Comparison of gene expression of epicardial and visceral adipocytes with regard to the differentiation stage

Jana Zdychova 1, Ivana Kralova Lesna 2, Jana Maluskova 3, Libor Janousek 4, Monika Cahova 1, Ludmila Kazdova 1

1 Institute for Clinical and Experimental Medicine, Department of Metabolism and Diabetes, Prague, Czech Republic
2 Institute for Clinical and Experimental Medicine, Laboratory for Atherosclerosis Research, Prague, Czech Republic
3 Institute for Clinical and Experimental Medicine, Department of Pathology, Prague, Czech Republic
4 Institute for Clinical and Experimental Medicine, Department of Transplant Surgery, Prague, Czech Republic

Correspondence to: Mgr. Jana Zdychova, PhD.
Department of Metabolism and Diabetes
Institute for Clinical and Experimental Medicine
Videnska 1958/9, Prague 14021, Czech Republic.
TEL: +420261365368; FAX: +420261363027; E-MAIL: jana.zdychova@ikem.cz

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Abstract

BACKGROUND: Our study focused on the ability of epicardial adipocytes to produce bioactive substances and compare the extent of this production with the production of adipokines in visceral adipocytes, which are well known endocrine cells capable of contributing to the development of atherosclerosis.

MATERIAL AND METHODS: The gene expression of human cytokines (IL-6, IL-8, IL-18, RANTES and MCP-1) and adipokines (leptin and adiponectin) was measured in primary cell lines of epicardial and visceral adipocytes, both in undifferentiated and mature statuses, after a 21-day-long differentiation protocol. Each condition was assayed in triplicate in two independent primary cell lines obtained from two different donors.

RESULTS: The epicardial preadipocytes showed an increased expression of IL-8 (3.25-fold, p<0.05) compared with visceral preadipocytes. The expression of the atheroprotective adiponectin in epicardial preadipocytes was minimal compared with the expression in visceral preadipocytes (p<0.0001). Moreover, the expression of the genes of interest was dependent on the differentiation degree and cell origin. We observed an altered expression of the proinflammatory genes IL-8 (0.016-fold, p<0.01) and MCP-1 (0.19-fold, p<0.05) in differentiated epicardial adipocytes compared with undifferentiated adipocytes. The epicardial adipocytes showed an increased expression of IL-6 (8.13-fold, p<0.05) compared with the visceral adipocytes.

CONCLUSION: Our results suggest that epicardial adipocytes substantially differ from visceral adipocytes and might locally contribute to the pathogenesis of coronary atherosclerosis.
INTRODUCTION

Virtually all blood vessels are surrounded by various amounts of adipose tissue (Cinti 2002). The composition of the perivascular fat tissue (PVAT) varies by location, and its amount increases with increasing adiposity (Lehman et al. 2010; Britton & Fox 2011). For a long time, PVAT was considered to be rather a passive structural support for vessels, but the vasocrine function of PVAT has been extensively studied recently (Gao 2007).

Based on recent data, perivascular adipocytes appear, similar to visceral adipose tissue, which is a well-known producer of various bioactive substances with apocrine, paracrine and endocrine effects, to be powerful endocrine cells capable of responding to metabolite cues (Chartterjee et al. 2009) and transducing signals to adjacent blood vessels through a paracrine or vasocrine signaling pathway (Gustafson 2010). Several findings even suggest an outside-to-inside signaling paradigm that justifies a pathologic role for PVAT-produced biologically active mediators in the development of atherosclerosis (Mazurek et al. 2003; Yudkin et al. 2005).

Epicardial adipose tissue (EAT) is a special type of PVAT that can be found near the adventitia of coronary arteries. Recent clinical findings, together with ex vivo and in vitro studies, suggest an important role of this adipose tissue in the process of the pathogenesis of atherosclerosis (Mazurek et al. 2003; Zhou et al. 2011; Chartterjee et al. 2009). Moreover, there is evidence demonstrating an epidemiological relationship between epicardial and visceral fat masses (Iacobellis et al. 2003). The expansion of visceral adipose tissue is now well established to lead to a high risk for metabolic syndrome and a high incidence of cardiovascular disease (Després et al. 2008; Piché et al. 2008). Several findings have even showed an association of pericardial fat accumulation, defined as EAT plus paracardial fat, with coronary atherosclerosis, as assessed by the calcified and non-calcified plaque burden (Ding et al. 2008; Taguchi et al. 2001).

The aim of our study was to determine the production of selected adipokines and cytokines in both pre- and fully differentiated epicardial and perirenal adipocytes.

METHODS

Cell culture

The experiments were approved by the Ethical Committee of the Institute for Clinical and Experimental Medicine. Perirenal adipose tissue was selected as the representative for visceral fat, as it easily accessible and available in sufficient quantities. These samples were collected exclusively from healthy volunteers during kinship transplantations. The EAT samples were obtained from the explanted hearts of patients whose hearts were transplanted for the diagnosis of cardiomyopathy. The EAT samples were sampled from the following locations: the right distance of a. coronalis, ramus circumflexus and anterior descending branch of the coronary artery. For ethical reasons, obtaining EAT from healthy donors is impossible; however, to minimize the influence of preexisting pathologies, only samples obtained from patients without atherosclerosis were included in the study. All EAT and perirenal fat samples used in our study were obtained from middle-aged men. The average BMI of the EAT sample donors (N=2) was 30.6±0.71 kg/m², and the BMI of the perivascular sample volunteers (N=2) was 28.35±1.2 kg/m².

The preparation of the adipose tissue cultures was performed as previously described (Fried & Moustaid-Moussa 2001). Briefly, the EAT samples were subsequently pooled and, similar to the visceral samples, were minced with scissors under aseptic conditions into small pieces (2–4 mm²). Per 1–3 grams of minced tissue, the samples were washed in PBS, placed in digestion solution (2 mg/ml collagenase, 2% fatty acid-free BSA in PBS) and incubated in a shaker (200 rpm) at 37°C for 1 hr. The collagenase-digested samples were separated from undigested tissue by filtration through a 200 μm mesh fabric. Floating fat cells were separated from the stromal vascular fraction (SVF) by centrifugation at 200 g × 10 min. The SVF containing the preadipocytes was subsequently washed several times and finally resuspended in selective preadipocyte growth medium (Promo Cell, Heidelberg, Germany), cultivated in a T25 culture flask and maintained in an incubator at 37°C and 5% (v/v) CO₂. The medium was changed every 2–3 days unless otherwise stated. At subconfluence, the cultured cells were trypsinized and plated in triplicate for each condition into 15-mm dishes at 5 × 10⁵ cells/dish.

When the cell monolayer cultures reached ~90% confluence, some of the cells were used for RNA isolation, and others were induced to differentiate. The preadipocytes were always cultured minimally 8 days before the RNA was isolated. The cells were subsequently cultured for a total of 21 days, with the first 3 days in adipocyte differentiation medium and later in adipocyte growth medium (Promo Cell, Heidelberg, Germany), to obtain mature adipocytes. The lipid accumulation in the differentiated adipocytes was assessed through Oil Red O dye staining (Sigma Aldrich, St. Louis, MO, USA). In brief, the differentiated adipocytes were fixed in 4%
formaldehyde for 10 min and then stained for 30 min with the Oil Red O dye dissolved in isopropanol. The stained adipocytes were washed with PBS. All experiments were performed on cells at passage 4 or less, and each condition was assayed in triplicate in two independent experiments – i.e. in two independent primary cell lines sampled from two volunteers during kinship transplantations or from explanted hearts.

**RT-PCR analyses**

Total RNA was isolated from each separate dish (total number n=6 per each condition) using the RNeasy Mini kit (Qiagen, Venlo, Netherlands). After DNase I (Sigma Aldrich, St. Louis, MO, USA) treatment to eliminate any DNA from the RNA preparations, cDNA was generated according to the manufacturer's instructions using 400 ng of total RNA and the High Capacity RNA-to-cDNA master mix (Life Technologies, Carlsbad, CA, USA). The gene expression level of the cytokines and adipokines of interest was then determined in the Corbett Life Science Rotor Gene 3000 (Qiagen, Venlo, Netherlands) using the QuantiTect SYBR Green PCR Kit (Qiagen, Venlo, Netherlands). The nucleotide sequences of the primer pairs used in this study are given in Table 1. The levels of B2M were used as endogenous controls for normalization. The relative gene expressions were calculated by the ΔΔCt method as described previously (Pfaffl 2001).

**Statistical analysis**

The data are presented as the means±standard deviation (SD). The statistical analysis was performed using the Student t-test. Differences were considered statistically significant at the level of p<0.05.

**RESULTS**

The formation of lipid droplets in the differentiated adipocytes was confirmed by Oil Red O staining. Even though both the epicardial and visceral preadipocytes were subjected to the differentiation protocol, more than 80% of visceral adipocytes and approximately 60% of epicardial adipocytes contained multiple lipid droplets (data not shown). Given that the accumulation of cytoplasmic lipid droplets is viewed as an index of adipocyte differentiation, our results show a lower degree of differentiation in the group of epicardial adipocytes compared with their visceral counterparts.

The gene expression levels of cytokines (IL-6, IL-8, IL-18, RANTES and MCP-1) and adipokines (leptin and adiponectin) were measured in primary cell lines of epicardial and visceral adipocytes, both undifferentiated and mature, using qPCR. Our results demonstrated a 3.25-fold increased expression of IL-8 in the epicardial preadipocytes compared with the visceral preadipocytes (p<0.05) (Figure 1A and 1B). Although not significant, IL-18 also showed an increased expression in the epicardial preadipocytes compared with the visceral preadipocytes (15.7-fold, p=0.064). In contrast, the expression level of atheroprotective adiponectin together with the expression of inflammatory leptin were significantly decreased in epicardial preadipocyte cultures compared with visceral preadipocytes (p<0.0001). Moreover, our results determined that the levels of gene expression were affected by the degree of adipocyte differentiation and cell origin. We observed altered expression levels of the proinflammatory genes leptin (825-fold, p<0.05), IL-8 (0.19-fold, p<0.05) and MCP-1 (0.19-fold, p<0.05) in the epicardial adipocytes compared with their undifferentiated counterparts together with increased expression level of IL-6 (8.13-fold p<0.05) in the epicardial adipocytes compared with the visceral adipocytes. Conversely, leptin showed a significantly increased expression in visceral adipocytes compared with epicardial adipocytes (13.47-fold p<0.05). Compared with the visceral preadipocytes, our results showed a 19.16-fold increased expression of leptin (p<0.05) in the visceral adipocytes.

**DISCUSSION**

In a pilot study, we demonstrated that the epicardial preadipocytes tend to express proinflammatory cytokines at higher levels concomitantly with the minimal expression of the atheroprotective adiponectin compared with the visceral preadipocytes. Moreover, the pro-inflammatory phenotype is affected by the adipocyte degree of differentiation. The increased expression of MCP-1, IL-6, and IL-18 in the epicardial preadipocytes and adipocytes suggests that these cells could be under basal conditions, i.e., in the absence of atherosclerosis, they are an important source of proinflammatory mediators and could play an important role in epicardial adipose tissue inflammation. The significantly decreased level of the atheroprotective adiponectin in the abovementioned cell lines further exacerbates the pro-inflammatory phenotype of these cells.
In the context of perivascular adipose tissue inflammation, massive infiltration with inflammatory cells is often mentioned. These cells are considered to be an important source of a variety of cytokines and mediators that may significantly contribute to the development of a proinflammatory milieu and to the final progression of atherosclerosis (Mazurek et al. 2003; Baker et al. 2006). Our results suggest that under basal conditions not only infiltrating cells but also the epicardial adipocytes themselves are meaningful endocrine cells expressing several proinflammatory cytokines having relevant relationships with the pathophysiology of atherosclerosis. Our measurement could be potentially affected by the macrophage gene expression. Nevertheless, a study by Mangan & Wahl (1991) showed that the life-span of un-stimulated macrophages, co-isolated together with preadipocytes, should be a maximum of 5–6 days in culture. In our experimental setting, the preadipocytes were always cultured for a minimum of 8 days before the RNA isolation, which excludes potential macrophage contamination.

Based on our knowledge, research comparing the gene expression of preadipocytes and in vitro differentiated epicardial adipocytes under basal conditions has not been performed. Similar research conducted by Fain et al. (2008) used the omental adipose tissue of extremely obese patients. Their results suggested that proinflammatory cytokines, such as MCP-1, IL-6, IL-8, PAI-1 and IL-1 beta, are predominantly expressed in omental preadipocytes. However, the result of that study could likely have been affected by the extreme obesity of the donors as obesity has been previously shown to be almost always associated with visceral adipose tissue inflammation (Dolinkova et al. 2008). Epicardial fat shares a common embryological origin with omental fat (Ho & Shimada 1978). However, a study by Fain et al. (2010) demonstrated that the gene expression profiles of appointed fat depots are different.

The authors are aware of the limitations of the present study. For practical and ethical reasons, obtaining EAT from healthy donors is not possible, and we cannot exclude the possibility that the inflammatory state in the donor’s EAT was affected by the underlying pathology (cardiomyopathy). Nevertheless, as shown previously (Sacks & Fain 2007; Hug & Lodish 2005; Silaghi et al. 2007; Baker et al. 2006; Cheng et al. 2008; Eiras et al. 2008; Mazurek et al. 2003), the most prominent cause of the pro-inflammatory phenotype in EAT is the presence of coronary artery disease (CAD). To avoid this problem, we used cell cultures of epicardial and visceral preadipocytes and adipocytes derived exclusively from donors without the presence of atherosclerosis. Another point that should be discussed is the fact that cultures of epicardial and visceral adipocytes did not come from the same donor. This limitation could not be surmounted as simultaneous sampling during surgery procedures is not, for technical and ethical reasons, performed in our institute. We attempted to overcome this obstacle by careful matching of the donors.

In conclusion, the results of our pilot study suggest that epicardial adipocytes substantially differ from visceral adipocytes and that the former exhibit a pro-
inflammatory phenotype even under basal conditions. As such, the epicardial adipocytes might contribute locally to the pathogenesis of coronary atherosclerosis.

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