Coronary Atherosclerosis Is Associated With Macrophage Polarization in Epicardial Adipose Tissue

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Objectives

The purpose of this report was to assess the link between macrophage polarization in epicardial adipose tissue and atherosclerosis in patients with coronary artery disease (CAD).

Background

Macrophage accumulation enhances chronic inflammation in adipose tissue, but macrophage phenotypic change in human epicardial adipose tissue and its role in atherogenesis are unknown.

Methods

Samples were obtained from epicardial and subcutaneous adipose tissue during elective cardiac surgery (CAD, n = 38; non-CAD, n = 40). Infiltration of M1/M2 macrophages was investigated by immunohistochemical staining with antibodies against CD11c and CD206, respectively. Expression of pro- and anti-inflammatory adipocytokines in adipose tissue was evaluated by real-time quantitative polymerase chain reaction.

Results

Infiltration of macrophages and expression of pro- and anti-inflammatory cytokines were enhanced in epicardial fat of patients with CAD compared with that in non-CAD patients (p < 0.05). The ratio of M1/M2 macrophages was positively correlated with the severity of CAD (r = 0.312, p = 0.039). Furthermore, the expression of pro-inflammatory cytokines was positively correlated, and the expression of anti-inflammatory cytokines was negatively correlated with the ratio of M1/M2 macrophages in epicardial adipose tissue of CAD patients. By contrast, there was no significant difference in macrophage infiltration and cytokine expression in subcutaneous adipose tissue between the CAD and non-CAD groups.

Conclusions

The ratio of M1/M2 macrophages in epicardial adipose tissue of CAD patients is changed compared with that in non-CAD patients. Human coronary atherosclerosis is associated with macrophage polarization in epicardial adipose tissue.

Accumulating evidence suggests that adipose tissue not only stores energy but also secretes various bioactive substances called adipocytokines. It was reported that serum levels of pro-inflammatory cytokines are increased in overweight people with enhanced accumulation of visceral fat. Dys-regulated secretion of adipocytokines is assumed to be critically involved in the pathogenesis of obesity-associated diseases (1).

The adventitial and peri-adventitial tissues are composed of various cell types, including adipocytes, vascular cells, macrophages, T cells, mast cells, and fibroblasts (2–4). It was demonstrated that peri-adventitial adipose tissue releases a transferable adventitia-derived relaxing factor that acts by tyrosine kinase-dependent activation of potassium channels in vascular smooth muscle cells (5). Recent reports showed that peri-adventitial fat secretes various chemokines and might contribute to the progression of obesity-associated atherosclerosis (6). Epicardial adipose tissue (EAT) is located in close proximity to coronary arteries. Epicardial adipose tissue has been reported to be a source of inflammatory mediators (7). It was also reported that EAT...
thickening is an indicator of cardiovascular risk (8). Chatterjee et al. (9) reported that perivascular adipocytes exhibit reduced differentiation and a pro-inflammatory state, suggesting that dysfunction of perivascular adipose tissue induced by fat feeding might link metabolic signals to inflammation in the blood vessel wall. These results suggest that perivascular fat, particularly EAT, might play a role in increased risk of cardiovascular disease in obese individuals. However, the physiological and/or pathological role of perivascular adipose tissue in the maintenance of vascular homeostasis and in pathological vascular remodeling remains to be elucidated.

Recent studies suggest that adipose tissue macrophages play a pivotal role in the establishment of the chronic inflammatory state associated with obesity-linked diseases, such as type 2 diabetes and atherosclerosis (10). Furthermore, adipose tissue macrophages can exist in different activation states: either pro-inflammatory classically activated by interferon-gamma or lipopolysaccharide, known as M1; or anti-inflammatory alternatively activated by interleukin (IL)-13 or IL-4, known as M2. The M1- and M2-activated macrophages fulfill different functions through production of pro- or anti-inflammatory factors (11), depending on microenvironmental stimuli. The M1 macrophages are potent effector cells that kill microorganisms and produce primarily pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-6, and macrophage chemotactic protein (MCP)-1 (12,13). In contrast, M2 macrophages dampen these inflammatory and adaptive Th1 responses by producing anti-inflammatory factors—such as IL-10, transforming growth factor-β, and alternative macrophage activation-associated CC chemokine (AMAC)-1—scavenging debris, and promoting angiogenesis, tissue remodeling, and repair (12,13). Thus, inflammatory diseases such as atherosclerosis might be caused not only by a sustained pro-inflammatory reaction but also by failure of anti-inflammatory regulatory mechanisms.

Recently, Lumeng et al. (14) used CD11c as an M1 marker in flow cytometric analysis and reported that diet-induced obesity leads to a shift in the activation state of adipose tissue macrophages from an M2-polarized state to an M1 pro-inflammatory state that contributes to insulin resistance. Bourlier et al. (15) reported that macrophages in human subcutaneous fat that accumulated with fat mass development exhibit a particular M2 phenotype, with CD206 as a M2-macrophage marker. However, the relationship between M1/M2 macrophage polarization in adipose tissue, especially in EAT, and vascular remodeling remains to be elucidated.

To further characterize the phenotype of macrophages in EAT, we evaluated cell surface markers and cytokine expression in epicardial and subcutaneous adipose tissue (SCAT) obtained during cardiac surgery from patients with or without coronary artery disease (CAD). Our findings suggested that pro-inflammatory M1 macrophages are increased and anti-inflammatory M2 macrophages are decreased in EAT in patients with CAD.

### Methods

#### Study population.

The protocol of this study was approved by the institutional review boards of the University of Tokushima Hospital and Sakakibara Heart Institute. Samples were obtained from 38 patients who underwent elective coronary artery bypass graft surgery (CAD group) and 40 patients who underwent surgery for aortic or mitral valve replacement (non-CAD group). Written informed consent was obtained from each patient. The clinical characteristics of patients and laboratory measurements are summarized in Tables 1 and 2. At the beginning of the surgical intervention, EAT was taken from the anterior wall of the left ventricle. Subcutaneous adipose tissue was taken from the subcutaneous fat on the sternum.

#### Immunohistochemical staining.

Each adipose tissue sample was cut into 2 pieces. One-half of the sample was fixed in formalin and embedded in paraffin. Antigen retrieval was performed in Tris-ethylenediaminetetraacetic acid buffer (pH 9.0) with a microwave oven for 10 min. Immunohistochemical staining of 5-μm-thick thin sections was performed with primary antibodies against CD11c (1:100, clone EP1347Y, Abcam, Cambridge, Massachusetts), CD206 (1:100, clone KP-1, Dako, Glostrup, Denmark) followed by

### Table 1 Patient Characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>CAD Group (n = 38)</th>
<th>Non-CAD Group (n = 40)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>68.3 ± 10.9</td>
<td>65.4 ± 12.1</td>
<td>0.2632</td>
</tr>
<tr>
<td>Male</td>
<td>32 (84.2)</td>
<td>21 (52.5)</td>
<td>0.0035</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.8 ± 2.48</td>
<td>22.9 ± 3.02</td>
<td>0.54</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>84.9 ± 9.8</td>
<td>83.4 ± 10.1</td>
<td>0.54</td>
</tr>
<tr>
<td>BP (systole: mm Hg)</td>
<td>127.6 ± 19.5</td>
<td>124.3 ± 19.0</td>
<td>0.44</td>
</tr>
<tr>
<td>BP (diastole: mm Hg)</td>
<td>67.4 ± 12.7</td>
<td>65.6 ± 14.2</td>
<td>0.56</td>
</tr>
<tr>
<td>CAD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-vessel disease</td>
<td>1 (2.6)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>2-vessel disease</td>
<td>8 (21.1)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>3-vessel disease</td>
<td>29 (76.3)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Complications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>30 (78.9)</td>
<td>26 (65.0)</td>
<td>0.21</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>28 (73.7)</td>
<td>16 (40.0)</td>
<td>0.0033</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>17 (44.7)</td>
<td>5 (12.5)</td>
<td>0.0023</td>
</tr>
<tr>
<td>Medication</td>
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<tr>
<td>β-blocker</td>
<td>23 (60.5)</td>
<td>10 (25.0)</td>
<td>0.0026</td>
</tr>
<tr>
<td>ACEI</td>
<td>7 (18.4)</td>
<td>8 (20.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>ARB</td>
<td>24 (63.2)</td>
<td>18 (45.0)</td>
<td>0.12</td>
</tr>
<tr>
<td>Statin</td>
<td>26 (68.4)</td>
<td>13 (32.5)</td>
<td>0.0030</td>
</tr>
</tbody>
</table>

Values are mean ± SD or n (%).

ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker; BMI = body mass index; BP = blood pressure; CAD = coronary artery disease.
incubation with an avidin-biotin-blocking system (Dako, code X0590) and secondary antibody and peroxidase-labeled avidin-biotin complex system (LSAB + System-HRP; Dako). Localization of the primary antibody was visualized with 3,3′-diaminobenzidine and counter-stained with hematoxylin. The number of macrophages was counted within a circle of 200 μm diameter in 3 random fields with the FLOVEL Filing System (FLOVEL Company, Ltd., Tokyo, Japan) in a double-blind fashion.

Ribonucleic acid isolation and quantification. Ribonucleic acid was extracted from one-half of the adipose tissue sample with an RNAeasy Lipid Tissue Mini kit (QIAGEN, Valencia, California). Expression of adipokines was evaluated by real-time quantitative polymerase chain reaction with a TaqMan Gold RT-PCR kit and PRISM 7500 Sequence Detection System (Applied Biosystems, Carlsbad, California). Primers were purchased from Takara Bio, Inc. (Kyoto, Japan). The following primers were used: 5′-AAGCCAGAGCTGTGAGATGAGTA-3′ and 5′-TGTCTCGAGCACCACCTGTTC-3′ for IL-6, 5′-GACAGCTTGAGCCCATGTGTA-3′ and 5′-CAGCTTGGCCTTGAGAG-3′ for TNF-α, 5′-CCCTCAGGCATTCAGCTTCA-3′ and 5′-GGCTTGGCAACCAGGTAAC-3′ for IL-10, 5′-AAAGACTCCTGCTGCTGTCTA-3′ and 5′-CCCTCAGGCACTGCTTCA-3′ for AMAC1, and 5′-GCTTGGCAGAGGCACTCTTCA-3′ and 5′-GCCCTGAGGCATTCAGCTTCA-3′ for CD68. Data were analyzed with the ΔΔCT method. Relative gene expression was normalized to β-actin level.

Gensini score. To assess the severity of CAD, we used the Gensini scoring system (16). Coronary artery score equals the sum of all segment scores (where each segment score equals segment weighting factor multiplied by severity score). Severity scores assigned to the specific percentage luminal diameter reduction of the coronary artery segment are 32 for 100%, 16 for 99%, 8 for 90%, 4 for 75%, 2 for 50%, and 1 for 25%.

Statistical analysis. All results are expressed as mean ± SD. We compared the valuables between groups by 2-way analysis of variance, and correlations were assessed with Spearman’s rank correlation for non-normally distributed data (SPSS [version 14.0, SPSS, Chicago, Illinois]; IBM [Tokyo, Japan]; and Prism 5, GraphPad Software [La Jolla, California]). With regard to patient characteristics and laboratory measurements, we compared the groups with Student t tests and chi-square tests. Differences were considered significant when p < 0.05.

Table 2 Laboratory Measurements

<table>
<thead>
<tr>
<th></th>
<th>CAD Group</th>
<th>Non-CAD Group</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mg/dl)</td>
<td>163.0 ± 89.9</td>
<td>128.1 ± 70.2</td>
<td>0.039</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>91.7 ± 24.3</td>
<td>113.2 ± 28.7</td>
<td>0.0018</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>43.6 ± 13.3</td>
<td>57.8 ± 16.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL/HDL ratio (mg/dl)</td>
<td>2.24 ± 0.73</td>
<td>2.11 ± 0.82</td>
<td>0.47</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.14 ± 1.03</td>
<td>5.63 ± 0.71</td>
<td>0.013</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>0.34 ± 0.79</td>
<td>0.24 ± 0.42</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Values are means ± SD.

CRP = C-reactive protein; HbA1c = hemoglobin A1c; HDL = high-density lipoprotein cholesterol; LDL = low-density lipoprotein cholesterol; TG = triglyceride.

Patient characteristics. Patient characteristics are summarized in Table 1. There was no significant difference in age, body mass index, waist circumference, and blood pressure between the CAD group and the non-CAD group. The majority of patients in the CAD group were male (84.2%) and had multivessel CAD (97.4%) with a high prevalence of dyslipidemia (73.7%) and diabetes mellitus (44.7%). Table 2 summarizes the laboratory values for serum lipid profile and inflammatory biomarkers. In the lipid profile, low-density and high-density lipoprotein cholesterol levels were significantly lower in the CAD group than in the non-CAD group (p < 0.001, respectively), presumably due to the higher morbidity of dyslipidemia and statin treatment in the CAD group. Hemoglobin A1c was also significantly higher in the CAD group. As for medication, β-blockers and statins were more frequently administered in the CAD group than in the non-CAD group.

Macrophage infiltration in EAT and SCAT. The number of infiltrating macrophages in EAT as determined by anti-CD68 immunostaining was significantly larger in the CAD group than in the non-CAD group. Interestingly, the number of not only M1 macrophages but also M2 macrophages was significantly larger in EAT in the CAD group, as determined by immunohistochemical staining against CD11c and CD206, respectively (Fig. 1). A double-immunofluorescent study against CD11c and CD68 revealed that all of the CD11c-positive cells or CD206-positive cells were also positive for CD68 (Online Fig. 1). In other words, there was no CD11c(+)CD68(−) cell or CD206(+)CD68(−) cell in adipose tissues of our study. These results support our definition that CD11c acts as a marker of M1 macrophages and CD206 acts as a marker for M2 macrophages. The ratio of CD11c/CD68-positive cells was significantly increased, and the ratio of CD206/CD68-positive cells was significantly decreased in EAT in the CAD group (Fig. 2A). By contrast, there was no significant difference in the number of macrophages in SCAT between the 2 groups. These data indicate that M1 macrophages were relatively increased and M2 macrophages were relatively decreased in EAT in the CAD group. Then we assessed the ratio of CD11c/CD206-positive cells, as a marker of macrophage polarization, in the 2 groups. Intriguingly, the CD11c/CD206 ratio was significantly higher in EAT in the CAD group compared with that in the
non-CAD group, which might reflect a “shift” of macrophages from a resting state toward an inflammatory state (Fig. 2B).

Next, to evaluate the relation between macrophage polarization in EAT and the severity of CAD, we investigated the correlation between Gensini score and the number of infiltrating cells in EAT of CAD patients (Fig. 2C). The ratio of CD11c/CD206-positive cells was positively correlated with the Gensini score ($r = 0.312$, $p = 0.035$). These data suggest that macrophage infiltration in EAT is not a simple indicator of the inflammatory state in EAT and that the ratio of M1/M2 macrophages in EAT might be associated with enhanced coronary artery atherosclerosis.

**Expression of M1/M2 cytokines in EAT and SCAT.** We evaluated messenger ribonucleic acid expression of adipocytokines in EAT and SCAT. The IL-6, TNF-$\alpha$, and MCP-1, pro-inflammatory cytokines secreted by M1-macrophages, were upregulated in EAT in the CAD group compared with those in the non-CAD group (Fig. 3A). These differences were not observed between the 2 groups in SCAT. Furthermore, IL-10 and AMAC1, anti-inflammatory cytokines secreted by M2-macrophages, were also upregulated in EAT in the CAD group compared with those in the non-CAD group (Fig. 3B). These data indicate that both pro- and anti-inflammatory cytokines in EAT were relatively upregulated in the CAD group compared with those in the non-CAD group.

**Relationship between expression of M1/M2 cytokines and polarization of infiltrating macrophages.** To examine the relationship between the expression of cytokines and the polarization of infiltrating macrophages, we investigated the correlation between the number of infiltrating macrophages in EAT in the CAD group and the expression of cytokines. First, expression levels of IL-6, TNF-$\alpha$, and MCP-1 were positively correlated with the ratio of CD11c/CD206-positive cells (Fig. 4A). Moreover, expression levels of IL-10 and AMAC1 were negatively correlated with the ratio of CD11c/CD206-positive cells (Fig. 4B). These data suggest that the polarization of infiltrating macrophages influences the expression of pro-and anti-inflammatory cytokines in the EAT of CAD patients.

Pro-inflammatory cytokine levels had positive correlation with Gensini score, and anti-inflammatory cytokines had negative correlation with Gensini score (Online Fig. 2). It is assumed that there are many inherent confounding factors that influence macrophage polarization, cytokine profile, and CAD severity. Adiponectin expression in EAT of the CAD group was relatively low compared with that in EAT.
of the non-CAD group (Online Fig. 3A). The difference was not statistically significant. Adiponectin expression in SCAT of CAD patients was also decreased compared with that in SCAT of non-CAD patients. In other words, the lower adiponectin expression in CAD patients was not specific to epicardial adipose tissue. Adiponectin expression in EAT seemed to be negatively correlated with the M1/M2 ratio in CAD patients, but the correlation was not statistically significant (Online Fig. 3B).

Discussion

In this study, we found that infiltration of macrophages and expression of pro- and anti-inflammatory cytokines were enhanced in the epicardial fat of patients with CAD, compared with those in non-CAD patients. Furthermore, the expression of pro-inflammatory cytokines was positively correlated and the expression of anti-inflammatory cytokines was negatively correlated with the ratio of M1/M2 macrophages in EAT in the CAD group. By contrast, there was no significant difference in macrophage infiltration and cytokine expression in SCAT between the CAD and non-CAD groups.

Atherosclerosis has been recognized to be a chronic inflammatory disease (17–22). Most of the previous studies focused on inflammatory changes in the vessel wall. However, few studies have investigated the relationship between atherosclerosis and perivascular adipose tissue inflammation. The human coronary arteries are surrounded by abundant EAT. In contrast to visceral fat, little attention has been paid to the role of EAT in the pathogenesis of CAD. Payne et al. (23) reported that EAT impaired endothelial-dependent vasodilation to bradykinin in swine with metabolic syndrome but not in lean animals. Greif et al. (24) reported that fat depots localized around the heart are highly variable and that an elevated pericardial adipose tissue volume was associated with coronary atherosclerosis. It was also reported that epicardial fat in patients who are candidates for coronary artery bypass graft surgery seems to be more inflammatory than the subcutaneous fat located in the legs of the same patients (7). However, to confirm the hypothesis that an inflammatory state of epicardial fat could
lead to aggravation of vascular inflammation and coronary atherosclerosis, EAT in CAD patients should be compared with that in non-CAD patients.

Here, we characterized the infiltrating macrophages in EAT of patients with or without CAD. It is reported that the number of macrophages infiltrating into adipose tissue is increased in obesity (25). Recent reports suggest that a vicious cycle between infiltrating macrophages and adipocytes augments the inflammatory response in adipose tissue in obesity (26). Adipose tissue macrophages consist of at least 2 different phenotypes; classically activated M1 macrophages and alternatively activated M2 macrophages. Lumeng et al. (14) reported that diet-induced obesity leads to a “shift” in the activation state of adipose tissue macrophages from an M2-polarized state to an M1 pro-inflammatory state, contributing to insulin resistance. Furthermore, it was reported that adiponectin, a major adipocytokine, functions as a regulator of macrophage polarization (27). Conditions of high adiponectin expression might deter metabolic and cardiovascular disease progression by favoring an anti-inflammatory phenotype in macrophages (28). The most important finding in this study is that the ratio of M1/M2 macrophages was positively correlated with the severity of CAD (Fig. 2B). This finding suggests that the most important factor for the inflammatory state of EAT could be not the number of infiltrating macrophages but the macrophage polarity in EAT. The present report showed a phenotype switch of M1/M2 macrophages in epicardial fat of CAD patients, which might potentially influence the inflammatory status of EAT and the pathogenesis of coronary atherosclerosis.

**Study limitations.** Our sample size is small, and patient selection bias is likely present. This study is based on 2 methodologies: counting of immunohistochemically stained macrophages in the adipose tissue, and the use of real-time quantitative polymerase chain reaction to quantitate the expression of various cytokines. We acknowledge that flow cytometry is more suitable for the evaluation of macrophage infiltration in EAT. Although we tried the isolation of macrophages from adipose tissue with collagenase in the preliminary study, we could not obtain enough adipose tissue to perform flow cytometry. On the basis of the large number of previous studies (14,25,29), we believe that immunohistochemical study enables us to estimate macro-

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**Figure 3 mRNA Expression of M1/M2 Cytokines in Adipose Tissue**

(A) Expression of messenger ribonucleic acid (mRNA) of pro-inflammatory (M1 type) cytokines in EAT and SCAT. Each point represents the expression level assessed by real-time quantitative polymerase chain reaction. Expression level was normalized to each β-actin level. (B) Expression of mRNA of anti-inflammatory (M2 type) cytokines in EAT and SCAT. Bar indicates mean. *p < 0.05. AMAC = alternative macrophage activation-associated CC chemokine; IL = interleukin; MCP = monocyte chemotactic protein; TNF = tumor necrosis factor; other abbreviations as in Figure 1.
phage polarization in adipose tissue sufficiently. Another limitation of this study is that the expression of cytokines was evaluated on “whole tissue biopsy,” and thus it cannot be assumed that the findings represent only the production by inflammatory cells. Moreover, it is difficult to speculate that the inflammatory response in the epicardial fat is the cause or result of coronary atherosclerosis.

Conclusions

We found that infiltration of macrophages in epicardial adipose tissue was enhanced in epicardial adipose tissue of patients with CAD. The polarization of M1/M2 macrophages in epicardial adipose tissue was shifted toward a pro-inflammatory state in the CAD group. These results suggest that macrophage polarization might influence atherogenesis in human coronary arteries.

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REFERENCES


Key Words: atherosclerosis • biopsy • coronary disease • immune system • inflammation.

APPENDIX

For supplementary figures, please see the online version of this article.