Enhanced Inflammation in Epicardial Fat in Patients With Coronary Artery Disease

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SUMMARY

It has been hypothesized that epicardial fat, a local visceral fat depot with close proximity to coronary arteries, may serve as a source of inflammatory cytokines and cells in coronary atherosclerotic lesions. Here, we characterized infiltration of inflammatory cells and expression of adipocytokines in epicardial adipose tissue in patients with and without coronary artery disease (CAD). Pare samples were obtained from epicardial and subcutaneous adipose tissue during elective cardiac surgery (CAD, $n = 8$; non-CAD, $n = 9$). Inflammatory cell infiltration was investigated by immunohistochemical staining using antibodies against CD3, CD4, CD8 and CD68. Expression of adipocytokines was evaluated by real-time quantitative reverse transcription-polymerase chain reaction. Infiltration of macrophages and CD8-positive T cells in the epicardial adipose tissue in the CAD group was greater than that in the non-CAD group. In contrast, there was no significant difference between the two groups in the number of inflammatory cells in subcutaneous adipose tissue. No statistical difference could be found between the CAD group and the non-CAD group in the expression levels of adiponectin and inflammatory cytokines in epicardial adipose tissue. Our findings suggest that inflammatory cell infiltration is enhanced in epicardial adipose tissue, but not in subcutaneous fat, in patients with coronary artery disease. Chronic inflammation in epicardial fat may influence the pathogenesis of coronary atherosclerosis. (Int Heart J 2011; 52: 139-142)

Key words: Atherosclerosis, Adipose tissue, Inflammation

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 proinflammatory cytokines are increased in overweight people with enhanced accumulation of visceral fat. Dysregulated secretion of adipocytokines is assumed to be critically involved in the pathogenesis of obesity-associated diseases.

Adventitial and periadventitial tissue are composed of various cell types including adipocytes, vascular cells, macrophages, T cells, mast cells, and fibroblasts.^{1,2)} It was demonstrated that perivascular adventitial adipose tissue releases a transferable adventitia-derived relaxing factor that acts by tyrosine kinase-dependent activation of K^+ channels in vascular smooth muscle cells.³⁾ Recent reports showed that periadventitial fat secretes various chemokines that might contribute to the progression of obesity-associated atherosclerosis.⁴⁾ Epicardial adipose tissue (EAT) is located in close proximity to coronary arteries. EAT has been reported to be a source of inflammatory mediators.⁵⁾ It was reported that epicardial adipose tissue thickness is an indicator of cardiovascular risk.⁶⁾ Chatterjee, *et al* re-

ported that perivascular adipocytes exhibit reduced differentiation and a proinflammatory state, suggesting that dysfunction of perivascular adipose tissue induced by fat feeding may link metabolic signals to inflammation in the blood vessel wall.⁷⁾ These results suggest that perivascular fat, particularly epicardial adipose tissue, may play a role in the 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.³⁾

Here, we evaluated inflammatory cell infiltration and cytokine expression in epicardial and subcutaneous adipose tissue obtained during cardiac surgery from patients with and without coronary atherosclerosis. The results suggested that inflammatory cell infiltration is enhanced in epicardial adipose tissue in patients with coronary artery disease.

METHODS

Patients: The protocol of this study was approved by the insti-

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tutional review boards of The University of Tokushima Hospital, Tokushima Prefectural Central Hospital, and Ehime Prefectural Central Hospital. Data were obtained from 8 patients who underwent elective coronary artery bypass graft surgery (CAD group) and 9 patients who underwent surgery for aortic or mitral valve replacement (non-CAD group). Written informed consent was obtained from each subject. The clinical characteristics of the patients are summarized in Table I. At the start of the surgical intervention, EAT was taken from the anterior wall of the left ventricle. Subcutaneous adipose tissue (SCAT) was taken from the subcutaneous fat on the sternum.

Immunohistochemical staining: Each adipose tissue sample was cut into two pieces. Half of the sample was fixed in formalin and embedded in paraffin. Immunohistochemical staining was performed on 5- μ m-thick thin sections using primary antibodies against CD4 (clone IF6, Nichirei, Japan), CD8 (clone C8/144B, Nichirei), and CD68 (clone KP-1, DAKO), followed by incubation with secondary antibody, an avidin-biotin-blocking system (DAKO, code X0590), and a peroxidase-labeled avidin-biotin complex system (LSAB+ System-HRP; DAKO). Localization of the primary antibody was visualized with 3, 3'-diaminobenzidine (DAB), with hematoxylin counter-staining. A lymph node biopsy sample taken at The Tokushima University Hospital was used as a positive control of immunostaining for inflammatory cells. The number of macrophages and T cells was counted within a circle of 200 μ m diameter in three random fields, using a FLOVEL Filing System (Flovel Co., Ltd.).

RNA isolation and quantification: RNA was extracted from half of the adipose tissue sample using an RNeasy Lipid Tissue Mini kit (QIAGEN). Expression of adipocytokines was evaluated by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) with a TaqMan Gold RT-PCR kit and PRISM 7500 Sequence Detection System (Applied Biosystems). Primers were purchased from Takara Bio Inc. (Kyoto, Japan). The following primers were used: 5'-TGGCTATGCTCACAGTCTCACATC-3' and 5'-CTCTGTGCCTCTGGTTCACAA-3' for adiponectin, 5'-AAGCCAGAGCTGTGCGATGAGTA-3' and 5'-TGTCCTGCAGCCACTGGTTC-3' for IL-6, 5'-GACAAGCCTGTAGCCCCATGTTGTA-3' and 5'-CAGCCTTGGCCCTTGAAGA-3' for TNF- α , 5'-CGCTCAGCCAGATGCAATCA-3' and 5'-TGTGGCCAAGGAGATCTGTG-3' for MCP-1, and 5'-GCCCTGAGGCACTCTTCCA-3' and 5'-GCGGATGTCCACGTCACA-3' for β actin. We analyzed the data using the $\Delta\Delta$ CT method. Relative gene expression was normalized to β -actin level.

Statistics: All results are expressed as the mean $\pm$ SEM. Differences between the groups were evaluated for statistical significance using Student's *t* test and one-way ANOVA method. Values of *P* < 0.05 were considered significant.

RESULTS

Patient characteristics: Patient characteristics are summarized in Table I. There were no significant differences between the CAD group and the non-CAD group in age, body mass index, and lipid profile except for the serum level of HDL-cholesterol. HbA1c level was significantly higher in the CAD group. There was no significant difference in medication between the two groups.

Macrophage and T cell infiltration in EAT and SCAT: The number of infiltrating macrophages in EAT in the CAD group was significantly larger than that in the non-CAD group as determined by anti-CD68 immunostaining (Figure 1). The number of CD8-positive T cells in EAT was significantly larger in the CAD group (Figure 2). On the other hand, there was no significant difference between the two groups in the number of macrophages and T cells in SCAT. CD4-positive cells were not detected in EAT and SCAT in either group.

Figure 1. Increased macrophage infiltration in EAT and SCAT. Immunohistochemical staining for macrophages (CD68) in epicardial adipose tissue (EAT) from coronary artery disease (CAD) and non-CAD patients. Bar, 100 μ m. The number of macrophages in EAT and subcutaneous adipose tissue (SCAT) is expressed as mean $\pm$ SEM. * indicates *P* < 0.05.

Table I. Clinical Characteristics of the Patients

	Non-CAD group (n = 9)	CAD group (n = 8)	<i>P</i>
Age (years)	63.4 $\pm$ 4.9	65.3 $\pm$ 2.9	NS
Male (%)	2 (22.2)	4 (50.0)	NS
Body mass index (kg/m ²)	24.2 $\pm$ 1.3	21.1 $\pm$ 1.3	NS
TG (mg/dL)	109.5 $\pm$ 17.4	118.4 $\pm$ 16.0	NS
LDL-cholesterol (mg/dL)	124.0 $\pm$ 11.7	99.6 $\pm$ 13.8	NS
HDL-cholesterol (mg/dL)	51.9 $\pm$ 4.6	34.0 $\pm$ 4.0	< 0.01
HbA1c (%)	5.7 $\pm$ 0.3	6.9 $\pm$ 0.4	< 0.05
Medication (%)			
β -Blocker	4 (44.4)	2 (25.0)	NS
CCB	1 (11.1)	2 (25.0)	NS
Statin	2 (22.2)	2 (25.0)	NS
ACEI/ARB	2 (22.2)	4 (50.0)	NS
Diuretic	4 (44.4)	2 (25.0)	NS
Anti-diabetes	1 (11.1)	3 (37.5)	NS

CCB indicates calcium channel blocker; ACEI, angiotensin converting enzyme inhibitor; and ARB, angiotensin receptor blocker. Values are mean $\pm$ SEM or n (%). * indicates *P* < 0.05.

Figure 2. Increased CD8-positive T cell infiltration in EAT of CAD patients. Immunohistochemical staining was performed for CD4 and CD8 in lymph node (positive control) and EAT. Bar, 100 μ m. The number of CD8-positive T cells in EAT and subcutaneous adipose tissue (SCAT) is expressed as mean $\pm$ SEM. * indicates $P < 0.05$.

Adipocytokine expression in EAT and SCAT: We evaluated mRNA expression of adipocytokines in EAT and SCAT (Table II). Adiponectin expression in EAT was slightly down-regulated in the CAD group compared to that in the non-CAD group. The inflammatory cytokines IL-6, TNF- α and MCP-1 in EAT were slightly up-regulated in the CAD group compared with those in the non-CAD group, however, the differences were not statistically significant. There was no difference between the CAD group and the non-CAD group in adipokine expression in SCAT.

DISCUSSION

In this study, we found that infiltration of macrophages and T cells was enhanced in the epicardial fat of patients with coronary artery disease, when compared with that of non-CAD patients. Increased inflammatory cell infiltration was associated with a tendency for down-regulation of adiponectin and up-regulation of inflammatory cytokines. On the other hand, there was no significant difference between the CAD and non-CAD groups in terms of inflammatory cell infiltration and adipocytokine expression in subcutaneous fat.

Atherosclerosis has been recognized as a chronic inflammatory disease.⁸⁻¹¹ Most previous studies focused on inflammatory changes in the vessel wall. On the other hand, fewer studies investigated the relationship between atherosclerosis and perivascular adipose tissue inflammation. The human cor-

Table II. Comparison of Adipocytokine Expression Between the Non-CAD and CAD Patients in Epicardial and Subcutaneous Adipose Tissue

	Epicardial fat			Subcutaneous fat		
	Non-CAD	CAD		Non-CAD	CAD	
Adiponectin	0.86 ± 0.12	0.60 ± 0.12	NS	0.98 ± 0.18	0.88 ± 0.16	NS
IL-6	1.78 ± 0.76	2.24 ± 1.27	NS	0.82 ± 0.38	1.10 ± 0.96	NS
TNF- α	1.26 ± 0.43	2.33 ± 0.82	NS	1.12 ± 0.18	1.36 ± 0.29	NS
MCP-1	2.17 ± 0.65	3.50 ± 1.37	NS	1.57 ± 0.68	1.04 ± 0.0	NS

β -Actin was used as an internal control. Values represent the ratio of cytokine expression to β -Actin expression in arbitrary units.

onary arteries are surrounded by abundant epicardial adipose tissue (EAT). In contrast to visceral fat, little attention has been paid to the role of EAT in the pathogenesis of coronary artery disease. Recently, Greif, *et al* reported that fat depots localized around the heart are highly variable, and that an elevated pericardial adipose tissue volume was associated with coronary atherosclerosis.¹² It was reported that epicardial fat in obese patients who are candidates for coronary artery bypass graft appears to be more inflammatory than the subcutaneous fat located in the legs of the same patients.⁵ To confirm the hypothesis that the inflammatory state of epicardial fat could lead to aggravation of vascular inflammation and coronary atherosclerosis, epicardial adipose tissue in CAD patients should be compared with that in non-CAD patients.

Here, we characterized EAT in patients with CAD. Inflammatory cell infiltration was enhanced in EAT of CAD patients. It was reported that adipose tissue macrophage number is increased in obesity and participates in inflammatory pathways that are activated in adipose tissue of obese individuals.¹³ Recent reports suggest that a vicious cycle between infiltrating macrophages and adipocytes augments the inflammatory response in adipose tissue in obesity.¹⁴ Our data suggest that enhanced infiltration of inflammatory cells into EAT may account for inflammatory cytokine expression in EAT of CAD patients.

Obesity generates a proinflammatory environment in adipose tissue, but the factors that initiate this inflammatory cascade are unclear. Nishimura, *et al* reported that in obese individuals, adipose tissue activates CD8-positive T cells and that CD8-positive cells promote the recruitment and activation of macrophages in adipose tissue.¹⁵ Kintcher, *et al* evaluated T lymphocyte infiltration in visceral adipose tissue in obese patients¹⁶ and reported that they could not detect CD-8-positive cells in human visceral adipose tissue. The number of CD-4-positive cells was correlated with the BMI of patients. Our study demonstrated infiltration of a larger number of CD8-positive lymphocytes in epicardial adipose tissue of CAD patients compared with that of non-CAD patients.

Winer, *et al* reported that CD4-positive T cells in visceral adipose tissue control insulin resistance in mice with diet-induced obesity. CD4-positive T cell transfer into lymphocyte-free Rag1-null DIO mice reversed weight gain and insulin resistance.¹⁷ They suggested that the progression of obesity-associated metabolic abnormalities is under the pathological control of CD4-positive T cells. In our present study, no CD4-positive T cell was detected in adipose tissue by immunohistochemical study, although CD4-positive cells were readily de-

tected in lymph nodes. It is plausible that there are few CD-4-positive cells in human epicardial and subcutaneous adipose tissue.

In our study, we could detect no significant difference in mRNA expression of inflammatory cytokines between the two groups. There have been a few reports on cytokines in epicardial adipose tissue.^{5,18-23} Iacobellis, *et al* reported that mRNA expression of adiponectin in epicardial adipose tissue was significantly lower in subjects with severe CAD than in those without CAD.²¹ Our data suggest that enhanced infiltration of inflammatory cells into EAT may account for inflammatory cytokine expression in EAT of CAD patients, although our sample size was too small to draw a definitive conclusion. Further investigation is needed to clarify the influence of adipokines on the formation of atherosclerotic lesions and inflammation in epicardial adipose tissue.

Recent evidence suggests that an inflammatory response in the adipose tissues plays a role in the pathogenesis of insulin resistance and dyslipidemia. Natali, *et al* reported that insulin resistance is associated with vascular dysfunction/damage, impaired fibrinolysis, and low-grade inflammation independently of obesity and poor glycemic control.²⁴ More severe insulin resistance was associated with being overweight, central fat distribution, hypertension, and dyslipidemia. In our study, the CAD group had a statistically significant lower HDL-cholesterol level and higher HbA1c level compared with those of the non-CAD group (Table I). Thus, it would be plausible that insulin resistance status led to a lower HDL-cholesterol level, higher HbA1c level, and enhanced inflammatory cell infiltration in epicardial adipose tissue in the patients with CAD. In the CAD group, inflammatory cell infiltration was much more enhanced in epicardial than in subcutaneous adipose tissue, suggesting that insulin resistance might have a greater influence on epicardial adipose tissue than on subcutaneous fat.

Conclusion: In summary, our findings demonstrated enhanced infiltration of CD8-positive T lymphocytes as well as macrophages in epicardial fat of patients with coronary artery disease. These changes were associated with alteration of adipocytokine expression. Our findings suggest that inflammation in epicardial adipose tissue may potentially influence the pathogenesis of coronary atherosclerosis.

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REFERENCES

- Sartore S, Chiavegato A, Faggin E, *et al*. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. *Circ Res* 2001; 89: 1111-21. (Review)
- Bot I, de Jager SC, Zernecke A, *et al*. Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* 2007; 115: 2516-25.
- Löhn M, Dubrovskaya G, Lauterbach B, Luft FC, Gollasch M, Sharma AM. Periadventitial fat releases a vascular relaxing factor. *Faseb J* 2002; 16: 1057-63.
- Henrichot E, Juge-Aubry CE, Pernin A, *et al*. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? *Arterioscler Thromb Vasc Biol* 2005; 25: 2594-9.
- Mazurek T, Zhang L, Zalewski A, *et al*. Human epicardial adipose tissue is a source of inflammatory mediators. *Circulation* 2003; 108: 2460-6.
- Iacobellis G, Ribaudo MC, Assael F, *et al*. Echocardiographic epicardial adipose tissue is related to anthropometric and clinical parameters of metabolic syndrome: a new indicator of cardiovascular risk. *J Clin Endocrinol Metab* 2003; 88: 5163-8.
- Chatterjee TK, Stoll LL, Denning GM, *et al*. Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding. *Circ Res* 2009; 104: 541-9.
- Ramshaw AL, Parums DV. Immunohistochemical characterization of inflammatory cells associated with advanced atherosclerosis. *Histopathology* 1990; 17: 543-52.
- Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999; 340: 115-26. (Review)
- Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; 352: 1685-95. (Review)
- Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002; 105: 1135-43. (Review)
- Greif M, Becker A, von Ziegler F, *et al*. Pericardial adipose tissue determined by dual source CT is a risk factor for coronary atherosclerosis. *Arterioscler Thromb Vasc Biol* 2009; 29: 781-6.
- Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003; 112: 1796-808.
- Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arterioscler Thromb Vasc Biol* 2005; 25: 2062-8.
- Nishimura S, Manabe I, Nagasaki M, *et al*. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat Med* 2009; 15: 914-20.
- Kintscher U, Hartge M, Hess K, *et al*. T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. *Arterioscler Thromb Vasc Biol* 2008; 28: 1304-10.
- Winer S, Chan Y, Paltser G, *et al*. Normalization of obesity-associated insulin resistance through immunotherapy. *Nat Med* 2009; 15: 921-9.
- Kremen J, Dolinkova M, Krajickova J, *et al*. Increased subcutaneous and epicardial adipose tissue production of proinflammatory cytokines in cardiac surgery patients: possible role in postoperative insulin resistance. *J Clin Endocrinol Metab* 2006; 91: 4620-7.
- Sacks HS, Fain JN. Human epicardial adipose tissue: a review. *Am Heart J* 2007; 153: 907-17. (Review)
- Thalmann S, Meier CA. Local adipose tissue depots as cardiovascular risk factors. *Cardiovasc Res* 2007; 75: 690-701. (Review)
- Iacobellis G, Pistilli D, Gucciardo M, *et al*. Adiponectin expression in human epicardial adipose tissue in vivo is lower in patients with coronary artery disease. *Cytokine* 2005; 29: 251-5.
- Baker AR, Silva NF, Quinn DW, *et al*. Human epicardial adipose tissue expresses a pathogenic profile of adipocytokines in patients with cardiovascular disease. *Cardiovasc Diabetol* 2006; 5: 1.
- Stern N, Marcus Y. Perivascular fat: innocent bystander or active player in vascular disease? *J Cardimetab Syndr* 2006; 1: 115-20. (Review)
- Natali A, Toschi E, Baldeweg S, *et al*. Clustering of insulin resistance with vascular dysfunction and low-grade inflammation in type 2 diabetes. *Diabetes* 2006; 55: 1133-40.