BRIEF REPORT

The density of crown-like structures in epicardial adipose tissue could play a role in cardiovascular diseases

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Abstract

Purpose The visceral fat of patients affected by abdominal obesity is inflamed, and the main histopathologic feature is the high density of crown-like structures (CLS). Epicardial adipose tissue (EAT) is a visceral fat of paramount importance for its relationships with coronary vessels and myocardium. Its inflammation in patients with abdominal obesity could be of clinical relevance, but histopathological studies on CLS density in EAT are lacking. This study aimed to assess the histopathology of EAT biopsies obtained from patients undergoing open-heart surgery.

Methods We collected EAT biopsies from 10 patients undergoing open-heart surgery for elective coronary artery bypass grafting (CABG) ($n=5$) or valvular replacement (VR) ($n=5$). Biopsies were treated for light microscopy and immunohistochemistry. We quantify the CLS density in each EAT sample.

Results Despite all patients having abdominal obesity, in EAT samples, no CLS were detected in the VR group; in contrast, CLS were detected in the CABG group (about 17 CLS/10^4 adipocytes vs. 0.0 CLS/10^4 adipocytes, CABG vs. VR group, respectively). An impressive density of CLS (100 times that of other patients) was found in one patient (LS) in the CABG group that had a relevant anamnestic aspect: relatively rapid increase of weight gain, especially in abdominal adipose tissue, coincident with myocardial infarction.

Conclusions CLS density could be an important predictive tool for cardiovascular diseases. Furthermore, the LS case implies a role for timing in weight gain.

Level of evidence No level of evidence; this is a basic science study.

Keywords Epicardial adipose tissue · Crown-like structures · Inflammation · Cardiovascular diseases · Open-heart surgery

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Introduction

Excess of visceral fat is characterized by chronic low-grade inflammation [1]. This inflammation can cause metabolic consequences, such as insulin resistance and type 2 diabetes mellitus, and local consequences, including cancers [1]. The cause of inflammation seems to be linked to adipocytes death, probably due to their pathologic hypertrophy engaging mechanisms ending with death for pyroptosis at a critical size [2]. Dead adipocytes are surrounded by scavenger macrophages reabsorbing their debris and forming the classic crown-like structures (CLS) [3]. Visceral fat in mice shows a critical death size lower than that of subcutaneous adipocytes, suggesting that the excess of visceral fat has worse metabolic consequences than subcutaneous fat [4]. Epicardial adipose tissue (EAT) is a visceral fat with a peculiar anatomical contiguity with the myocardium. A positive correlation between EAT thickness and cardiac diseases has been shown [5, 6].

This fat depot's morphological and molecular characteristics are different from those of other visceral depots, and adipocytes have been described as intermediate between white and brown [7]. Although inflammation of EAT can be suspected by molecular approaches [8] and different imaging techniques [6], histopathologic studies focusing on CLS density are scarce [9]. Thus we studied the histopathology of ten EAT biopsies from patients undergoing open-heart surgery.

Methods

We analysed EAT from 10 with obesity or overweight abdominal obesity undergoing open-heart surgery: five patients for elective coronary artery bypass grafting (CABG) and five for valve replacement (VR). VR patients did not show any sign of coronary artery disease (CAD) in the pre-operative coronary angiographic examination. EAT biopsy samples were harvested adjacent to the proximal right coronary artery prior of cardiopulmonary bypass pumping and immediately fixed in paraformaldehyde 4% in phosphate saline buffer (PBS) at pH 7.4. After overnight fixation, tissue was dehydrated in ethanol, cleared in xylene, and embedded in paraffin.

All samples were stained with H&E and immunostained with antibody anti-CD68: to reveal the presence of macrophages widespread in adipose parenchyma and/or organized to form CLS (3). In brief, 3 μm paraffin tissue sections were obtained for each sample, and immunohistochemical staining was performed. Sections were rehydrated, reacted with 3% H_2O_2 (in dH_2O for 5 min), rinsed with PBS, and incubated with 2% blocking solution (in PBS for 20 min). Then they were incubated overnight at 4 °C with the primary CD68 antibody (Dako #M0814; 1:200; antigen retrieval method by citrate buffer pH6). After some rinsing in PBS, they were incubated with the biotinylated secondary antibody (in PBS for 30 min), rinsed in PBS, and incubated in Vectastain ABC kit (Vector Laboratories) for 60 min, washed several times in PBS and finally incubated with 3,3'-diaminobenzidine tetrahydrochloride (0.05% in 0.05 M Tris with 0.03% H_2O_2; 5 min) as the substrate. Sections were finally counterstained with haematoxylin, dehydrated, and mounted in Eukitt (Merck). Negative control was included in each reaction by omitting the primary antibody, to assess the specificity of the antibody. All observations were performed using Nikon Eclipse E800 light microscope.

The size of adipocytes (area) was measured in all subjects. Five fields from each stained slide were captured at $10 \times$ magnification with a Nikon DXM 1220 camera. One hundred adipose cells for each paraffin tissue slide were counted using the morphometric program ImageJ (RRID:SCR-003070).

The density of CLS identified for their specific aspect (defined as a large lipid droplet mimicking an adipocyte [3] surrounded by CD68 + cells for at least 50% of its circumference) was calculated as previously described [10]. In brief, for each patient CLS density per 10^4 adipocytes was determined using ImageJ.

Data are presented as mean value ± standard error (SEM); the Student's t test was used to compare CLS density of patients for VR and those for CABG. In addition, one-way ANOVA compared the CLS density between the CABG patient (SL) alone and the other two groups. We used the GraphPad Prism 6.0 software and considered significant a $p < 0.05$.

We used Fisher's exact test for categorical variables and the t test or Wilcoxon rank-sum test to compare unpaired means in normally or non-normally continuous distributed variables for clinical data comparisons.

Results and discussion

The main demographic, anthropometric, clinical, and biochemical characteristics of the 10 patients (5 CABG and 5 VR) are reported in Table S1 (Supplementary Information). The mean age was 65.7 ± 12.7 years. Even if CABG patients were older than VR patients (71.6 ± 6.8 years vs. 59.8 ± 14.3 years, respectively), no statistical difference was seen. The majority of patients were males ($n = 9$).

No significant difference in the measurements of weight, height, body mass index (BMI), and waist circumferences was observed between CABG and VR patients.

According to BMI, an indicator of general fatness, eight patients were affected by obesity and two by overweight; while adopting waist circumference as an indicator of abdominal fat distribution, all patients were affected by abdominal obesity.

No difference was observed in smoking status, and only one patient in the CABG group was affected by type 2 diabetes mellitus. The percentages of antiplatelet, statins, angiotensinogen-converting enzyme inhibitors/angiotensin receptor blockers and oral glucose-lowering drugs were similar in the two groups. It is noteworthy that beta-blockers were taken by all patients in the CABG group and only by one patient in the VR group, with a borderline significant difference.

It is interesting to note that beta2-adrenergic receptor interactions may have pleiotropic effects in cardiac myocytes, such as inhibition of apoptosis and inhibition of inflammation by blocking the pro-inflammatory transcription factor nuclear factor-kappaB, a crucial mediator of the expression of key pro-inflammatory cytokines [11, 12]. Therefore, the use of nonselective beta1–beta2 blockers could counteract the inflammatory effect given by the paracrine or vasocrine crosstalk between epicardial adipose tissue and myocardium. However, all patients in our study who were on beta blockers, used selective beta-1 blockers, in particular four patients were taking atenolol and two patients were taking bisoprolol. Consequently, the anti-inflammatory and anti-apoptotic effect on myocardium of beta-2-adrenergic receptors was not hampered.

No difference was observed in echocardiographic EAT thickness between the two groups. Adipocytes have a dynamic endocrine role by expressing and secreting many factors, including hormones and cytokines, that depend on cell size [6]. Several studies have recently paid attention to EAT, because its specific anatomical location. In this way, regional differences in adipocyte hypertrophy and inflammatory function might suggest a different metabolic response in patients with cardiovascular disease. The size of adipocytes in EAT is significantly smaller than in other adipose tissues [6].

In line with data from other studies [6], in our study, the size of adipocytes of both groups was around 3000 μm^2 (2940 ± 188 vs. 3260 ± 299 VR vs. CABG group, respectively, $p = 0.39$). Thus, the size was about one-half found in visceral fat (omentum) of patients with obesity of a different case series but measured with the same methods [10].

Inflamed visceral adipose tissue is marked by the presence of CLSs, where scavengers' macrophages surround dead adipocytes [3, 4]. Although all patients had abdominal (visceral) obesity, in EAT samples, no CLS were detected in the VR group who did not show any sign of CAD in the preoperative coronary angiographic examination; in contrast, CLS were detected in three patients of the CABG group (about 17 CLS/10^4 adipocytes vs. 0.0 CLS/10^4 adipocytes, CABG vs. VR group, respectively), although no significant differences in adipocyte size were seen in the two groups (Fig. 1). Interestingly, the density of CLS was about three times higher than that found in visceral fat (omentum) of patients with obesity in the above-cited study [10].

One of the CABG patients (SL) had an extraordinary density of CLS (Fig. 2); therefore, to avoid a false picture of the degree of inflammation in the CABG group, his data were not included. CLS density in SL was about 100 times the average value observed in other CABG patients. We

Fig. 1 Crown-like structures (CLS) density in epicardial adipose tissue of patients undergoing open-heart surgery for valvular replacement (VR) or elective coronary artery bypass grafting (CABG). **A** Number of CLS per 10^4 adipocytes in VR group ($n = 5$) vs. CABG group ($n = 4$) without patient SL. **B** CLS density/10^4 adipose cells in two groups of patients and in CABG patient (LS) with peculiar clinical data different from the other patients examined (recent heart attack after a period of relatively rapid increase of abdominal visceral fat). ****$p < 0.0001$
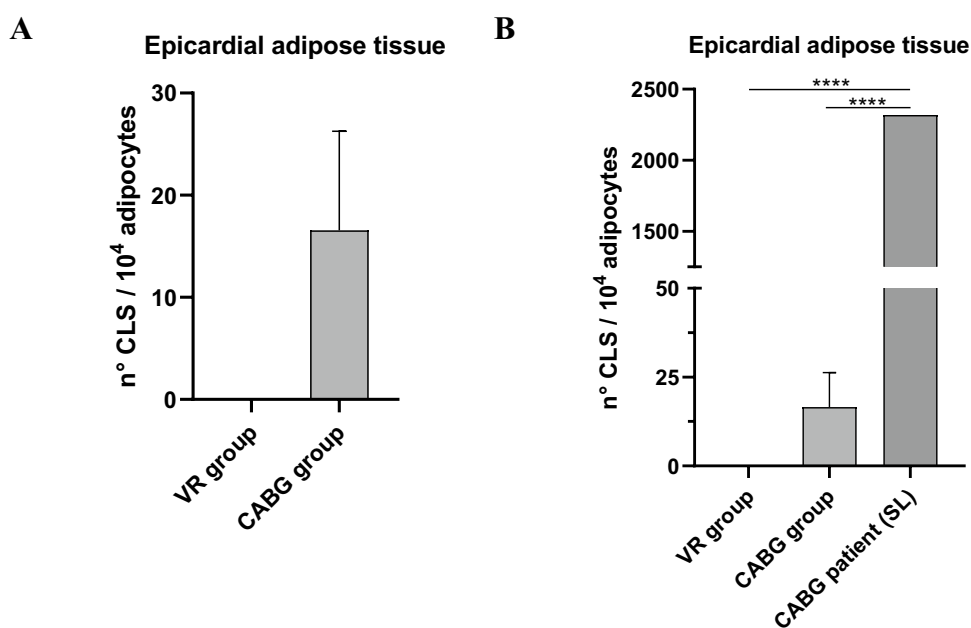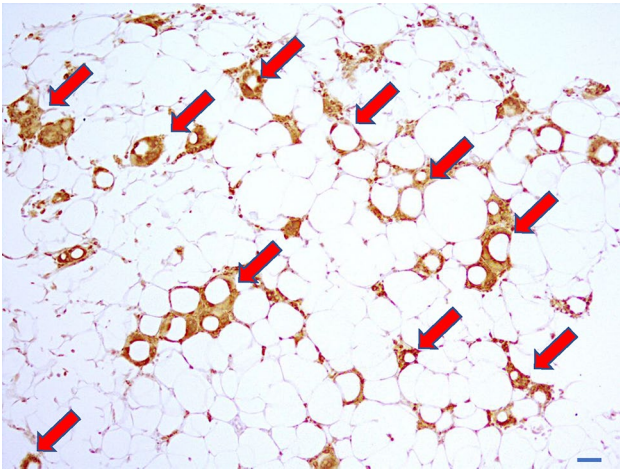
Fig. 2 Light microscopy. Immunohistochemistry for CD68 +. Representative picture of epicardial adipose tissue of CABG patient LS. An impressive amount of classic CLS (single and groups) are visible (arrows, some indicated). Bar: 30 μm

observed such a density of CLS only in visceral fat of mice with severe obesity [13 and unpublished data], but never in humans [10]. Of note, the clinical data of LS differed from all other cases mainly for one important clinical aspect: he had a recent episode of myocardial infarction and a quite rapid (few years) weight gain of about 13 kg, almost entirely in the abdominal region, with an increase in abdominal circumference of 9 cm.

The specific unique nature of EAT with intermediate characteristics between white and brown adipocytes, also known as beige or brite adipose tissue, [6, 7] could explain the higher inflammation found in EAT when compared with omental fat of another case series studied with the same methods [10]. In addition, the age of LS (78 years) must also be taken into account as, with ageing, epicardial adipocytes become more susceptible to environmental, metabolic, and haemodynamic factors, which gradually change the function of EAT from thermogenesis to energy storage [6].

Indeed, EAT brown fat-like activity decreases substantially with age. The changes are not only functional but also structural. The proportion of brown adipocytes decreases in favour of more unilocular white adipocytes in older individuals. This finding suggests that the transition from brown fat to beige fat is a feature of EAT in adults [6]. In line with these data, we recently showed that whitening of brown fat induces a high degree of inflammation with a high density of CLS in this depot [14].

The absence of CLS in the VR group could be explained by the small adipocytes size difference (about 10%), but mainly by the different ages of the two study groups, as the CABG group was more than 10 years older than the VR group, who did not show any sign of CAD in the pre-operative coronary angiographic examination. Aging has been

recently reported as an important factor worsening EAT chronic inflammation [15].

An alternative, plausible explanation of the higher presence of CLS in EAT from CABG than VR patients is the downregulation of the EAT housekeeping genes transcriptome that we observed and reported previously [16]. The hypothesis might be that end/stage or advanced coronary artery disease (CAD) causes downregulation of EAT genes due to fibrotic and apoptotic changes or, better, to the mounting pyroptotic changes following the continuous and chronic inflammatory insult [2]. These processes would lead to adipocytes' death and consequent CLS formation. Based on this observation, we suggest that the EAT adipocytes are "burned-out" (pyroptosis) in advanced CAD. CAD more than obesity plays a role in the higher EAT inflammation and related morphological changes. This study clearly confirms this.

In line with previous data showing that EAT plays a role in the progression and development of CAD, inflammation is the main feature of EAT in patients with obesity and CAD, showing dense macrophage infiltrates [5, 6, 8], as observed among adipocytes in all our studied patients with abdominal obesity and CAD (not shown).

To the best of our knowledge, only one recent paper described CLS in EAT of patients with obesity, CAD and type 2 diabetes mellitus [9]. They did not quantify the CLS density in each patient but described only if CLS were present or not. Surprisingly, in their case series of EAT biopsies studied by immunohistochemistry ($n = 16$ patients affected by obesity and $n = 28$ patients without obesity), the CLS were found only in 14% of patients without obesity, but with CAD; interestingly the prevalent phenotype of macrophages was the pro-inflammatory M1 [9]. It would be interesting to know if the patients without obesity but with CLS in EAT, in that case series, had an excess of visceral fat. Data presented show only an average (including patients without CLS) waist circumference within normal values.

Conclusions

Although our data cannot be considered significant from a statistical point of view for obvious numerical reasons, we thought worthwhile their description to stimulate the study of EAT as an important cardiovascular risk factor and therapeutic target for drugs with cardiovascular benefits, such as glucagon-like peptide 1 receptor (GLP1R) agonists and sodium–glucose co-transporter 2 (SGLT2) inhibitors. Of note, it is well known that these drugs induce EAT reduction. If confirmed in a larger number of patients, CLS density could be an important predictive tool for cardiovascular diseases. Furthermore, the LS case outlines the importance of

timing in weight gain as recently described also by another study [17].

Strength and limits

This study is the first analysis of CLS density in the human EAT sample, as far as we know. Furthermore, all patients in our study who were on beta-blockers were using beta1-selective blockers, not compromising the observation of differences in CLS density between the CABG and VR groups.

Nonetheless, some limitations are evident. First, our patients' number was small and underpowered; therefore, our data cannot be considered significant from a statistical point of view. Second, we did not collect subcutaneous adipose tissue (SAT) from the substernal site during cardiac surgery to explore any potential difference among various fat depots. However, our and other studies clearly showed the difference between EAT and SAT [10, 16].

What is already known on this subject?

The visceral fat of patients affected by abdominal obesity is inflamed, and the main histopathologic feature is the crown-like structures (CLS) density. Epicardial adipose tissue (EAT) is a visceral fat of paramount importance for its relationships with coronary vessels and myocardium. Its inflammation in patients with abdominal obesity could be of clinical relevance, but histopathology studies focusing on CLS density in EAT are lacking.

What does this study add?

This report is the first study that describes and quantifies the CLS density in human EAT samples. CLS density, found only in patients with abdominal obesity and CAD, could be an important predictive tool for cardiovascular diseases.

Declarations

Conflict of interest All authors declare that they have no conflicts of interest.

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