Imaging Mass Spectrometry Reveals Unique Lipid Distribution in Primary Varicose Veins


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Abstract
Background: The lipid metabolism of varicose veins (VVs) remains unknown. To elucidate the pathogenesis of VV, we utilized the novel technique of imaging mass spectrometry (IMS).

Materials and methods: We obtained VV tissues from 10 limbs of 10 VV patients who underwent great saphenous vein stripping. As control vein samples, we harvested segmental vein tissues from 6 limbs of 6 patients with peripheral artery occlusive disease who underwent infrainguinal bypass with reversed saphenous vein grafting. To identify the localization of lipid molecules in the VV tissues, we performed matrix-assisted laser desorption/ionization IMS (MALDI-IMS). We also performed MS/MS analyses to identify the structure of each molecule.

Results: We obtained mass spectra directly from control vein tissues and VV tissues and found a unique localization of lipid molecules in the VV tissues. We localised lysophosphatidylcholine (LPC) (1-acyl 16:0), phosphatidylcholine (PC) (1-acyl 36:4) and sphingomyelin (SM) (d18:1/16:0) at the site of the VV valve.

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Conclusion: MALDI-IMS revealed the distribution of various lipid molecules in normal veins and VVs both. Accumulation of LPC (1-acyl 16:0), PC (1-acyl 36:4) and SM (d18:1/16:0) in the VV tissues suggested that inflammation associated with abnormal lipid metabolism may contribute to the development of VV.

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Introduction

Primary varicose vein (VV) is a common venous disease,\(^1,2\) and various risk factors have been reported for the same.\(^3\)\(^-\)\(^5\) Several studies have reported accompanying hyperlipidaemia in many of the VV patients.\(^6\)\(^-\)\(^8\) However, lipid metabolism in local VV tissues is yet to be analysed. This study aimed to investigate the lipid profile in the local region of VV tissues.\(^9\) To gain insights into exact the pathogenesis in VV tissues, we used the novel technique of matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS)\(^10,11\) to analyse the lipid metabolism in VV tissues. At present, few methods exist that can be used for the holistic study of the distribution of lipid molecules in vein walls. Lipid staining—such as Oil red O staining—is available for visualising high accumulation of lipids; however, this stain by itself cannot assess differences in the molecular structures of lipids. In this respect, MALDI-IMS is the only methodology that can clarify the distribution of lipid molecules at the molecular species level. MALDI-IMS can distinguish between different lipid molecular species by simultaneously determining the differences in the mass-to-charge ratios (m/z).\(^12\)\(^-\)\(^14\) Furthermore, tandem mass spectrometry (MS\(^n\)) on tissue surfaces enables us to identify the molecules in tissues by providing detailed information on their structures.\(^12,15\)\(^-\)\(^17\) In this study, we applied IMS to analyse lipid metabolism in VV tissues.

Materials and Methods

Sample collection

All the procedures used in this study were approved by the ethical committee of clinical research in Hamamatsu University School of Medicine. We obtained VV tissues from 10 limbs of 10 patients who underwent great saphenous vein (GSV) stripping. The clinical information of these patients is summarized in Table 1. Clinical disease severity was graded according to the standard CEAP classification according to the recommendations of an International Consensus Committee on Chronic Venous Disease. Before surgical treatment, 4 limbs were assigned to class 2 (simple VV), 1 limb to class 3 (VV with ankle swelling), 4 limbs to class 4 (VV with skin changes), and 1 limb to class 5 (VV with healed ulcer). In order to compare among the clinical phase groups, the limbs were grouped according to dermatological symptoms into the C2,3 and C4,5 groups. As control vein samples, segmental vein tissues were harvested from 6 limbs of 6 patients with peripheral artery occlusive disease who underwent infrainguinal bypass with reversed GSV grafting (Table 1). Although the levels of serum total cholesterol (TC) and triglycerides (TG) did not differ significantly between VV and control patients (Table 1), the distribution of the lipid molecules in the vein tissues differed considerably between the two groups. Our data indicates that it is important to analyse the lipid profile in the affected area in addition to the serum lipid profile. We analysed two regions, i.e. the proximal GSV tissue located close to the sapheno-femoral junction (SFJ), including a terminal valve (Fig. 1a), and the more distal GSV tissue located in the mid-thigh area, including a valve. The red square in Fig. 1b highlights the analysed region mentioned above. Fig. 1c shows a cross-section of the frozen tissue before IMS was performed, and this corresponds to the red square in Fig. 1b. All patients underwent duplex ultrasound scan with a 7.5-MHz transducer (LOGIC 500; GE Yokogawa Medical, Tokyo, Japan) to assess the venous haemodynamics. Duplex scanning was performed in the standing position. An analysis of the venous systems in the saphenous systems, its junctions, and their varicose tracts was carried out along with an analysis of the presence of non-saphenous reflux. ‘Reflex’ was defined as a flow in a reverse direction to the physiological flow for a duration greater than 0.5 s after a provocation manoeuvre. All VV patients had retrograde flow in the sapheno-femoral junction and in the GSV in the calf region; however, the control group patients showed no reflux in the GSV. The sample tissues were immediately frozen in liquid nitrogen and stored at −80 °C in order to maintain tissue morphology.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Men/Female</th>
<th>Age (Mean SD)</th>
<th>Disease (Limbs)</th>
<th>Serum TC (mg/dL)</th>
<th>Serum TG (mg/dL)</th>
<th>BMI (Mean SD)</th>
<th>CEAP Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vein graft (Control)</td>
<td>4/2</td>
<td>73.5 ± 7.1</td>
<td>4/2</td>
<td>173.0 ± 32.1</td>
<td>69.7 ± 10.8</td>
<td>20.4 ± 2.3</td>
<td>C1/C2/C3/C4/C5/C6</td>
</tr>
<tr>
<td>Varicose vein (VV)</td>
<td>3/7</td>
<td>66.7 ± 14.0</td>
<td>4/6</td>
<td>206.5 ± 42.6</td>
<td>84.6 ± 22.6</td>
<td>21.5 ± 1.90</td>
<td>0/4/1/4/1/0</td>
</tr>
</tbody>
</table>
and minimize molecular degradation until MALDI-IMS analyses were performed.

**Imaging mass spectrometry (IMS)**

We cut the frozen tissues, including valves, into 8 μm-thick sections in the direction of the long axis by using a cryostat (CM1950; Leica, Wetzler, Germany). Fig. 1c shows a cross-section that includes a terminal valve. Sections were thaw-mounted onto an indium-tin-oxide (ITO)-coated glass slides (Bruker Daltonics, Bremen, Germany) and dried at room temperature. A total of 500 μL of 2,5-dihydroxybenzoic acid (2,5-DHB) solution (50 mg/mL in 70% methanol/0.1% trifluoroacetic acid) was sprayed to on the sample sections by using a 0.2-mm nozzle caliber airbrush (Procon Boy FWA Platinum; Mr. Hobby, Tokyo, Japan). During spraying, a 15-cm distance was maintained between the nozzle and the tissue surface. IMS was performed using a MALDI-hybrid quadrupole time-of-flight mass spectrometer (QSTAR XL; Applied Biosystems/MDS Sciex, Foster City, CA) equipped with an orthogonal MALDI source and an Nd:YAG laser at a repetition rate of 100 Hz. All analyses were performed in positive-ion mode after an external calibration with human angiotensin II ([M + H]+, m/z 1046.54) and bradykinin peptide fragment 1–7 ([M + H]+, m/z 757.40 Da). MS spectra were acquired in the mass range of m/z 400–1000.[26] IMS was carried out with using the software MALDI server version 4 by defining a region of interest on the tissue slice. Two-dimensional ion density images were created using the BioMap software (Novartis, Basel, Switzerland).[18,19]

**Identification of biomolecules**

To identify the molecular-related peaks obtained from IMS, we conducted tandem mass spectrometric (MSn) analyses by using a MALDI-quadrupole ion trap (QIT)-TOF mass spectrometer (AXIMA-QIT; Shimadzu, Kyoto, Japan). Ionization was performed with using a 337 nm-pulsed N2 laser. The pulsed argon gas was used for collision-induced dissociation (CID). Precursor and fragment ions obtained by CID were ejected from the ion trap and analysed by a reflectron TOF detector. The conditions of data acquisition (i.e. the laser power, collision energy, and number of laser irradiations) were modified in order to obtain efficient mass spectra. Specific fragment patterns of lysophosphatidylcholine (LPC), phosphatidylcholine (PC) and sphingomyelin (SM) (Fig. 2) were also confirmed by previous reports.[13,20–23]

**Figure 1** Sample collection and analysis region for IMS. a, Intraoperative picture. b, Schema of the VV (varicose vein) tissue. The red square represents the analysed region, including the terminal valve in the giant saphenous vein (GSV). c, Cross-section of frozen tissue before performing IMS, corresponding to the red square in Fig. 1b.

**Statistical analysis**

All data were expressed as mean and SD. Differences in the means between the two groups were assessed using paired Student’s t-test. Significances of differences in the ion intensity ratio of the valve to the proximal region among groups were determined by one-way analysis of variance (ANOVA) followed by Tukey’s test. All statistical analyses were performed using StatView 5.0 (SAS Institute, Tokyo, Japan).

**Results**

The mass spectra obtained from control vein tissues and VV tissues are shown in Fig. 3. Among them, we identified 9 peaks, namely, m/z 496, 725, 741, 760, 780, 782, 798, 808 and 820, that we estimated to correspond to LPC, PC and SM (Table 2) according to previous reports by using IMS.[11,13,18–26] The accumulative spectra did not show

**Figure 2** Representative example for molecular species of lysophosphatidylcholine (LPC), phosphatidylcholine (PC) and sphingomyelin (SM). a, LPC (1-acyl 16:0); b, SM (d18:1/16:0); c, PC (1-acyl 34:1).
a marked difference among the three groups. With regard to the spatial distribution of the molecules, however, we found characteristic distribution patterns of the molecules around the valvular regions in VV tissues (Fig. 4). Ion images created by the BioMap software showed that LPC (1-acyl 16:0) was localised around valves in the VV tissues, while LPC (1-acyl 16:0) was uniformly distributed in the control tissues (Fig. 4a). The ratio of LPC (1-acyl 16:0) ion intensity in the valvular area to that in the proximal area of SFJ was 1.09 SD 0.18, 4.26 SD 1.61 and 4.50 SD 1.50 in the control, C2,3 and C4,5 groups, respectively. In the mid-thigh area, the ratio of LPC (1-acyl 16:0) ion intensity in the valvular area to that in the proximal area of the great saphenous vein was 3.52 SD 1.07 in the C2,3 group and 3.56 SD 0.76 in the C4,5 group. Similarly, SM (d18:1/C16:0) was mainly distributed around the valves in the VV tissues, but uniformly distributed in the control tissues. The ratio of SM ion intensity in the valvular area to that in the proximal area of SFJ was 1.06 SD 0.18, 2.84 SD 0.40 and 2.50 SD 0.47 in the control, C2,3 and C4,5 groups, respectively (Fig. 4b). The ratio of SM (d18:1/C16:0) ion intensity in the valvular area to that in the proximal area in the mid-thigh area was 2.09 SD 0.74 in the C2,3 group and 2.09 SD 0.74 in the C4,5 group (Fig. 4b). Interestingly, the distribution pattern of PC depended on the type of the PC molecule. PC (1-acyl 34:2) was ubiquitously distributed among all groups. On the other hand, PC (1-acyl 36:4) was accumulated around the valves in the VV tissues, but not in the control tissues. The ratio of PC (1-acyl 36:4) ion intensity in the valvular area to that in the proximal area of SFJ was 0.95 SD 0.06, 3.08 SD 1.00 and 2.88 SD 1.07 in the control, C2,3 and C4,5 groups, respectively (Fig. 4b). The ratio of PC (1-acyl 36:4) ion intensity in the valvular area to that in the proximal area in the mid-thigh area was 2.22 SD 0.88 in the C2,3 group and 2.43 SD 0.36 in the C4,5 group. Comparing the C2,3 and C4,5 groups, there were no differences in the lipid species (Fig. 4b). However, other numerous lipid molecules were noted, which need to be studied in order to clarify the lipid metabolism in VV tissues.

**Discussion**

IMS, which is a mass spectrometry method used for two-dimensional samples, is becoming a popular imaging
Figure 4  Imaging mass spectrometry (IMS) of human VV revealed a characteristic distribution of phospholipid molecules in the valvular region as compared to that in the control vein tissue. a, IMS and optical images of haematoxylin–eosin (HE) staining. Scale bar = 200 µm. b, Ratio of valve (v) to proximal (p) region. Significances of differences in the ion intensity ratio of the valve to the proximal region among the three groups were determined by one-way analysis of variance followed by Tukey’s test. *P < 0.05 indicates a significant difference.
both vascular cell adhesion molecule-1 (VCAM-1) and inter-lymphocytes, and is known to induce the expression of inflammation. LPC is a chemotactic factor for macrophages lipid profile. It is well known that LPC is associated with the lipid profile in the affected area in addition to the serum damage.34,35 Although the mechanism that initiates the injured endothelial cells trigger leucocyte infiltration, acti- the smooth muscle components of the vein wall. Then, the metallocproteinases (MMPs) in damaging the endothelium and studies and basic scientific research suggest a role of matrix of the endothelium that express ICAM-1. Recent clinical studies have reported direct lipid analyses in mammalian tissues, histopathological materials and pharmacokinetics in rat whole body sections.28 These studies have identified the importance of molecular imaging techniques in understanding the pathophysiology and/or pathological biochemistry of individual diseases.9 In this study, we report for the first time the use of MALDI-IMS in the analysis of VV tissues. We found a characteristic distribution of lipid species (LPC, SM and PC) around the damaged valvular area in VV tissues. Although the levels of serum lipid parameters did not differ significantly between control and VV patients (Table 1), the localisation of lipid molecules differed considerably between the two groups (Fig. 3b). Our data indicates that it is important to analyse the lipid profile in the affected area in addition to the serum lipid profile. It is well known that LPC is associated with inflammation. LPC is a chemotactic factor for macrophages and lymphocytes, and is known to induce the expression of both vascular cell adhesion molecule-1 (VCAM-1) and inter-cellular adhesion molecule-1 (ICAM-1) in endothelial cells.29 Moreover, LPC is capable of lysing cells at high concentra-tions due to its detergent-like property. The LPC molecule is wedge-shaped, consisting of a long hydrophobic fatty acyl chain and a large hydrophilic polar choline head group, which are attached to a glycerol backbone.30 The amphipathic nature of LPC endows it a detergent-like property. The specific localisation of LPC at the valves of VV tissues sug-gested that the valves might be damaged by LPC-induced inflammation or the detergent-like property of LPC. Dysfunction of the valve causes venous reflux, which leads to VV development.2,3 However our finding is compatible with those of previous reports, which state that venous valve incom-petence is related to inflammation.32,33 Monocytes and macrophages infiltrate valve leaflets in chronic venous disease, and such infiltration has been associated with areas of the endothelium that express ICAM-1. Recent clinical studies and basic scientific research suggest a role of matrix metalloproteinases (MMPs) in damaging the endothelium and the smooth muscle components of the vein wall. Then, the injured endothelial cells trigger leucocyte infiltration, activation and inflammation, which lead to further vein wall damage.34,35 Although the mechanism that initiates the inflammation in venous valves is not known, the accumula-tion of pro-inflammatory lipid molecules around the valvular region may be involved in the pathogenesis of the tissue inflammation process and valvular incompetence. Further studies are needed to clarify the mechanisms of accumula-tion of the lipid molecules. Since PC (1-acyl 36:4) is one of the major substrates of PL2A, LPC accumulation around the valvular region in VV tissue may reflect its increased production via phospholipase A2 (PL2A)-catalyzed PC hydrolysis. Considering the abundance of fatty acids in human tissue, the major fatty acid composition of PC (1-acyl 36:4) would be C16:0 (palmitic acid) and C20:4 (arachidonic acid (AA)). PLA2 produces LPC and free fatty acids by the hydrolysis of PC. Therefore, AA production may be increased by the hydrolysis of PC (1-acyl 36:4) at the valves of VV tissues. AA is a precursor of lipid mediators, such as prosta-glandins and leukotrienes, which induce chronic inflamma-tion. Therefore, inflammation triggered by the AA cascade should be considered as one of the mechanisms involved in the formation of VV. SM (d18:1/16:0) may also be involved in such tissue inflammation. Since SM (d18:1/16:0) has been observed to be localised around valves in VV tissues, it may also be involved in tissue inflammation. SM is a precursor of sphingolipid mediators.36 Several studies have suggested that sphingolipid mediators, including ceramide and ceramide-1-phosphate, play integral roles in the process of inflammation. The accumulation of PC (1-acyl 36:4) and SM (d18:1/16:0) around the valvular region may critically aggravate tissue inflammation around the valves in VV. Characteristic lipid distribution was observed uniformly in each clinical stage, suggesting that lipid molecules-related inflammation occurs from the early clinical stage of the disease and continues until the advanced stages. However, the initiating mechanism of such abnormal lipid metabolism is yet to be elucidated. Therefore, further studies are required in order to validate the cascade described in our hypothesis. Moreover, other unknown abnormal lipid metabolisms could exist in the disease development of VV. Since chronic venous insufficiency (CVI) is caused by multiple factors, multivariate analyses should be performed to verify the importance of the characteristic distributions of the lipid molecules in VV tissues. With the application of MALDI-IMS, further studies on a larger scale are needed for performing multivariate analyses, which could determine the role of the accumulation of the lipid molecules on disease progress in VV tissues. With regard to the effectiveness of statin administration in treating CVI, we currently have no information. Because the serum TC content did not differ statistically between the control and VV patients, the effectiveness of statins in lowering systemic TC levels for improving CVI is doubtful. However, as recently reported,37 statins possess anti-inflammatory effects which may ameliorate lipid mole-cules-mediated VV tissue inflammation. In summary, in this study, we identified the characteristic localisation of lipid molecules in the human VV wall using MALDI-IMS. Our data provides the first evidence of the accumu-lation of lipid molecules, such as LPC (1-acyl 16:0), PC (1-acyl 36:4) and SM (d18:1/16:0) around the damaged valvular region, which suggests the association of these molecules with tissue inflammation and resultant valvular incompetence. Conflict of interest None. Acknowledgements We are grateful to Yukiko Sugiyama for technical assistance. This work was supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented
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