Cardiac Lymphatic Dysfunction Causes Drug-Eluting Stent–Induced Coronary Hyperconstricting Responses in Pigs In Vivo

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Objective—We have previously demonstrated that coronary adventitial inflammation plays important roles in the pathogenesis of coronary vasomotion abnormalities, including drug-eluting stent (DES)–induced coronary hyperconstricting responses. Importantly, the adventitia also harbors lymphatic vessels, which may prevent inflammation by transporting extravasated fluid and inflammatory cells. We thus aimed to examine the roles of coronary adventitial lymphatic vessels in the pathogenesis of DES-induced coronary hyperconstricting responses in a porcine model in vivo.

Approach and Results—We performed 2 experimental studies. In protocol 1, 15 pigs were divided into 3 groups with or without DES and with bare metal stent. Nonstented sites 20 mm apart from stent implantation also were examined. In the protocol 2, 12 pigs were divided into 2 groups with or without lymphatic vessels ligation followed by DES implantation at 2 weeks later (n=6 each). We performed coronary angiography 4 weeks after DES implantation, followed by immunohistological analysis. In protocol 1, the number and the caliber of lymphatic vessels were greater at only the DES edges after 4 more weeks. In protocol 2, coronary hyperconstricting responses were further enhanced in the lymphatic vessels ligation group associated with adventitial inflammation, Rho-kinase activation, and less adventitial lymphatic vessels formation. Importantly, there were significant correlations among these inflammation-related changes and enhanced coronary vasoconstricting responses.

Conclusions—These results provide evidence that cardiac lymphatic vessel dysfunction plays important roles in the pathogenesis of coronary vasoconstrictive responses in pigs in vivo.

Key Words: coronary vasospasm ■ drug-eluting stent ■ inflammation ■ lymph ■ Rho kinase

Despite recent advances in the medical management for ischemic heart disease, intractable vasospastic angina and unremitting angina because of coronary hyperconstricting responses even after successful percutaneous coronary intervention with drug-eluting stent (DES) are emerging clinical issues.1–6 We have previously demonstrated that activation of Rho-kinase, a molecular switch for vascular smooth muscle contraction, plays a key role in the pathogenesis of coronary vasospasm and hyperconstricting responses after DES implantation in animals and humans.2,3,7,8 We also have demonstrated that adventitial inflammatory changes play important roles in the pathogenesis of coronary spasm in pigs and humans.9–14 Importantly, the adventitia harbors a variety of components, including not only vasa vasorum (VV) but also lymphatic vessels.15

The lymphatic vasculature collects interstitial fluid, macromolecules, and extravasated leukocytes from tissues.16 Lymphatic vessels are essential for the development of inflammation, and edema occurs in inflammation when the rate of plasma leakage from blood vessels exceeds the drainage through lymphatic vessels.17 Indeed, airway inflammation leads to bronchial lymphedema and exaggerates airflow obstruction in mice.17 In addition, VEGF (vascular endothelial growth factor)-C (lymphangiogenic factor) reduces chronic skin inflammation in mice.18 Notably, recent studies suggested that lymphatic vessels play important roles in the cardiovascular system in various diseases including inflammation, edema, and vasospasm.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2019;39:741-753. DOI: 10.1161/ATVBAHA.119.312396.)
The number of adventitial lymphatic vessels increases associated with intimal thickness of human carotid arteries. Lymphatic vessels are also involved in the development of atherosclerosis. However, since the discovery of the cardiac lymphatic system by Rudbeck in 1653, little attention has been paid to the roles of cardiac lymphatic vessels in the pathogenesis of coronary vasomotion abnormalities.

In the present study, we thus performed experimental studies to examine the roles of cardiac lymphatic vessels in the pathogenesis of coronary spasm using an established porcine model with hypercontracting responses after DES implantation in vivo. We performed 2 experimental protocols. All animal care and experimental studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication, 8th Edition, 2011) and were approved by the Institutional Committee for Use of Laboratory Animal of Tohoku University (No 2017MdA-139).

In the protocol 1, we examined the changes in adventitial lymphatic vessels in a porcine model of DES-induced coronary hypercontracting responses in vivo (Figure 1A). In the protocol 2, we examined the effects of cardiac lymphatic vessels ligation on coronary vasomotion (Figure 1B).

**Nonstandard Abbreviations and Acronyms**

- **BMS** bare metal stent
- **CAG** coronary angiography
- **CD68** cluster of differentiation 68
- **DES** drug-eluting stent(s)
- **IC** intracoronary injection
- **ICG** indocyanine green
- **IL-1β** interleukin-1β
- **LVVE-1** lymphatic vessel endothelial hyaluronan receptor 1
- **p-MYPT1** phosphorylated myosin phosphatase target subunit 1
- **ROCK** Rho-associated coiled-coil containing protein kinase
- **VV** vasa vasmor

**Materials and Methods**

All data and supporting materials have been provided with the published article.

**Study Protocol**

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**Protocol 1**

In our animal facility, the light is turned on at 8 o’clock and turned off at 18 o’clock. Pigs can freely drink water at any time and eat normal diet (Grandeal B; Zennoh Feed Mills of the Tohoku Distict, Miyagi, Japan) with the photodynamic eye (PDE) near-infrared camera system (Hamamatsu Photonics, Hamamatsu, Japan). The speed was calculated by the following formula: [the length of ICG diffusion divided by time constant]. After these studies, the animals were euthanized with a lethal dose of potassium chloride (0.25 mEq/kg, IV) under deep anesthesia with inhaled 5% sevoflurane.

**Coronary Angiographic Study**

After control CAG, we examined coronary vasoconstricting responses to serotonin (10 and 100 μg/kg, IC) before and after intracoronary hydroxyfasudil (30 and 300 μg/kg, slow IC for 3 min), a specific Rho-kinase inhibitor (Asahi Kasei Pharma, Tokyo, Japan), and the nonstented sites (20 mm apart from the stent edges; DES+20) in the protocol 1 were cut into a 3-μm-thick slice. They were stained with Masson’s trichrome staining and hematoxylin eosin staining. Histomorphometric parameters were manually measured by using Image-J (US National Institute of Health, Bethesda, MD). Adipocyte size was evaluated as mean diameter (μm) in the perpendicular maximum and minimum axes, and the data were represented as an average of each adipocyte in 3 random high-power fields. In the protocol 2, myocardium slices were stained with Masson’s trichrome staining to assess the influence of lymphatic vessels ligation on myocardial fibrosis. Collagen volume fraction, expressed as percentage of fibrosis area per microscopical field by using Image J, was used to express the extent of myocardial fibrosis. Overall thickness of the atrioventricular valve leaflets was averaged...
over 5 equally distributed length measurements throughout the valve and was quantified by using Image J, as previously described.  

Immunohistological Analysis

Immunohistological staining was performed on paraffin-embedded tissue sections using rabbit anti-LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1; 1:10000, RF2219633; Thermo Fisher Scientific, Waltham, MA) antibody for lymphatic vessels (Major Resources Table in the online-only Data Supplement). Immunohistological staining was performed with rabbit anti-human VWF (von Willebrand factor) antibody (N1505; DAKO, Copenhagen, Denmark) for VV and rabbit anti-tyrosine hydroxylase (BIO RAD; Hercules, CA) for sympathetic nerve fibers. The densities of lymphatic vessels, VV, and sympathetic nerve fibers were expressed as a number of LYVE-1-positive, VWF-positive vessels, and tyrosine hydroxylase–positive fibers divided by the adventitial area (mm²), respectively.  

Figure 1. In the protocol 1 (A), 10 pigs were pretreated orally with aspirin (200 mg/day, PO) and clopidogrel (225 mg/day, PO). Two days after the start of the dual antiplatelet therapy, a stent was implanted into the left coronary artery in 5 pigs, and the remaining 5 pigs did not undergo stent implantation as a control group. At 1 mo, all animals underwent coronary angiography followed by histological analysis. In the protocol 2 (B), 12 domestic pigs underwent left thoracotomy and were randomly divided into 2 groups, cardiac lymphatic vessels ligation group and the sham group (N=6 each). For 12 days after the operation, the animals were treated with aspirin (200 mg/day, PO) and clopidogrel (225 mg/day, PO). Two days after the start of the dual antiplatelet therapy, all animals underwent drug-eluting stent (DES) implantation into the left anterior descending (LAD) coronary arteries. At 1 mo after DES implantation, all animals underwent coronary angiography followed by histological analysis.
were significantly more and larger at the DES edges compared with the control sites and BMS edges (Figure 2A through 2D, 2G, and 2H). Indeed, quantitative analysis showed that the number and the caliber of lymphatic vessels were significantly more and larger at the DES edges compared with the control and DES+20 sites (Figure 2K and 2L). There were significant correlations between the number of lymphatic vessels and IL-1β-positive cells (Figure 2M) and vasoconstriction to serotonin in control sites and DES edges (Figure 2N).

Protocol 2. Effects of Cardiac Lymphatic Vessels Ligation on Coronary Hyperconstricting Responses

In the protocol 2, we performed cardiac lymphatic vessels ligation, in which the ligation points of cardiac lymphatic vessels were clearly visualized with Evans blue (Figure 3A). At 1 month after DES implantation, we performed ICG injection study followed by CAG study. In the ICG study, lymph transport of ICG was clearly noted from the apex to the base in the sham group, whereas ICG remained at the apex in the ligation group (Figure 3B). Indeed, lymph transport speed was markedly reduced in the ligation group than in the sham group (Figure 3C). In the CAG study, no in-stent restenosis was noted in both groups (Figure 4A and 4D). Notably, coronary vasoconstricting responses to intracoronary serotonin were enhanced at the proximal and distal DES edges in the ligation group compared with the sham group, which were abolished by intracoronary pretreatment with hydroxyfasudil (Figure 4B, 4C, 4E, and 4F). Quantitative CAG analysis demonstrated that coronary vasoconstricting responses to serotonin were significantly enhanced in the ligation group compared with the sham group (Figure 4G). In contrast, endothelium-independent and -dependent coronary vasodilating responses to nitroglycerin and bradykinin (regardless of the presence or absence of N0-monomethyl-L-arginine) were comparable between the 2 groups (Table II in the online-only Data Supplement).

Effects of Cardiac Lymphatic Vessel Ligation on Coronary Adventitial Inflammation and Rho-Kinase Activity

Immunohistological and subsequent quantitative analyses showed that LYVE-1-positive lymphatic vessels at the DES edges appeared less and smaller in the ligation group compared with the sham group (Figure 5A, 5B, 5E, 5F, 5I, and 5J). Masson’s trichrome staining showed that adipocyte size was significantly larger in the ligation group compared with the sham group (Figure 5C, 5G, and 5K). In contrast, adiponectin-positive areas (%) were significantly reduced in the ligation group compared with the sham group (Figure 5D, 5H, and 5L). There was a significant negative correlation between the number of lymphatic vessels and adipocyte size (Figure 5M).

Inflammatory cells (macrophages and IL-1β-positive cells) in the adventitia at the DES edges were noted in the ligation group more than in the sham group (Figure 6A through 6H). In contrast, there was no significant difference in the number of VV or sympathetic nerve fibers between the 2 groups (VV, sham 34.9±2.7/mm² versus ligation 30.8±4.2/mm², P=0.43;...
sympathetic nerve fibers, sham 9.9±1.3/mm² versus ligation 8.2±2.3/mm², P=0.52). Immunoreactivities for ROCK1/2 and pMYPT1 were predominantly noted in the medial vascular smooth muscle cells (Figure 6I through 6P), and semiquantitative analysis showed that the expressions of ROCK1/2 and pMYPT1 at the DES edges were significantly enhanced in the
There was a significant positive correlation between adipocyte size and the number of IL-1β-positive cells (Figure 6T). Furthermore, significant positive correlations were noted between the number of IL-1β-positive cells and pMYPT1 expression (Figure 6U) and between pMYPT1 levels and the extent of coronary vasoconstriction to serotonin (Figure 6V).

Effects of Cardiac Lymphatic Vessels Ligation on Myocardium and Atrioventricular Valves
Masson’s trichrome staining showed that the extent of myocardial fibrosis was significantly increased in the ligation group compared with the sham group (Figure IIA, IIB, and IIG in the online-only Data Supplement). In addition, the thicknesses of tricuspid and mitral valves were also significantly greater in
the ligation group than in the sham group (Figure IIC through IIIF, IIH, and II-I in the online-only Data Supplement).

**Negative Controls for Immunohistochemistry**

Negative controls of immunohistochemistry were performed for LYVE-1, adiponectin, CD68, IL-1β, ROCK1, ROCK2, and pMYPT1 (Figure IIIA through IIIN in the online-only Data Supplement).

**Discussion**

The major findings of the present study were as follows: (1)

In the present study with pigs, enhanced lymphatic vessel
formation was noted at the DES edges associated with coronary vasoconstricting responses but not at the BMS edges or DES+20 mm sites. (2) Lymphatic vessels ligation further enhanced coronary hyperconstricting responses after DES implantation. (3) Lymphatic vessels ligation decreased the number of lymphatic vessels, although there were no significant differences in the number of VV between the sham and the ligation groups. (4) Lymphatic vessels ligation caused adipocyte hypertrophy associated with enhanced inflammation in the adventitia and Rho-kinase activation. This study demonstrates the important roles of cardiac lymphatic vessels in the pathogenesis of coronary hyperconstricting responses and cardiac structural changes.

**Effects of Stent Implantation on the Density and Caliber of Lymphatic Vessels**

We examined the density and the caliber of lymphatic vessels at the control sites and at the DES and BMS edges. Histological

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**Figure 5.** Representative pictures at the drug-eluting stent (DES) edges for Masson’s trichrome staining (A and E) and immunostainings of LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1; B and F), adipocytes with Masson’s trichrome staining (C and G), and adiponectin (D and H). Black arrow heads indicate lymphatic vessels, and arrows indicate the diameters in the perpendicular maximum and minimum axes. Quantitative histological analysis between the 2 groups (I–L) and correlation between the number of LYVE-1 vessels and adipocyte size is shown M. Results are expressed as mean±SEM. *P<0.05, **P<0.001 sham vs ligation.
Figure 6. Representative pictures at the drug-eluting stent (DES) edges for Masson’s trichrome staining (A, D, I, and M) and immunostainings of CD68 (cluster of differentiation 68; B and E), IL (interleukin)-1β (C and F), ROCK1 (J and N), ROCK2 (K and O), and pMYPT1 (L and P). Quantitative histological analysis and correlations among the histological results and the extent of coronary vasoconstriction are shown (G, H, and Q–V). Results are expressed as mean ± SEM. *P<0.05, **P<0.01, Sham vs. Ligation. HPF indicates high-power field; p-MYPT1, phosphorylated myosin phosphatase target subunit 1; and ROCK, Rho-associated coiled-coil containing protein kinase.
analysis showed that the number and the caliber of lymphatic vessels were significantly greater at the DES edges compared with the control and the BMS edges, whereas there was no significant difference between the control and the BMS sites. These results indicate that DES, but not BMS, affect lymphatic vessels in the coronary adventitia. Histological analysis also showed that the number and the caliber of lymphatic vessels were significantly greater at the DES edges compared with the control and DES+20 sites, whereas there was no significant difference between the control and the DES+20 sites. These results indicate that DES affect the number and the caliber of lymphatic vessels at the DES edges in the coronary adventitia in pigs.

Effects of Cardiac Lymphatic Vessel Ligation on Coronary Vasomotion in Pigs In Vivo

We have previously demonstrated that adventitial inflammation associated with Rho-kinase activation plays important roles in the pathogenesis of coronary artery spasm and DES-induced coronary hyperconstricting responses in pigs and humans.1,8,12–14,25,26 DES implantation enhances chronic adventitial inflammation associated with enhanced VV formation, sympathetic nerve fiber formation, macrophage infiltration, and Rho-kinase activation.1,7 Notably, the adventitia harbors 2 types of vessels, VV and lymphatic vessels.15 In the protocol 1, enhanced formation of cardiac lymphatic vessels at the DES edges, but not at the BMS edges or nonstented sites, suggests that lymphatic vessels may be involved in the pathogenesis of coronary hyperconstricting responses after DES implantation. In the protocol 2, cardiac lymphatic vessels ligated enhanced coronary hyperconstricting responses associated with decreased coronary lymphatic vessels, hypertrophic adipocytes, enhanced inflammation in the adventitia, and Rho-kinase activation, whereas there was no significant difference in the number of VV or sympathetic nerve fibers between the 2 groups. Discrepancy between the coronary lymphatic vessels and VV formation in the protocol 2 may be because of their different healing processes in response to lymphatic vessels ligation injury and prolonged need of lymphatic vessels to drain excessive proteins and fluid compared with VV.40

In line with the present findings, lymphatic vessels were markedly decreased in lymphatic edematous tail of mice.41 Adipocyte hypertrophy was initiated by lymphatic dysfunction,42 and hypertrophic adipose tissue in obese mice mobilize inflammatory cells.43 These structural and functional alterations of lymphatic vessels in responses to inflammation may also be the case for the heart in our body.44 Indeed, inhibition of endogenous lymphangiogenesis increases cardiac inflammation in mice.45 We previously demonstrated that reduced adiponectin level is associated with enhanced macrophage infiltration in visceral adipose tissue in mice.46 We have recently demonstrated that reduced adiponectin and enlarged adipocyte size are associated with enhanced macrophage infiltration in the coronary adventitia and enhanced coronary hyperconstricting responses in pigs in vivo.25

In the present study, the number of lymphatic vessels and adipocyte size were associated with the extent of local inflammation (IL-1β-positive cells) and Rho-kinase activity. Indeed, we have previously demonstrated that IL-1β increases Rho-kinase activity in human coronary vascular smooth muscle cells in vitro47 and that chronic treatment with IL-1β in the coronary adventitia induces coronary vasospasm through Rho-kinase activation in pigs in vivo.25 Taken together, in the protocol 1, although compensated lymphangiogenesis occurred in the coronary adventitia, it is possible that the extent of compensatory lymphangiogenesis was not enough to suppress adventitial inflammation. In the protocol 2, it is conceivable that reduced cardiac lymphatic vessels with hypertrophic adipocytes lead to chronic adventitial inflammation and enhances Rho-kinase activity, resulting in coronary hyperconstricting responses. Coronary adventitia harbors several components, including VV, lymphatic vessels, macrophages, and adipocytes. When lymphatic dysfunction occurs, lymph stasis and decreased number of lymphatic vessels are noted, associated with adipocyte hypertrophy and infiltration of inflammatory cells, such as macrophages and IL-1β–positive cells, in the coronary adventitia. IL-1β can enhance Rho-kinase activity in the medial vascular smooth muscle cells, resulting in enhanced coronary hyperconstricting responses. Taken together, cardiac lymphatic vessel dysfunction plays important roles in the pathogenesis of coronary spasm and cardiac structural changes (see Graphic Abstract).

We found that the lymphatic flow of ICG was obstructed even at 6 weeks after the lymphatic vessels ligation. This finding suggests that lymphatic flow remained impaired in the ligation group for at least 6 weeks after the operation. Indeed, in the present study, we also confirmed that myocardial fibrosis and thickening of tricuspid and mitral valves were evident in the ligation group compared with the sham group. Consistent with the present findings, it was previously reported that fibrosis was commonly noted in lymphedematous tissue48–51 and in the rabbit heart with cardiac lymph obstruction52 and that cardiac lymphatic obstruction also induced thickening of atrioventricular valves in dogs.53 Our findings are consistent with the previous findings, suggesting that our ligation technique was enough to obstruct lymphatic flow.

Clinical Implications

Lymphatic vessels may play important roles in the pathogenesis of many disorders, including lymphedema, inflammation, transplant rejection, cancer, and cardiovascular disease.44,54 Accelerated cardiac lymphangiogenesis with VEGF-C improved myocardial fluid balance, cardiac inflammation, fibrosis, and functions.55,56 Moreover, increased lymphatic vessels were noted with cardiac pathological changes, including myocarditis and myocardial hypertrophy in the human autopsy hearts.37 However, the roles of cardiac lymphatic vessels in the pathogenesis of coronary vasomotion abnormalities remain to be elucidated. In the present study, we were able to provide evidence that functional and structural alterations of cardiac lymphatic vessels are substantially involved in the pathogenesis of coronary vasomotion abnormalities in pigs in vivo. Recent large-scale trial demonstrated that ≥30% of patients have unremitting angina until 1 year after successful percutaneous coronary intervention with DES.2 Notably, some patients suffer from enhanced coronary hyperconstricting responses after DES implantation.2,6 In addition, a lack of hemodynamically significant coronary artery obstruction is noted in about 20% to 40% of patients with
angina and a positive stress test.57 Intractable vasospastic angina is also an emerging therapeutic target.58 Based on the present findings, coronary lymphatic vessels could be regarded as a new therapeutic target for coronary vasomotion disorders.

Study Limitations
Several study limitations should be mentioned for the present study. First, it was previously reported that not only cardiac lymphatic vessels but also pretracheal lymph nodes could be ligated in dogs.28 In the present study, for technical reasons, it was difficult to ligate pretracheal lymph nodes in pigs in vivo. However, our technique of cardiac lymphatic vessels ligation was enough to create lymph flow obstruction as confirmed in the ICG study at 6 weeks after the procedure. Second, because we were unable to clearly see the lymphatic vessels because of their small size without blood color, it was difficult to determine the orientation of lymphatic vessels when making paraffin sections. Third, it was also difficult to perform immunostainings of resin-embedded sections of the stent implantation site, which makes it hard to reveal the whole topology of lymphatic vessels of the stented arterial segment. However, it is important to address the morphometry of lymphatic vessels at the stented coronary arteries for further mechanistic insights in future studies. Fourth, ligation of lymphatic vessels could injure the adventitial sympathetic nerve fibers because we were unable to clearly recognize adventitial nerves in vivo during the ligation procedure. We have recently demonstrated that there are significant correlations between sympathetic nerve fibers and coronary hyperconstricting responses.26 However, in the present study, quantitative histological analysis showed that there was no significant difference in the number of adventitial sympathetic nerve fibers between the sham and the ligation groups in the protocol 2. Fifth, we used only male pigs in the present study to eliminate potential hormonal changes in the ligation groups in the protocol 2. Sixth, we used only male pigs in the present study for technical reasons, it was difficult to perform immunostainings of resin-embedded sections of the stent implantation site, which makes it hard to reveal the whole topology of lymphatic vessels of the stented arterial segment. However, it is important to address the morphometry of lymphatic vessels at the stented coronary arteries for further mechanistic insights in future studies. Fourth, ligation of lymphatic vessels could injure the adventitial sympathetic nerve fibers because we were unable to clearly recognize adventitial nerves in vivo during the ligation procedure. We have recently demonstrated that there are significant correlations between sympathetic nerve fibers and coronary hyperconstricting responses.26 However, in the present study, quantitative histological analysis showed that there was no significant difference in the number of adventitial sympathetic nerve fibers between the sham and the ligation groups in the protocol 2. Fifth, we used only male pigs in the present study to eliminate potential hormonal changes during menstruation period and to consider coronary risks.59–61 However, we previously demonstrated that this male pig model has coronary vasomotion abnormalities.37,13,25–27,62–65

Conclusions
The present study provides evidence that cardiac lymphatic vessels dysfunction plays important roles in the pathogenesis of coronary hyperconstricting responses.

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Disclosures
None.

References

### Highlights

- The major findings of the present study were as follows: (1) Enhanced lymphatic vessel formation was noted at the drug-eluting stent edges associated with coronary vasoconstricting responses but not at the bare metal stent edges or sites apart from drug-eluting stent. (2) Lymphatic vessels ligation further enhanced coronary hyperconstricting responses after drug-eluting stent implantation. (3) Lymphatic vessels ligation decreased the number of lymphatic vessels, although there were no significant differences in the number of vasa vasorum between the sham and the ligation groups. (4) Lymphatic vessels ligation caused adipocyte hypertrophy associated with enhanced inflammation in the adventitia and Rho-kinase activation. The present study provides evidence that cardiac lymphatic vessels dysfunction plays important roles in the pathogenesis of coronary spasm and cardiac structural changes.