, P-2091, and P-2378, which were shown to be associated with leg ischemia symptoms in this study, are fragments of their parent proteins, kininogen, fibrinogen-α and complement C4, respectively. Although there was a weak but significant correlation between fibrinogen and P-2091 levels in the blood (Spearman's rank correlation coefficient: 0.185 [p = 0.018]), there was no significant difference in fibrinogen levels of subjects with grades 1 and 2 of the Rutherford classification (grade 1: 283.9 ± 74.1 mg/dl; grade 2: 299.5 ± 65.3 mg/dl [p = 0.157]). In addition, fibrinogen levels did not show a significant correlation with ABI or IMT (Pearson's correlation coefficient: ABI, –0.045 [p = 0.569]; IMT_{max}, 0.062 [p = 0.432]; IMT_{mean} 0.055 [p = 0.491]). Moreover, the association between P-2091 and leg ischemia symptoms was not altered in multivariable analyses with adjustment for fibrinogen (data not shown). Therefore, the association of P-2091, a fragment of fibrinogen, with symptoms of leg ischemia is independent of blood fibrinogen levels. Unfortunately, data for blood levels of the other parent proteins, kininogen and complement C4, were not available in the present study. One possible explanation for the associations between the peptide levels and leg ischemia is the involvement of changes in protease activities that affect peptide levels in LEAD since the peptides are fragments of their parent proteins. Further studies are needed to clarify the relationships between protease activities and leg ischemia in patients with LEAD.

Study limitations

There are limitations to this study. The subjects of this study had already received medication therapy for LEAD, and they were classified into only two categories (1 and 2) of the Rutherford classification. Therefore, further studies using a database of subjects with severe symptoms of leg ischemia are needed to confirm the findings of this study. The degree of carotid atherosclerosis was evaluated by IMT since stroke is an important cardiovascular complication of patients with LEAD. However, the IMT of the leg arteries was not investigated in the present study. Because of the small population size in the present study, we did not perform analyses using male and female subjects separately. It would be interesting to investigate whether the relationships between the HDP-related peptides and leg ischemia are different in male and female patients with LEAD, although the above relationships were not altered in the multivariable analyses with adjustment for gender in the present study. The mean age of the subjects in this study was 74.3 years, and further studies using

a database of younger subjects are also needed to confirm the findings of this study. Because the AUCs in the ROC analysis were not large enough (0.6–0.7) for the determination of cutoff values (**Supplementary Table 1**), further studies are needed to confirm a more accurate cutoff value for each peptide. In multivariable analyses, adjustment was performed for age, gender, BMI, blood pressure, LDL cholesterol, habits of smoking and alcohol drinking, and histories of diabetes and anticoagulation therapy. However, there are other possible confounding factors including physical activity, nutrition, and socioeconomic factors (e.g., education and occupation), of which information was not available in this study. Since the design of this study is cross-sectional, future prospective studies using large cohorts are needed to discuss causal relationships between the HDP-related peptides and LEAD.

Conclusion

The grade of the Rutherford classification was inversely associated with ABI but did not show a significant association with IMT_{max} or IMT_{mean}. The grade of the Rutherford classification was positively associated with levels of P-2091 and P-2378 and was inversely associated with the level of P-2081. Among the seven HDP-related peptides, no peptide showed a significant relationship with IMT_{max} or IMT_{mean}. Thus, P-2081, P-2091, and P-2378 are thought to be blood biomarkers of leg ischemia but are not associated with carotid atherosclerosis in patients with LEAD.

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Disclosure Statement

All the authors have stated they have no relevant relationships to disclose about the contents of this paper.

IRB Information

This study was conducted according to the principles of the Declaration of Helsinki. The protocol and informed consent of this study were approved by the Ethics Committee of Yamagata Saisei Hospital (approval number 199 at the ethics committee since 2013), and all of the subjects gave informed consent.

Author Contributions

Study conception: IW, YS, and YA
Data collection: YS, SH, and HO
Analysis: IW and YS
Methodology: YS, MY, and YA
Investigations: all authors
Writing: IW
Critical review and revision: all authors
Final approval of the article: all authors
Accountability for all aspects of the work: all authors.

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