

Atherosclerotic Coronary Plaque Is Associated With Adventitial Vasa Vasorum and Local Inflammation in Adjacent Epicardial Adipose Tissue in Fresh Cadavers

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Background: The coronary adventitia has recently attracted attention as a source of inflammation because it harbors nutrient blood vessels, termed the vasa vasorum (VV). This study assessed the link between local inflammation in adjacent epicardial adipose tissue (EAT) and coronary arterial atherosclerosis in fresh cadavers.

Methods and Results: Lesion characteristics in the left anterior descending coronary artery of 10 fresh cadaveric hearts were evaluated using integrated backscatter intravascular ultrasound (IB-IVUS), and the density of the VV and levels of inflammatory molecules from the adjacent EAT were measured for each of the assessed lesions. The lesions were divided into lipid-rich, lipid-moderate, and lipid-poor groups according to percentage lipid volume assessed by IB-IVUS. Higher expression of inflammatory molecules (i.e., vascular endothelial growth factor A [VEGFA] and VEGFB) was observed in adjacent EAT of lipid-rich ($n=11$) than in lipid-poor ($n=11$) lesions (7.99 ± 3.37 vs. 0.45 ± 0.85 arbitrary units [AU], respectively, for VEGFA; 0.27 ± 0.15 vs. 0.11 ± 0.07 AU, respectively, for VEGFB; $P<0.05$). The density of adventitial VV was greater in lipid-rich than lipid-poor lesions ($1.50\pm0.58\%$ vs. $0.88\pm0.23\%$; $P<0.05$).

Conclusions: Lipid-rich coronary plaques are associated with adventitial VV and local inflammation in adjacent EAT in fresh cadavers. This study suggests that local inflammation of EAT is associated with coronary plaque progression via the VV.

Key Words: Coronary plaque; Epicardial adipose tissue; Fresh cadaver; Inflammatory molecules; Vasa vasorum

The adventitia, the outermost layer of the vessel wall, has received considerable attention in recent years. Previous studies suggested that neovascularization of the adventitial vasa vasorum (VV) plays a key role in the development of human atherosclerotic plaques.^{1–4} Adipocytokines secreted from perivascular adipose tissue have direct access to the adjacent arterial wall via diffusion or the VV.^{5,6} Previously, we reported that human coronary atherosclerosis is associated with inflammation in epicardial adipose tissue (EAT).⁷ In that study we found that infiltration of macrophages, and expression of pro- and anti-inflammatory cytokines, was increased in the epicardial fat of patients with coronary artery disease (CAD) compared with non-CAD patients.⁷ However, little is known about the relationship between the VV and local inflammation in EAT around coronary plaques in humans.

Recently, the characteristics of coronary plaques have

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been evaluated by intravascular imaging in the clinical field. Invasive imaging modalities, such as intravascular ultrasound (IVUS), are considered the gold-standard method to measure the progression of atherosclerotic plaques.^{8,9} Integrated backscatter (IB)-IVUS, which uses the average power of backscatter radiofrequency signals, enables analysis of tissue components of coronary plaques in vivo.^{10,11} However, little is known about the relationship between coronary plaque characteristics and inflammation in EAT in humans. Because Tokushima University Graduate School of Biomedical Sciences has a laboratory that uses fresh cadaveric human bodies, we decided to investigate this relationship in cadaveric human hearts.

The aim of the present study was to investigate the

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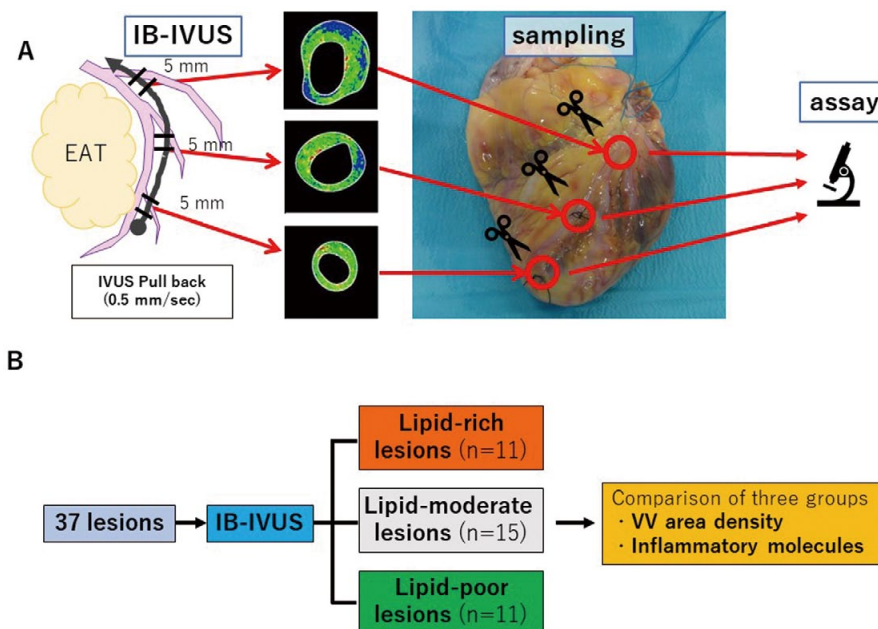


Figure 1. (A) Study design for using integrated backscatter intravascular ultrasound (IB-IVUS) and measuring the adventitial vasa vasorum (VV) area density and inflammatory molecules in adjacent epicardial adipose tissue (EAT) of cadaveric hearts. (B) Lesion classification. In all, 37 lesions were divided into 3 groups (lipid-rich, lipid-moderate, and lipid-poor groups) to measure the VV area density and inflammatory molecules.

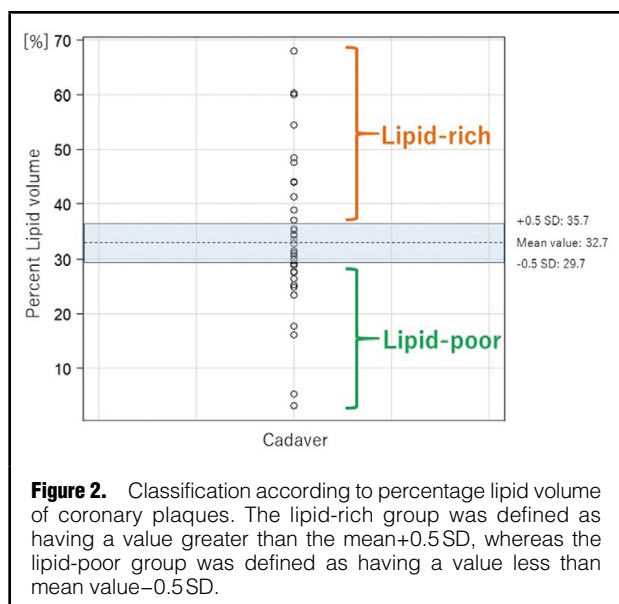


Figure 2. Classification according to percentage lipid volume of coronary plaques. The lipid-rich group was defined as having a value greater than the mean+0.5SD, whereas the lipid-poor group was defined as having a value less than mean value-0.5SD.

relationships among coronary plaque characteristics, adventitial VV, and inflammation in adjacent EAT using fresh cadaveric hearts.

Methods

Cadavers and Study Design

After receiving approval from the Institutional Review

Board of Tokushima University, we undertook this study using 10 fresh, frozen cadavers that had been stored at -20°C . Hearts were removed from the cadavers and cleaned with normal saline. Then, a guidewire was introduced into the left anterior descending artery (LAD) so that the LAD could be evaluated by IB-IVUS. For each heart, at least 2 samples were obtained in which the coronary plaque burden was over 25%. For each lesion, the area density of the adventitial VV was measured by immunohistological examination and the expression of inflammatory molecules in the adjacent EAT was determined (Figure 1A). Lesions were divided into 3 groups (lipid-rich, lipid-moderate, and lipid-poor groups) according to percentage lipid volume assessed by IB-IVUS, and VV area density and the expression of inflammatory molecules were compared between 2 groups (i.e., lipid-rich and lipid-poor groups; Figure 1B).

IVUS Data Acquisition

Following the passage of a 0.014-inch guidewire into the LAD, the IVUS catheter (40 MHz; ViewIT; Terumo, Tokyo, Japan) was introduced over the wire and positioned as distal to the LAD as possible. Saline was infused into the LAD via constant manual perfusion pressure to obtain clear images. The guidewire was removed from the vessel before IVUS in order to reduce associated artifacts. Data were collected at an auto pull-back rate of 0.5 mm/s and analyzed using an IVUS imaging system (VISIWARE; Terumo). For each lesion, 5 IB-IVUS images (5 mm in length) were captured at 1-mm intervals using a motorized pull-back system.

Conventional IVUS and IB-IVUS Parameters

IB-IVUS parameters were measured in the LAD of 10

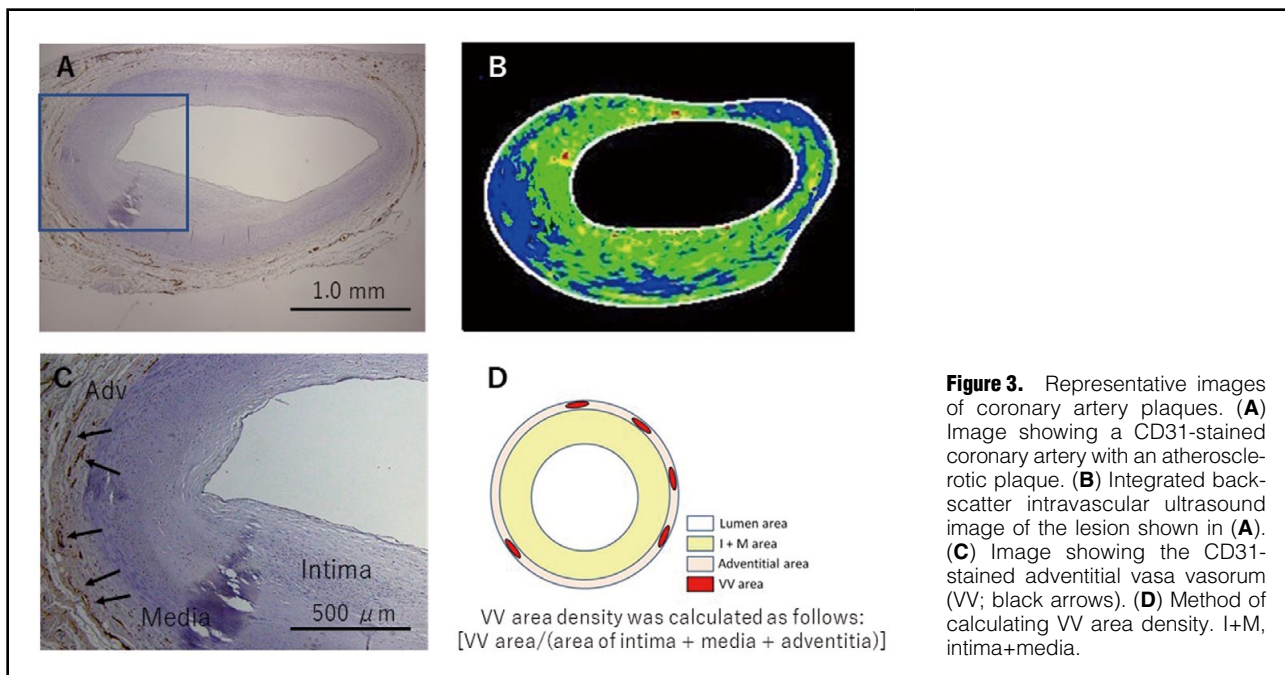


Figure 3. Representative images of coronary artery plaques. **(A)** Image showing a CD31-stained coronary artery with an atherosclerotic plaque. **(B)** Integrated backscatter intravascular ultrasound image of the lesion shown in **(A)**. **(C)** Image showing the CD31-stained adventitial vasa vasorum (VV; black arrows). **(D)** Method of calculating VV area density. I+M, intima+media.

fresh cadaveric hearts. The cross-sectional lumen area, cross-sectional vessel area within the external elastic membrane, and plaque area (external elastic membrane area minus lumen area) were calculated using software attached to the IVUS system. Plaque volume was calculated using integration. The distance from the ostium of the LAD to the proximal endpoint of the lesions was also measured.

The IB values for each histological category were defined by comparing histological images reported in a previous study.¹⁰ Plaque properties were classified into 4 types by combining spectral parameters of posterior scattering signals of IVUS: lipid pool, fibrosis, dense fibrosis, or calcification. The percentage area of each component was automatically measured in each plaque. The percentage lipid volume was calculated using integration. Lesions were divided into 3 groups (lipid-rich, lipid-moderate, and lipid-poor groups). The lipid-rich group was defined as having a percentage lipid volume greater than the mean value+0.5SD, whereas the lipid-poor group was defined as having a percentage lipid volume less than the mean value-0.5SD (**Figure 2**). The IVUS measurements were conducted independently by 2 physicians who were blinded to the cadaver's clinical characteristics.

Measurement of Inflammatory Molecules

After performing IB-IVUS, the vessels in which the coronary plaque burden was >25% and adjacent EAT for each lesion were trimmed from least 2 lesions from 1 LAD. The expression of inflammatory molecules (vascular endothelial growth factor A [*VEGFA*], *VEGFB*, *VEGFC*, C-C motif chemokine ligand 2 [*CCL2*], adiponectin [*ADIPOQ*], and glycerol-3-phosphate dehydrogenase [*G3PDH*]) was measured in each EAT. Total RNA was extracted using the illustra RNeasy spin RNA Isolation Kit (GE Healthcare). cDNA was synthesized from 100 ng total RNA extracted from tissues and cells using a QuantiTect Reverse Transcription kit (Qiagen). Real-time quantitative polymerase chain reaction (qPCR) was performed using an Mx3000P

(Agilent Technologies) and Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences used were as follows: *VEGFA*, 5'-AAGGAGGAGGGCA GAATCAT-3' (sense) and 5'-ATCTGCATGGTGATG TTGGA-3' (antisense); *VEGFB*, 5'-GGCTTAGAGCTC AACCCAGA-3' (sense) and 5'-GACAAGGGATGGCA GAAGAG-3' (antisense); *VEGFC*, 5'-AAAGAACCTG CCCCAGAAAT-3' (sense) and 5'-GAAAATCCTGGCT CACAAGC-3' (antisense); *CCL2*, 5'-CCCCAGTCACCT GCGTTAT-3' (sense) and 5'-AGATCTCCTTGGCCAC AATG-3' (antisense); *ADIPOQ*, 5'-GTGATGGCAGAG ATGGCAC-3' (sense) and 5'-ACACTGAATGCTGAGC GGTA-3' (antisense); *G3PDH*, 5'-TGGGTGTGAACCA TGAGAAG-3' (sense) and 5'-GCTAAGCAGTTGGTG GTGC-3' (antisense). Expression was normalized against that of *G3PDH* and is given in arbitrary units (AU).

Measurement of the Density of the Adventitial VV

Vessels trimmed from the LAD were cut into 5- to 7-mm slices and fixed in 10% neutralized formalin. Paraffin-embedded sections were cut into 5-μm slices. All sections were stained with hematoxylin-eosin. Immunohistochemical staining was performed using mouse anti-human CD31 antibody. Adventitial VV area density was calculated using the following formula (see **Figure 3**):

$$\text{Adventitial VV area density} = \text{VV area} / (\text{area of intima} + \text{media} + \text{adventitia})$$

Statistical Analysis

All data are expressed as the mean±SD. The significance of differences in each parameter between the lipid-rich and lipid-poor groups was determined using unpaired Student's t-test. Significance was set at 2-tailed $P < 0.05$. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (R Foundation for Statistical Computing, Vienna, Austria). More precisely,

Table 1. Baseline Characteristics of the Cadavers Used in the Present Study

Cadaver No.	Age, sex	Cause of death*
1	70, Female	Muscular dystrophy
2	80, Female	Caducity
3	96, Female	Pneumonia
4	70, Male	Amyloidosis
5	77, Male	Respiratory failure
6	95, Female	Caducity
7	69, Female	Multiple organ failure
8	74, Female	Brain cancer
9	93, Male	Chronic obstructive pulmonary disease
10	87, Female	Senile decay

*Causes of death are taken from death certificates.

EZR is a modified version of R commander designed to add statistical functions frequently used in biostatistics.¹²

Results

Baseline Characteristics

Ten fresh, frozen cadavers (5 male, 5 female) were used in this study. The mean age was 81.1 years (range 69–96 years). None of the cadavers had died of cardiovascular disease. The clinical characteristics and causes of death for all 10 cadavers are given in **Table 1**.

IVUS Measurements and Lesion Classification

Conventional and IB-IVUS parameters are given in **Table 2**. The 37 lesions were divided into 3 groups according to the percentage lipid volume assessed by IB-IVUS. The mean percent lipid volume was $32.7 \pm 6.0\%$. There were 11 lesions in each of the lipid-rich (percentage lipid volume $>35.7\%$) and lipid-poor (percentage lipid volume $<29.7\%$) groups (**Figure 2**). There were significant differences between these 2 groups in conventional and IB-IVUS parameters. Mean vessel area, mean lumen area, and plaque volume were larger in the lipid-rich than lipid-poor group, whereas percentage fibrous volume, percentage dense fibrous volume, and percentage calcified volume were higher in the lipid-poor than lipid-rich group.

Expression of Inflammatory Molecules

The expression of inflammatory molecules in adjacent EAT

is shown in **Figure 4**. Expression of *VEGFA* and *VEGFB* in adjacent EAT was higher for lipid-rich than in lipid-poor lesions (7.99 ± 3.37 vs. 0.45 ± 0.85 AU, respectively, for *VEGFA*; 0.27 ± 0.15 vs. 0.11 ± 0.07 AU, respectively, for *VEGFB*; $P < 0.05$). *CCL2* expression was slightly higher in lipid-rich lesions (0.50 ± 0.15 vs. 0.30 ± 0.26 AU; $P = 0.06$), whereas *ADIPOQ* expression was slightly higher in lipid-poor lesions (0.016 ± 0.012 vs. 0.009 ± 0.005 AU; $P = 0.08$).

Pathological VV Area Density

The density of adventitial VV was higher in lipid-rich than lipid-poor lesions (1.50 ± 0.58 vs. $0.88 \pm 0.23\%$; $P < 0.05$; **Figure 5**).

Discussion

Recent reports suggested that adipokines and inflammatory molecules secreted from the EAT significantly affect the myocardium and coronary arteries.¹³ The quality of the EAT, such as inflammatory status, determines cardiac and coronary vascular function.¹⁴ Several adipokines and inflammatory molecules secreted from the EAT can diffuse through interstitial fluid across the adventitia, media, and intima, and may interact with VV, endothelial cells, and vascular smooth muscle cells of the coronary vasculature, resulting in inflammation, endothelial and smooth muscle cell proliferation, atherogenesis, and destabilization of atherosclerotic plaques.^{5,6} However, little is known about the association between local inflammation in the EAT and the growth of coronary adventitial VV in vivo. In the present study we found that inflammatory molecules in adjacent EAT and the progression of VV are associated with the characteristics of coronary plaques.

Fresh Frozen Cadaveric Study

In this study we used fresh cadaveric hearts without formaldehyde fixation. The advantages of using fresh cadaveric hearts include the ability to measure the expression of inflammatory molecules under the same conditions as in living tissues and the absence of any adverse effects of formaldehyde on observers: formaldehyde is toxic, causes mucosal irritation, and respiratory damage, and is carcinogenic. In this study, the cadavers were safely and accurately observed in the laboratory.

Inflammation in Adjacent EAT

VEGF induces the migration and proliferation of endothelial cells, increases vascular permeability, and plays a role in tumor growth, adipose tissue expansion, age-related

Table 2. Conventional IVUS and Integrated Backscatter IVUS Parameters

	All (n=37)	Lipid-rich group (n=11)	Lipid-poor group (n=11)	P-value
Distance from the LAD ostium (mm)	34.0 ± 15.2	29.1 ± 13.9	39.1 ± 16.8	0.14
Mean vessel area (mm ²)	9.1 ± 1.3	11.0 ± 3.0	6.9 ± 3.0	0.005
Mean lumen area (mm ²)	4.4 ± 0.6	5.2 ± 1.2	3.6 ± 1.5	0.009
Plaque volume (mm ³)	18.8 ± 3.3	24.1 ± 8.8	13.5 ± 6.3	0.004
Fibrous volume (%)	55.1 ± 3.7	46.0 ± 8.9	62.0 ± 4.5	0.0003
Lipid volume (%)	32.7 ± 6.0	47.5 ± 11.8	19.2 ± 8.1	0.0002
Dense fibrous volume (%)	10.4 ± 2.8	5.4 ± 3.2	16.1 ± 8.3	0.0007
Calcified volume (%)	1.8 ± 0.7	1.0 ± 1.1	2.7 ± 2.1	0.03

LAD, left anterior descending artery; IVUS, intravascular ultrasound. Data are given as the mean \pm SD.

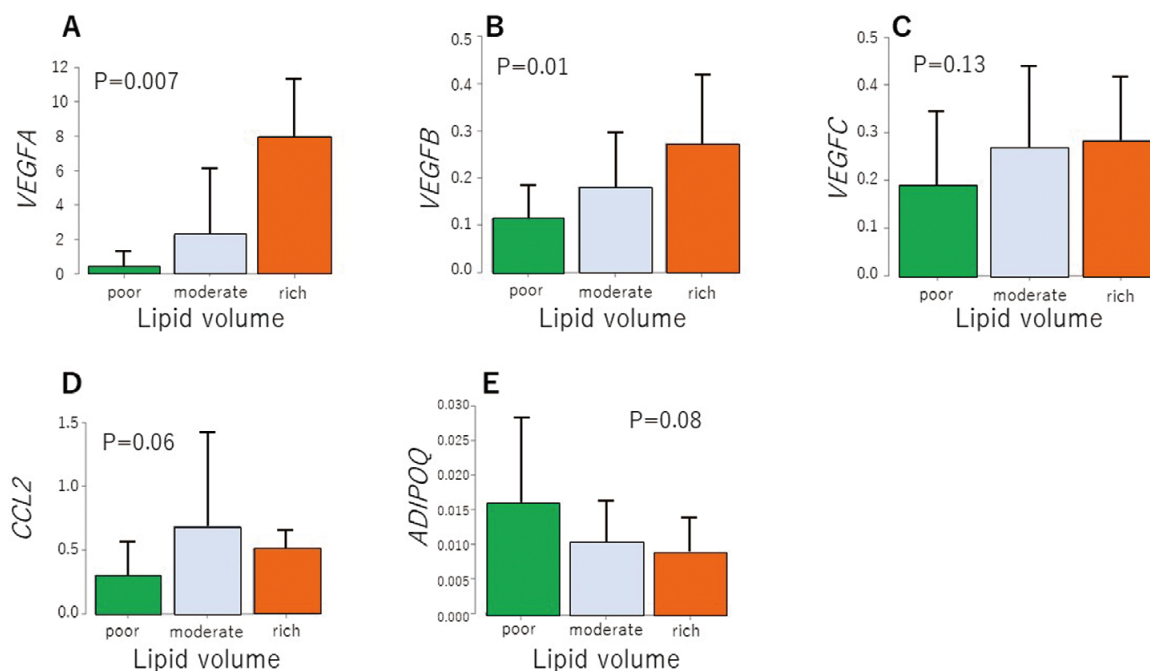


Figure 4. Expression of inflammatory molecules in adjacent epicardial adipose tissue (EAT). (A) Vascular endothelial growth factor A (VEGFA), (B) VEGFB, (C) VEGFC, (D) C-C motif chemokine ligand 2 (CCL2), and (E) adipocyte-specific protein (ADIPOQ) expression was compared among the 3 groups (lipid-rich, lipid-moderate, and lipid-poor groups). P-values are for comparisons between lipid-poor and lipid-rich lesions. There were no significant differences in the expression of inflammatory molecules in adjacent EAT between lipid-moderate lesions and other types of lesions.

macular degeneration, and diabetic retinopathy.^{15,16} CCL2 is expressed primarily by inflammatory and endothelial cells. Expression of CCL2 is upregulated by proinflammatory stimuli and tissue injury, which are associated with atherosclerotic lesions.^{17,18} A recent study reported that blood CCL2 concentrations were higher in patients with vulnerable coronary plaques.¹⁹ Previously, we reported on the relationship between CAD and EAT.^{6,7,20–22} In the present study the expression of VEGFs and CCL2 in focal adjacent EAT increased in the lipid-rich group, suggesting that the inflammation in focal adjacent EAT is associated with coronary plaque vulnerability. Conversely, ADIPOQ expression was slightly lower in lipid-rich coronary plaque lesions. Previous studies demonstrated a cardioprotective action of AdipoQ in vascular endothelial cells, smooth muscle cells, and cardiac myocytes.^{23–25} The findings of the present study suggest that AdipoQ inhibits the progression of vulnerable plaques.

Plaque Characterization Assessed by IVUS and Adventitial VV

In our conventional and IB-IVUS analyses, the large vessels had a higher lipid volume than small vessels. Previous studies reported that plaque in the proximal segment of the LAD has a significantly higher lipid content and lower fibrosis content than that in the distal segment.²⁶ One possible reason for this is destabilization of atherosclerotic plaques resulting from a change in composition caused by low shear stress.^{26,27} The present study suggests that another reason is related to the growth of adventitial VV.

Adventitial VV is associated with coronary plaque

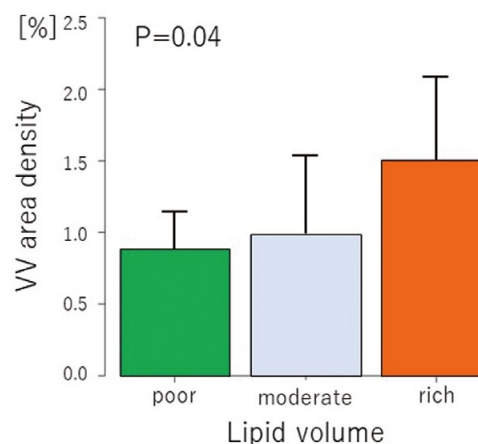


Figure 5. Association between the percentage lipid volume and pathological vasa vasorum (VV) area density in lesions in the lipid-rich, lipid-moderate, and lipid-poor groups. P-values are for comparisons between lipid-poor and lipid-rich lesions. There were no significant differences in VV area density between lipid-moderate lesions and other types of lesions.

progression and morphology.^{1–6,28} Adventitial focal VV formation coincides with early coronary atherosclerotic changes, such as focal coronary spasm.²⁹ In clinical settings, imaging devices, such as optical coherence tomography (OCT), are available to assess the neovessels of coronary

plaques. In this study, adventitial VV was observed during pathological examination. We found that the growth of adventitial VV was greater around lipid-rich coronary plaque lesions, and that the expression of inflammatory molecules in adjacent EAT was also higher in these lesions. These findings suggest that local inflammation in adjacent EAT affects plaque characteristics via the neovascularization of the VV.

Inward Progression Theory of Vascular Inflammation

Previous reports revealed an inward progression of vascular inflammation from the adventitia towards the media and intima.^{30–34} It was also suggested that prominent VV neovascularization in the adventitia occurs prior to the initiation of coronary lesion formation in apolipoprotein E-deficient mice.³⁵ In the present study, we demonstrated a significant relationship among lipid-rich coronary plaques, growth of adventitial VV, and higher expression of inflammatory molecules in adjacent EAT in fresh cadavers, supporting the important role of adventitial VV formation in the pathogenesis of atherosclerosis. We plan to assess the precise distribution of intraplaque neovessels and adventitial VV by OCT and pathological examination in order to clarify the mechanism of inward neovessel progression.

Clinical Implication

The findings of this study suggest that lipid-rich coronary lesions identified by IB-IVUS have inflammation in adjacent EAT and growth of adventitial VV. In clinical settings, it is important to follow the clinical course of such lipid-rich coronary lesions, even if they are non-stenotic. A previous study reported that the characteristics of plaques observed by IB-IVUS were consistent with the pathological findings of the coronary artery.³⁶ Recent IB-IVUS studies also report that lipid plaques of non-culprit lesions are associated with the atherosclerotic progression of CAD.³⁷ Our findings may explain one of the mechanisms underlying the clinical course and provide beneficial information about the future progression of CAD. Pathological examinations cannot be performed during an intervention in clinical settings. However, the findings of the present study suggest that IB-IVUS, which is readily available in clinical settings, can be used to examine inflammation in adjacent EAT and the characteristics of the VV. Thus, this method may provide useful information during interventions and in treatment planning.

Study Limitations

The present study has several limitations. First, the sample size was small because of the limited supply of fresh cadavers. In addition, the effects of repeated freeze and thaw cycles of cadavers on the qPCR results should not be discounted. We required freshly thawed cadavers to assay inflammatory molecules, restricting the sample size. We did not perform coronary angiography before using IVUS; therefore, it was difficult to introduce the guidewire into the LAD, and we sometimes removed the EAT to identify the route. Consequently, sampling sites were further restricted. Second, this study demonstrated an association among inflammatory molecules in EAT, the growth of VV, and coronary plaque vulnerability. However, it remains unclear whether the growth of VV directly affects the processing or healing of vulnerable plaques. It is necessary to accumulate clinical data, such as identification of VV using OCT, and observe plaque progression in order to clarify the mechanisms

involved. Third, we did not compare the pathological analyses and IB-IVUS findings. However, there was no significant pathological lipid core in our sampling sites of non-stenotic coronary arteries. Finally, we were unable to obtain clinical information for the cadavers other than the direct cause of death. Therefore, we cannot exclude the possibility that they underwent anti-inflammatory and/or immunosuppressive treatments that may have affected systemic inflammation.

Conclusions

In fresh cadavers, lipid-rich coronary plaques are associated with adventitial VV and local inflammation in adjacent EAT. This study suggests that local inflammation of the EAT is associated with coronary plaque progression via the VV.

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Conflict of Interest

None declared.

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