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Secretion of adipocytokines by perivascular adipose tissue near stenotic and non-stenotic coronary artery segments in patients undergoing CABG☆

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ABSTRACT

Objective: Perivascular adipose tissue (pvAT) may induce a local pro-inflammatory environment, possibly contributing to coronary atherosclerosis. We investigated whether there is a difference in adipocytokine production by pvAT near stenotic and non-stenotic coronary artery segments in patients with coronary artery disease (CAD).

Methods: In patients undergoing CABG with or without valve replacement ($n = 38$) pvAT near stenotic and near non-stenotic coronary segments was harvested. PvAT was incubated *ex vivo* for 24 h. Concentrations of 23 adipocytokines were measured in the supernatants with a Multiplex assay. The number of macrophages (CD68, CD11c, CD206) and lymphocytes (CD45) in pvAT was determined. Differences between stenosis and control pvAT were tested with Wilcoxon signed rank test corrected for multiple comparisons.

Results: Production of IL-5, IL-1α, IL-17, IL-18 and IL-23 was higher in control than stenosis pvAT samples ($p < 0.0021$). Macrophages were more abundant in stenosis than in control pvAT (median n/400× field: 2.3 IQR: 0.3–4.5 versus 1.2 IQR: 0.1–2.5). There was a predominance of M2 macrophages in both stenosis and control pvAT (median n/400× field: macrophages stenosis: M1: 0.0; M2: 1.0 $p = 0.004$; control: M1: 0.0; M2: 0.6 $p = 0.013$). The relation between adipocytokine production and macrophage infiltration was not different in stenosis and control pvAT.

Conclusion: In patients with CAD, multiple adipocytokines were secreted at higher levels by pvAT near non-stenotic than near stenotic coronary artery segments. Furthermore, pvAT macrophages are associated with stenosis of the adjacent vessel. M2 macrophages were more abundant than M1 macrophages in pvAT.

1. Introduction

Rather than being solely a storage depot for triglycerides, adipose tissue is able to secrete pro- and anti-inflammatory cytokines and adipokines [1,2]. Adipose tissue consists of adipocytes, macrophages and a stromal fraction, all contributing to the secretory function [3,4]. Large quantities of adiponectin, leptin and other adipcytokines secreted by adipocytes are released in the systemic circulation [5]. Increased plasma levels of these pro- and anti-inflammatory adipocytokines are thought to contribute to processes leading to atherosclerotic disease [6].

In addition to these systemic effects, with abdominal adipose tissue as the major source of systemic concentrations of adipocytokines, adipose tissue around arteries may influence the atherosclerotic process by direct paracrine signaling. Adipose tissue around arteries, often referred to as perivascular adipose tissue (pvAT), is situated close to the adventitia. PvAT may induce a local pro-inflammatory environment in the vascular wall by local secretion of adipocytokines [7,8]. Adipocytokines secreted by pvAT are able to diffuse into the vascular wall and attract macrophages as

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shown by development of intimal lesions after perivascular application of pro-inflammatory molecules in pigs [9,10]. Furthermore, pvAT is able to induce atherosclerosis in apo-E deficient mice [11].

In epicardial adipose tissue of patients with coronary artery disease (CAD), secretion of interleukin (IL)-6 and leptin is higher and adiponectin lower than of patients without CAD [12]. In addition, the ratio of pro-inflammatory M1 and anti-inflammatory M2 macrophages is more pronounced in epicardial adipose tissue of CAD patients compared to patients without CAD [13]. In these studies factors such as age, gender and body-mass index (BMI) may confound the relation between adipocytokine secretion of epicardial adipose tissue and the presence of CAD. A way to overcome these confounding factors is to investigate differences within the same patient. In 11 patients with CAD, secretion of adiponectin by pvAT near a stented artery was not significantly lower than by pvAT near a control coronary artery segment [14]. Aim of the present study was to evaluate differences in histologic markers of inflammation and differences in the *ex vivo* production of adipocytokine secretion from pvAT in the proximity of coronary atherosclerosis compared to pvAT near coronary artery segments without atherosclerosis in patients undergoing CABG, thus evaluating the potential contribution of pvAT to coronary atherosclerosis by direct paracrine signaling.

2. Methods

2.1. Patients

From December 2009 to July 2011, 38 patients scheduled for elective coronary artery bypass graft (CABG) surgery with or without valve replacement at the University Medical Centre Utrecht were included in the study. Inclusion criteria thus were presence of $\geq$50% stenosis of the right coronary artery, left anterior descending artery (LAD), diagonal branch of the LAD or marginal obtusis branches of the circumflex artery. Exclusion criteria were thyroid disease, a history of malignancy in the past 2 years, renal failure (MDRD $<$30 ml/min/1.73 m^2), a history of cardiothoracic surgery and use of thiazolidinediones or immune suppressive medication. The power calculation was performed using Altmans normogram for power calculations. The adiponectin concentration of adipose tissue conditioned medium of CAD patients is 1.67 (SD: 0.77) [15]. With a power of 90% ($\beta = 0.1$) and two sided α value of 0.05, the calculated sample size is 34 patients to detect a 50% difference in adiponectin secretion. Accounting for potential missing data, we aimed to collect adipose tissue samples of 40 patients. In four patients pvAT biopsies were not incubated due to the absence of qualified laboratory personnel at the time of sampling, leaving 34 patients for evaluation of *ex vivo* production of adipocytokine measurement and 38 patients for histological examination.

The Medical Ethics Committee approved the study. Written informed consent was obtained from each patient prior to study entrance. Cardiovascular history and blood pressure were obtained by the study physician. Blood was taken after an overnight fasting period. Insulin resistance was estimated using the homeostasis model of HOMA-IR: fasting glucose $\times$ fasting insulin/22.5. The left ventricular ejection fraction (LVEF) was assessed using a nuclear scan or echocardiogram at the referring centers. In the majority of patients LVEF was estimated by the echocardiographist (good LVEF: 60% ($n = 21$); reasonable to good LVEF: 55% ($n = 1$), reasonable LEVF: 50% ($n = 1$), moderate LVEF: 35% ($n = 2$), low LVEF: $<$35% ($n = 0$)).

2.2. PvAT biopsies and ex vivo incubation

The pvAT collection was performed right after implementation of the extra-corporal heart-lung system and administering of the cardioplegic fluid. Incision biopsies were obtained from adipose tissue surrounding the coronary arteries adjacent ($<$3 mm) to a coronary artery segment with stenosis, as was determined before the surgical procedure, and to a non-stenotic coronary artery segment in the same patient. The selection of the sampling site was determined by the attending surgeon based on the coronary angiogram. For pvAT samples near stenotic coronary artery segments a stenosis of $>$50% was present. In all patients also control pvAT samples were taken near coronary artery segments without irregularities of the vascular wall. During surgery, the biopsy-site was carefully verified by palpation. In 27 (71%) cases the control pvAT sample was taken in the same coronary artery or its associated branch. In other cases the control pvAT sample was taken near another coronary artery in the same patient. The median weight of stenosis pvAT samples was 16 mg (IQR 9–35) and of control pvAT samples 10 mg (IQR: 6–19) ($p = 0.01$). The difference in weight between the samples is most likely due to the presence of a larger amount of pvAT near stenosis compared to control segments.

Tissue biopsies from 34 patients were divided into two and incubated in 500 μl medium. Of both pvAT near stenosis and pvAT near non-stenotic coronary artery segments, the 2 pieces of adipose tissue were incubated in serum-free Dulbecco's modified Eagles medium (DMEM/F3 (31331-028)). Samples were stored at 37 °C (CO_2: 5%) for 24 h. After 24 h the supernatants from the pvAT cultures were centrifuged at 14,000 rounds per minute for 3 min. The centrifuged supernatants were stored at −80 °C until further processing.

2.3. Measurement of adipocytokines

Concentrations of adiponectin, cathepsin S, chemerin, soluble intercellular adhesion molecule-1 (sICAM-1), IL-1α, IL-5, IL-6, IL-13, IL-17, IL-18, IL-23, IL-33, leptin, macrophage inflammatory protein-1α (MIP-1α), macrophage colony stimulating factor (M-CSF), plasminogen activator inhibitor-1 (PAI-1), regulated upon activation normal T cell expressed and secreted (RANTES), retinol binding protein-4 (RBP-4), serum amyloid A1 (SAA-1), soluble vascular cell adhesion protein-1 (sVCAM-1), soluble vascular endothelial growth factor (sVEGF) and tissue inhibitor of metalloproteinases (TIMP-1) were measured in the undiluted supernatants with a multiplex assay (Biorad, Munich Gemany) [16]. The choice of antibodies was based on the availability of antibodies and whether proteins were encoding cytokines involved in atherosclerosis, in adipose tissue dysfunction or in M1 and M2 macrophage polarization. The adipocytokines IL-1β, IL-2, IL-4, interferon-γ induced protein 10 (IP10), granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α, were not detectable.

2.4. Histological examination

The pvAT samples from 38 patients were fixed in 10% formaldehyde and embedded in paraffin. Thin sections of 4 μm were processed for histological staining with hematoxylin and eosin (H&E). For quantification of macrophages (anti-CD68), lymphocytes (CD45) and capillary density (factor 8), immunohistochemistry stainings with 3,3′-diaminobenzidine (DAB) were performed. M1 macrophages were defined as CD11c positive cells (Abcam monoclonal antibody EP1347Y) and M2 macrophages as CD206 positive cells (Abcam monoclonal antibody AB52632) [17]. Macrophages and lymphocytes were counted with the researcher blinded for the location of the biopsy. The mean number of positive staining cells in 3 high power fields was used for analysis (HPF; magnification 400×). The adipocyte size was determined by calculating the mean adipocyte area of 10 adipocytes in 10 randomly selected fields at 200× magnification [18]. Capillary density (%) was calculated by