► **Original Article** ◀

Insufficient Lymph Drainage Causes Abnormal Lipid Accumulation and Vein Wall Degeneration

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Objective: Previously, we analyzed human varicose veins (VV) using imaging mass spectrometry (IMS) and detected the abnormal accumulation of lipid molecules in the walls of VV, possibly due to insufficient lipid drainage by the lymphatic vessels. In this study, we created an animal model of lymphatic insufficiency to investigate the effects of insufficient lymph drainage on vein walls.

Methods: In rats, the lymphatic collecting vessels surrounding the femoral vein were ligated on one side (the model tissue), which caused the local retention of lymphatic fluid in the perivascular tissue. The equivalent contralateral tissue was used as a control. A histological study of the femoral vein and the surrounding perivascular tissue was conducted. IMS was used to analyze the distribution of lipid molecules in the perivascular tissue.

Results: Fourteen days after the procedure, the lymphatic vessels in the model tissue were significantly dilated. Furthermore, IMS revealed that the composition of the lipid molecules in the perivascular regions of the model tissue had altered. Compared with the control tissue, the model tissue exhibited marked perivascular accumulation of lysophosphatidylcholine (1-acyl 16:0), phosphatidylcholine (16:0/20:4), and triglycerides (52:2). Interestingly, the walls of the femoral veins running through the model tissue were 3.4-fold thicker than those of the femoral veins running through the control tissue. The number of tumor necrosis factor α -positive adipocytes was increased in the perivascular regions of the model tissue.

Conclusion: The findings of this study indicated that the accumulation of lymphatic fluid due to insufficient lymph drainage changes the structure of vein walls, and such changes might be associated with chronic venous insufficiency. (This is a translation of Jpn J Phlebol 2015; 26: 227–235.)

Keywords: varicose veins, lymphedema, phlebolympology, indocyanine green fluorescence imaging

Introduction

The pathogenesis of chronic venous insufficiency-related lymphatic dysfunction remains unknown.^{1,2)} We should develop a deeper understanding of the concept of “phlebolympology.” Phlebolympedema is a condition involving a mixture of venous and lymphatic insufficiency. The presence of lymphatic insufficiency gradually impairs the function of the venous system.³⁾ However, we demonstrated that impaired lymphatic function recovered after the great saphenous vein was stripped in patients with varicose veins (VV).⁴⁾ Therefore, venodynamics and lymphodynamics might interact to form an inseparable and mutually dependent dual-outflow system in vivo. However, the mechanisms underlying such interactions are complex. Since our knowledge regarding the pathophysiology of venous/lymphatic disorders is solely based on clinical studies,^{4–6)} there has never been any basic research into the changes that occur in the venous and lymphatic systems over time. Therefore, the mechanisms responsible for “phlebolympological” interactions at the cellular or molecular level are poorly understood. In this study, we recreated an animal model of lymphatic insufficiency to investigate the effects of insufficient lymph drainage on vein walls.

Materials and Methods

Creation of lymphatic fluid stasis and sample collection

The animal care procedures and experiments were performed in accordance with the guidelines of the

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Hamamatsu University School of Medicine animal care committee at the Center for Animal Care. Male Sprague-Dawley rats (8-week old) were purchased from SLC (Shizuoka, Japan) and provided with food and water. The rats were anesthetized using diethyl ether and pentobarbital. In brief, 0.2 ml of Evans blue dye was subcutaneously injected into the rats' dorsalis pedis arteries (Fig. 1). Then, a few lymphatic vessels around the femoral arteries and veins were identified based on the flow pattern of the Evans blue dye. The analyzed region was located in the center of the femoral area, and included the femoral artery, femoral vein, and a few lymphatic vessels. The lymphatic vessels were ligated by placing 8-0 Prolene sutures at 10 mm proximal and distal from the target region, which was not isolated from the perivascular tissue (Fig. 1). The perivascular tissue surrounding the ligated lymphatic vessels was used to assess the leakage and accumulation of lymphatic fluid and that on the contralateral side was used as the control tissue. Both sets of perivascular tissue were harvested at 7, 14, or 28 days after the procedure. The tissues were frozen in isopentane and stored at -80°C . This method enabled an assessment of the distribution of lipid molecules in the tissues to be performed using matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS).

Immunohistochemistry

The perivascular tissues were harvested and cut with a cryostat (CM1950; Leica Microsystems GmbH, Wetzlar, Germany) into 8- μm thick sections. The sections were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH: 7.4) for 10 min at room temperature. For conventional immunostaining, slides containing the sections were incubated at room temperature with the following primary antibodies: rabbit anti-Smith-McCort dysplasia (SMC) (1:50; Thermo Fisher Scientific, Waltham, MA, USA), mouse anti-podoplanin (1:100; ReliaTech GmbH, Braunschweig, Germany), rabbit anti-tumor necrosis factor (TNF)- α (1:100; NOVUS Biologicals, CO, USA), or rabbit anti-cleaved caspase 3 (1:100; Cell Signaling Technology Inc., Danvers, MA, USA) for 120 min. The resultant immune complexes were then detected with a polymer reagent (Histofine Simple Stain MAX PO; Nichirei Bioscience, Tokyo, Japan). The binding of the primary antibodies to their specific antigens was visualized with the diaminobenzidine kit (Dako, Carpinteria, CA, USA) and imaged on a light microscope equipped with a Nikon Eclipse 80i camera using the Lumina Vision software (ver. 3.0, Mitani, Tokyo, Japan).

Immunofluorescent staining

After being subjected to fixation and then rinsed with PBS, the sections were pre-incubated with 10% normal

Fig. 1 Experimental animals and procedures. In brief, 0.2 ml of Evans blue dye was subcutaneously injected into the dorsalis pedis arteries of 8-week-old male Sprague-Dawley rats. The lymphatic vessels around the femoral veins in the mid-thigh were identified based on the Evans blue staining. The lymphatic vessels were ligated by placing 8-0 monofilament sutures at both the proximal and distal ends of the target region, and the surrounding perivascular tissue was not surgically manipulated. The target region was used as a model of lymphatic insufficiency, whereas the equivalent tissue in the contralateral thigh was used as the control tissue.

goat serum (Nichirei Bioscience) and incubated overnight at 4°C with mouse anti-podoplanin (1:100; ReliaTech GmbH) or rabbit anti-cleaved caspase 3 antibodies (1:100; Cell Signaling Technology Inc.). Immunoreactivity was visualized using Alexa Fluor 488-conjugated anti-mouse immunoglobulin G and Alexa Fluor 594-conjugated anti-rabbit immunoglobulin G (Invitrogen, Carlsbad, CA, USA). All Alexa Fluor-conjugated secondary antibodies were diluted 200-fold before use. The slides were stained with the appropriate secondary antibody. Cell nuclei were stained with 4-6-diamidino-2-phenylindole, and the cells were imaged using an inverted microscope (BZX-700; Keyence, Osaka, Japan).

IMS

The IMS protocol, which is briefly explained below, has been described previously.⁷⁾ Eight- μm -thick longitudinal frozen sections for the IMS were thaw-mounted onto indium tin oxide-coated glass slides (Bruker Daltonics, Bremen, Germany) and dried at room temperature. A total of 500 μL of 2,5-dihydroxybenzoic acid solution was sprayed onto the sections. We performed IMS using a MALDI-time-of-flight (TOF)/TOF-type instrument (Ultraflex II TOF/TOF; Bruker Daltonics) equipped with a 355-nm Nd:YAG laser at a repetition rate of 200 Hz. Data were acquired with a step size of 20 μm in positive-ion mode (reflector mode). All spectra were acquired automatically using the Flex Imaging software (Bruker Daltonics), which was also used to create ion images.

Statistical analysis

The results are summarized using descriptive statistics, and all data are expressed as the mean $\pm$ standard deviation.

Fig. 2 Intraoperative view. The white arrows indicate lymphatic vessels. Scale bar = 1 mm.

Fig. 3 Dilation and loss of lymphatic vessels in the experimental animals. **(A)** An immunohistological study showed that the dilated lymphatic vessels in the lymphatic insufficiency model tissue were positive for podoplanin. Scale bar = 5 μ m. **(B)** At 7, 14, and 28 days after the procedure, the lymphatic vessels in the lymphatic insufficiency model tissue exhibited significantly greater diameters than those in the control tissue (* $P < 0.05$). **(C)** At 28 days after the procedure, the number of lymphatic vessels in the lymphatic insufficiency model tissue was lower than that seen in the control tissue ($n = 5$, * $P < 0.05$). The diameters and number of lymphatic vessels differed significantly between the groups according to the Tukey–Kramer test; * $P < 0.05$ indicates a significant difference ($n = 5$, each group).

The significance of inter-group differences in the diameter or number of lymphatic vessels, wall thickness, or ion intensity was determined using the Tukey–Kramer test. P values of <0.05 were considered significant. All statistical analyses were performed using the StatView 5.0 software (SAS Institute, Tokyo, Japan).

Results

Presence of lymphatic insufficiency in the animal model

Upon exposing the femoral vein and subcutaneously injecting Evans blue dye into the dorsum of the foot, we

readily identified some lymph vessels around the vein (Fig. 2). During the immunohistological study, the model tissues were positively stained for podoplanin, which is a lymphatic vessel marker (Fig. 3A), and the ligated lymphatic vessels exhibited significantly larger diameters than the control vessels after the procedure (Fig. 3B). On the other hand, the number of lymphatic vessels was lower in the model tissue than in the control tissue at 28 days (Fig. 3C). This was considered to be indicative of insufficient drainage of the lymph that had flowed from the extracellular fluid into the lymph vessels.^{8,9)} Normal lymph vessels are straight in form, but in cases of lymphostasis, their lumens become enlarged, as is seen in

Fig. 4 Vein wall degeneration: Changes in vein wall thickness and the leakage and accumulation of lymphatic fluid. (A) and (B) Quantitative measurements of the thickness of the intima and media of the vein wall were carried out. At 7, 14, and 28 days, the intima and media of the veins in the lymphatic insufficiency model tissue were significantly thicker than those of the veins in the control tissue (* $P < 0.05$). EVG, Elastica van Gieson; Scale bar = 20 μm . Significant differences in vein wall thickness were detected between the groups according to the Tukey–Kramer test; * $P < 0.05$ indicates a significant difference ($n = 5$, each group).

Fig. 3A. As the dilation in this model persisted for 28 days, we used it as a model of lymphostasis around the venous system.

The thickness of the vein wall was affected by lymphostasis

Next, we performed a histological analysis to identify the vein wall changes caused by lymphostasis. After the procedure, the intima and media of the model veins were thicker than those of the control veins (Fig. 4). These histopathological changes were similar to those seen in great saphenous veins affected by venous reflux in patients with VV.

Characteristic distribution of lipid molecules around the vein wall

Lymphatic vessels carry out the important role of transporting proteins and lipids. Using IMS, which is uniquely capable of lipid imaging, we found that in the model tissue lipid molecules were localized in the vein wall and perivenous tissue after lymphostasis had been induced. In addition, IMS revealed the marked accumulation of

lysophosphatidylcholine (LPC) (1-acyl 16:0), phosphatidylcholine (PC) (diacyl 16:0/20:4), and triglycerides (TG) (52:2) in the walls of the model veins after the procedure (Fig. 5A). In the model tissue, inflammatory lipid molecules gradually accumulated in the vein wall and perivenous tissue (Fig. 5B). On the other hand, there were no differences in the localization of PC (diacyl 16:0/18:1) or other molecules (data not shown) between the model and control tissue.

Detection of TNF- α around hyperplastic adipocytes in the perivascular tissue

The prevalence of adipocytes in the model perivenous tissue increased markedly after the procedure (Figs. 6A and 6B), and the stroma surrounding these adipocytes was positive for TNF- α (Fig. 6C).

Cleaved caspase 3 expression in lymphatic vessels

An immunohistological study showed that cleaved caspase 3, which is an apoptotic marker, was specifically

Fig. 5 Imaging mass spectrometry. **(A)** Distribution of LPC (1-acyl 16:0), PC (diacyl 16:0/20:4), and TG (52:2) in the vein wall. The solid line indicates the inner edge of the vein wall, and the dotted line shows the outer edge of the vein wall. The white broken line corresponds to the outer circumference of the vein wall. **(B)** Ion intensity of LPC (1-acyl 16:0), PC (diacyl 16:0/20:4), and TG (52:2) in the vein wall. Significant differences in ion intensity were detected between the groups according to the Tukey–Kramer test; * $P < 0.05$ indicates a significant difference ($n = 5$, each group). LPC: lysophosphatidylcholine; Lu: lumen; PC: phosphatidylcholine; TG: triglycerides; V: vein wall

Fig. 6 TNF- α was detected around the hyperplastic adipocytes in the perivascular tissue. **(A)** The number of hyperplastic adipocytes was markedly increased in the TNF α -positive region at 7, 14, and 28 days. EVG, Elastica van Gieson; Scale bar = 50 μm . **(B)** At 7, 14, and 28 days, the total adipocyte area in the perivascular tissue located 200 μm from the vein wall was significantly greater than that in the control tissue (* $P < 0.05$). Significant differences in adipocyte area were detected between the groups according to the Tukey–Kramer test; * $P < 0.05$ indicates a significant difference ($n = 5$, each group). TNF: tumor necrosis factor

Fig. 7 Cleaved caspase 3 expression in lymphatic vessels. An immunohistological study detected cleaved caspase 3(→), which is an apoptotic marker, in the dilated lymphatic vessels in the lymphatic insufficiency model tissue at 7, 14, and 28 days after the procedure. Scale bar = 5 μ m (n = 5, each group).

expressed in the dilated lymphatic vessels in the model tissue (Fig. 7). The associated apoptosis could have been responsible for the reduction in the number of lymphatic vessels seen after the procedure (Fig. 3).

Discussion

We created an animal model of lymphatic insufficiency and performed an experimental investigation into the physiological relationship between the veins and lymph vessels of the lower extremities. As a result, it was demonstrated that lymphostasis causes venous wall inflammation by inducing adipocyte degeneration around vein walls. Furthermore, it was suggested that sustained inflammation could lead to lymph vessel apoptosis. Lymph vessel dilation is suggestive of a rise in lymphatic pressure, which is accompanied by an increase in the intra-duct lymph flow rate. Even at 28 days after the procedure, the lymph vessel diameter of the model tissue was significantly greater than that of the control tissue. We consider that this was due to high pressure in the tissue surrounding the lymph vessels, but the detailed dynamics (i.e., lymph flow rate and tissue pressure) responsible for the observed effects remain unclear. Therefore, this issue requires further study.

Lymph vessel dysfunction causes extracellular fluid and the lipid components contained in lymph to accumulate in the surrounding tissue.¹⁾ In the present study, IMS revealed not only the quantity, but also the distribution of lipid molecules. Specifically, it showed that in the model tissue lipid molecules accumulated in the vein wall and the surrounding tissue (Fig. 5). Also, because IMS makes

it possible to perform analyses at the level of molecular species, we were able to determine that the accumulating lipid molecules comprised LPC, an inflammatory mediator, and arachidonic PC, whose side chain arachidonic acid is known to accumulate in multiple chronic inflammatory diseases. Vein wall inflammation causes medial smooth muscle cell proliferation and excess production of fibrous matter, leading to hyperplasia, which in turn reduces the elastic compliance of the vein wall.^{10,11)} The vein wall hyperplasia shown in Fig. 4 was considered to have been caused by the effects of lymphostasis-induced inflammation. The ex vivo animal experiments conducted in the present study indicated that the sustained passage of an excess amount of lymph through the lymphatic vessels can adversely affect the vessels' pump function and overwhelm the lymphatic system. Thus, structural deterioration of the micro-lymphatic network might contribute to venous insufficiency.¹²⁾

The accumulation of toxins, exudate, the fine particles contained in lymph, and bacteria in tissue brings about a variety of changes, including inflammation, a low oxygen environment, and fibrosis.^{13,14)} In this study, we demonstrated that during lymphostasis, lipid molecules accumulate in the tissue surrounding veins. Based on these findings, we will attempt to discuss the mechanism responsible for lipid molecule accumulation and metabolic abnormalities. Small adipocytes secrete specific anti-inflammatory cytokines, such as adiponectin, which help to suppress inflammation. However, when excess lipids, such as fatty acids, are stored in adipocytes, hyperplasia, and phenotypically transformed adipocytes secrete

inflammatory promoters, such as $\text{TNF-}\alpha$.^{15,16)} Vascular wall cells are affected in a variety of ways by inflammation. For example, $\text{TNF-}\alpha$ promotes the proliferation of medial smooth muscle cells,^{17,18)} reduces vascular wall compliance, changes the inner lumen diameter of blood vessels, and causes abnormal blood flow. We considered that excessive accumulation of lipid molecules in lymph would cause adipocytes to degenerate and secrete pro-inflammatory cytokines, leading to adverse effects on the vascular wall.

The pathophysiological mechanisms responsible for VV and deep vein thrombosis involve lipid metabolism abnormalities.^{19,20)} Previously, we analyzed VV from patients with chronic venous insufficiency using IMS and detected the abnormal accumulation of lipid molecules in the walls of the VV, possibly due to insufficient lipid drainage by the lymphatic vessels.¹⁹⁾ Furthermore, we found that there are lymph vessels in the great saphenous vein wall, but the number of lymph vessels in the great saphenous vein wall was lower in the VV patients with prominent cutaneous symptoms.²¹⁾ Although the primary cause of inflammation in such tissues is not yet understood, the observed reduction in the number of lymphatic vessels could have been caused by lymphatic vessel cell apoptosis (Fig. 7). Such reductions in the number of lymphatic vessels could lead to lymphatic vessel dysfunction, and the drainage function of the surrounding tissues might subsequently deteriorate. Consequently, a vicious cycle could form in which interstitial fluid and lymph accumulate within the intracellular spaces. Vascular diseases involving chronic vascular wall inflammation, such as VV, might be related to localized lipid abnormalities. Specifically, lymph stagnation and lymph vessel dysfunction could be closely related to the inflammatory response.

It is thought that many vascular disorders are not simply diseases of the venous or lymph duct, as the functions of the venous and lymphatic systems are closely related, and hence, if the function of one declines then the other will also be affected. In this study, we sequentially observed the changes induced by artificially generated lymphostasis. In future studies, we will evaluate the effects of venous abnormalities on the adjacent lymph vessels.

Conclusion

This study indicated that the accumulation of lymphatic fluid due to insufficient lymph drainage could cause inflammation and the degeneration of the walls of adjacent veins.

Disclosure Statement

No financial assistance was received for this study. The authors have no conflicts of interest.

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