Macrophages of the subcutaneous and omental fatty tissue in obese patients: Immunohistochemical phenotyping of M2 subtypes in relation to type 2 diabetes

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Background and Aims. Macrophages are linked to the initiation of the chronic inflammation believed to underlie the changes taking place in the white fatty tissue of obese people. Both the number of macrophages, but their functional status, play an important role in the development of inflammation. Classically, macrophages are divided into two types: pro-inflammatory (M1) and anti-inflammatory (M2) types, and based on current immunological studies, further views on the functional distribution of macrophages are suggested. In this study, we evaluated the M1 and M2 macrophages ratio in obese subjects with, or without diabetes. To identify all macrophages, we used CD68 expression, while CD204 expression is typically used for the M2 macrophage.

Materials and Methods. During bariatric surgery, carried out in obese people with and without type 2 diabetes (T2D), we obtained subcutaneous adipose tissue from the navel and omental adipose tissue. We also obtained the same tissue from people with a physiological range of BMI from a judicial autopsy. Applying immunohistochemical staining anti-CD68 and anti-CD204, we carried out a quantitative evaluation of the number of macrophages.

Results. We found CD68+ and CD204+ positive macrophages in perivascular spaces and between fat cells, both isolated and in larger infiltrates. They were also present in so-called "crown-like structures" (CLS) around dying adipocytes. Quantitative analysis showed an increased number of macrophages in all obese patients compared to the control group of non-obese, individuals without T2D. The most striking observation was the macrophage increase in the visceral fatty tissue of diabetics. The number of CD68 and CD204 positive macrophages was statistically significantly smaller in patients without T2D.

Conclusion. We demonstrated a significantly greater number of macrophages in visceral adipose tissue, especially in patients with T2D. Our results also show a positive correlation between the presence of T2D and the total number of macrophages; a significantly greater number of macrophages were found in visceral adipose tissue, especially in patients with T2D.

Key words: adipose tissue, type 2 diabetes, macrophage, M1/M2 polarization, immunohistochemistry

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INTRODUCTION

Over the last decade, many articles have been published which confirm that white adipose tissue obesity leads to chronic low grade inflammation. Chronic inflammatory processes are also involved in the etiology of atherosclerosis, hypertension, insulin resistance, T2D and even in some cancers associated with obesity.

Of all of the immune cells present in the adipose tissue, macrophages are thought to be the key driver in the development of morphological and physiological changes connected with obesity. Macrophages, which play an important role in white adipose tissue homeostasis, accumulate in the fatty tissue of obese individuals. They demonstrate remarkable phenotypic heterogenity, with the ability to perform different tasks, depending on the biological situation.

Macrophages were traditionally subdivided into two main populations: classically activated M1, or alternatively activated M2 macrophages. Recent studies describe that, besides the two classically defined poles (M1/M2) of macrophages, there is a broad spectrum of phenotypically variable macrophages. It is generally accepted that M1 macrophages have a key role in the development of low-grade inflammation and insulin resistance.

MATERIALS AND METHODS

In this study, we evaluated expression of CD68 (a pan-macrophage marker) and CD204 expression (an M2 macrophage marker).

The studied material was obtained from intraoperative biopsy in diabetic and non-diabetic patients, undergoing

bariatric surgery for obesity. The study group included 42 men, age 21-66 years with BMI 34-58, and 64 women, age 21-60 years with BMI 30-54. All patients gave their written informed consent. The same tissues were obtained from judicial autopsies in non-obese non-diabetic patients (10 patients, average age 49, average BMI 23). The study was approved by the local ethics committee of Vitkovice Hospital a.s., Ostrava – Vitkovice, Czech Republic, and performed in accordance with the Helsinki Declaration (Fortaleza 2013).

We investigated both subcutaneous (from the umbilicus region) and omental white adipose tissues. Tissue samples were immediately fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Paraffin sections (5μm thick) were stained with hematoxylin-eosin. For immunohistochemical evaluation, a two-step immunohistochemical indirect method was used. The macrophages were detected using anti-CD68 (mouse monoclonal anti-human CD68, DakoCytomation, diluted 1:100). Anti-CD204 was produced in rabbit (Sigma, diluted 1:100). In the second step, the slices were incubated with EnVision (Dako) for 1 hour, and the color reaction was developed in 2% 3,3´-diaminobenzidine (DAB). Slices were counterstained with hematoxylin. Adipocytes and macrophages were initially identified by detailed examination at 40x magnification. The adipocyte count was then taken from five areas at 20x magnification (total investigated area 0.019 mm^2). The number of macrophages were recalculated to 100 adipocytes.

Statistical Analysis

The results were evaluated by the Mann-Whitney test to find differences between studied groups at the level of significance $P<0.05$. All calculations were performed by GraphPad Prism 6.1 software.

RESULTS

In the control group of non-obese patients without diabetes, sporadic macrophages were present in the interstitium (Fig. 1). CD204+ and CD68+ macrophages in obese samples were found, both in the subcutaneous and omental white adipose tissue, macrophages were

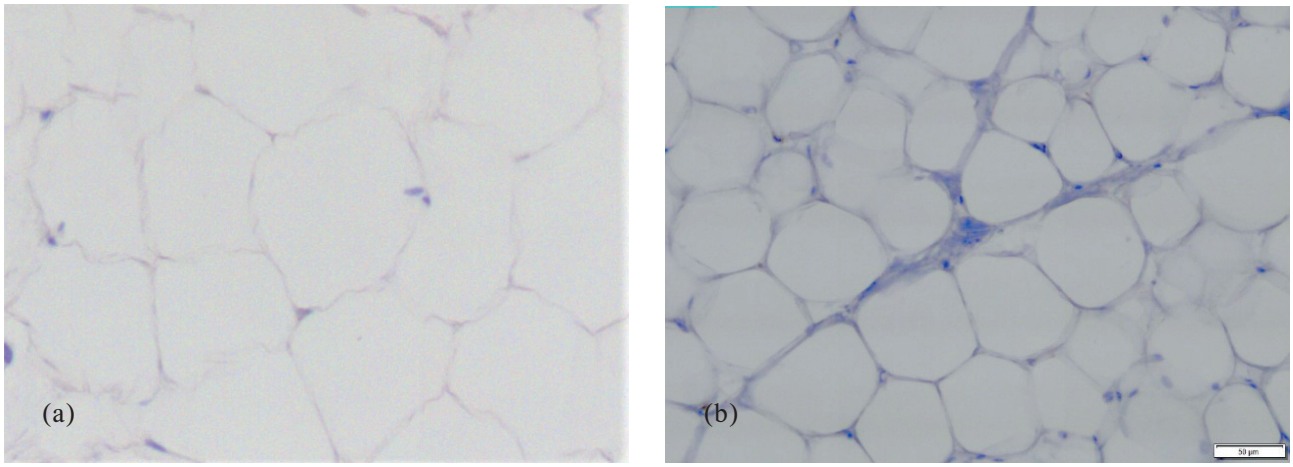

Fig. 1. Normal white adipose tissue in control group: (a) subcutaneous white adipose tissue (20×), (b) visceral white adipose tissue (20×). No macrophages. Immunohistochemical staining CD68.

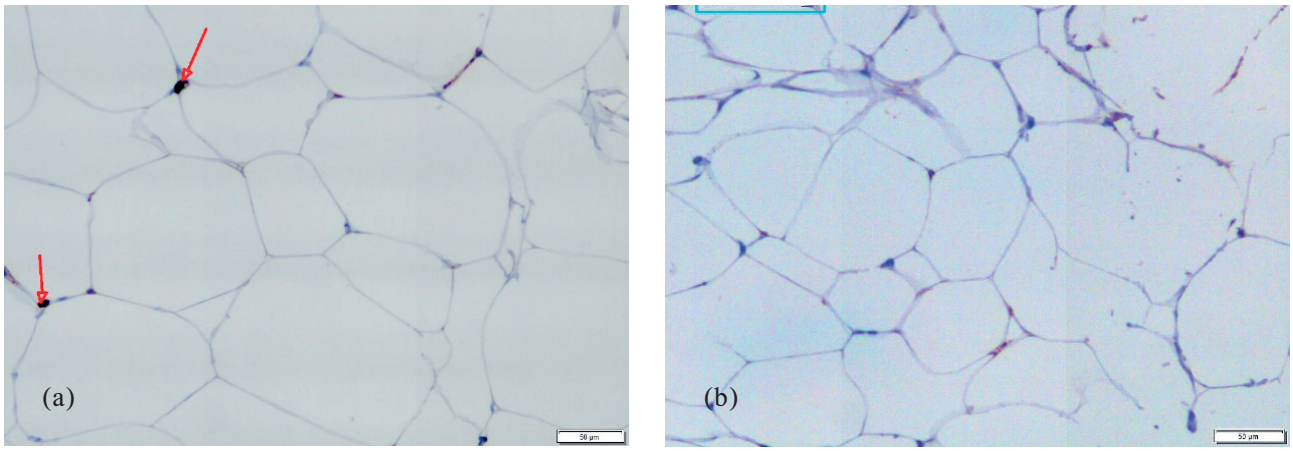

Fig. 2. White adipose tissue in patients without diabetes: (a) subcutaneous white adipose tissue (20×), the arrows indicate the CD204+ macrophages. (b) visceral white adipose tissue (20×). Immunohistochemical staining CD204.

localized in the interstitium with single cells in the thin septa between adjoining adipocytes while groups of macrophages were localized near the small blood vessels, or at the point of contact between more adipose cells. Macrophages surrounding the disintegrating adipocytes, form aggregates similar to macrophage syncytia which are found in a chronic inflammatory environment: known as the crown-like structures (CLS). CD204+ macrophages were found in the same locations as the CD68+ macrophages. Both were also involved in the formation of the CLS structures (Fig. 2, 3).

Quantitative analysis of macrophage numbers in all parameters in both obese patients with T2D and those without T2D demonstrated a statistically highly significant increase in comparison with the control group (Fig. 4). The greatest number of CD68 + and CD204+ macrophages were found in the visceral fat of obese patients with diabetes. The number of CD204+ macrophages were statistically significantly smaller than the CD68+ count. There were no statistically significant differences between the numbers of CD68+ and CD204+ macrophages in subcutaneous adipose tissue (Fig. 4).

DISCUSSION

The aim of this study was to determine the proportion of CD204+ macrophages within the entire population of macrophages in the adipose tissue of obese patients in relation to T2D. Immunohistochemical staining for CD68 is an accepted pan-macrophages marker, while CD204 serves as a useful marker differentiation associated with the M2 subtype of macrophages[1,2]. Most recent studies describe more complex forms of macrophage activation than the previous M1/M2 model. The macrophage phenotype is determined by the spectrum of stimuli in their microenvironment[3].

It has been confirmed that macrophages infiltrate both visceral and subcutaneous adipose tissue in obese patients[4]. We found significantly more CD68+ and CD204+ macrophages in omental, than subcutaneous, fat tissue. As the development of visceral adipocytes differs from the differentiation of subcutaneous adipocytes, it can be assumed that these adipocytes differ in their potential to attract macrophages by stimulating cytokine secretion[5].

Visceral adipose tissue expresses a higher level of cytokines, and participates in the activation of additional genes related to macrophages and inflammation had previously been described[5]. Harmann-Boehm and their collaborators established that, in humans, there is a higher

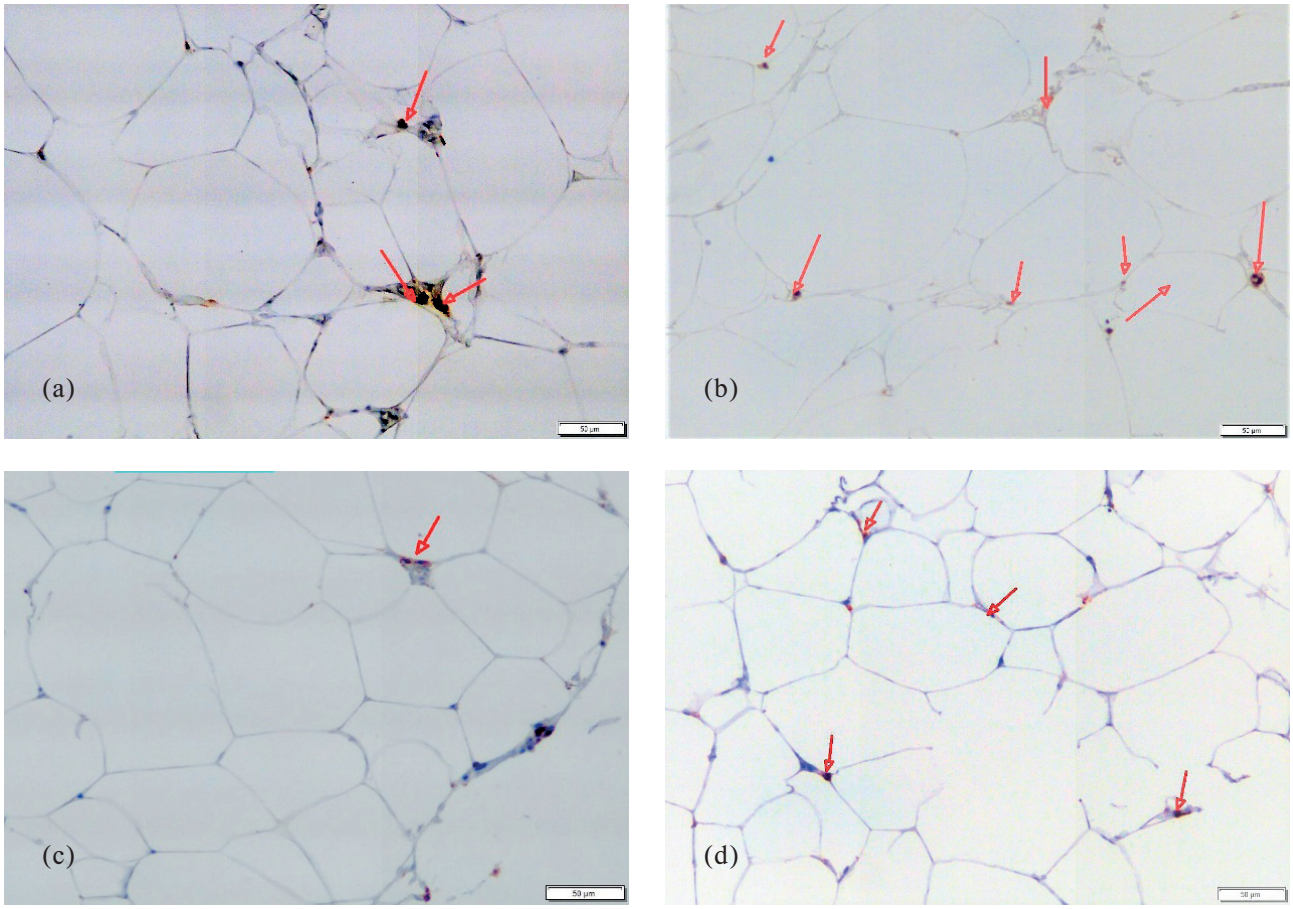

Fig. 3. White adipose tissue in patients with diabetes: (a) subcutaneous white adipose tissue (20×), (b) visceral white adipose tissue (20×). Immunohistochemical staining CD68, (c) subcutaneous white adipose tissue (20×), (d) visceral white adipose tissue (20×). Immunohistochemical staining CD204. The arrows indicate the macrophages.

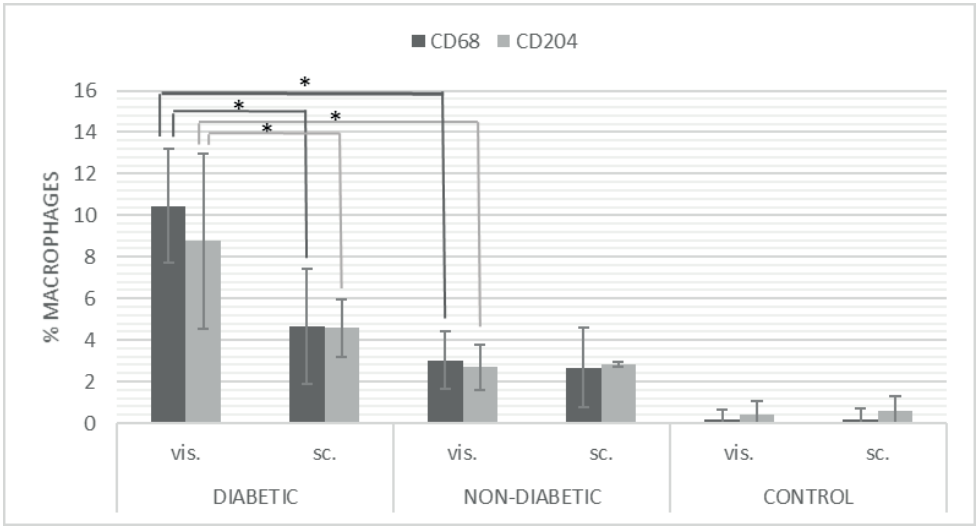

Fig. 4. Number of CD68+ and CD204+ of macrophages in control, diabetic patients and T2D patients. Statistically significant differences are marked with asterisks.

level of macrophage infiltration in omental fat in both non-obese and obese individuals. This is particularly evident in the central type of obesity[4,6].

Obesity, not only increases the number of macrophages within adipose tissue, there is also an evident switch from M2 anti-inflammatory macrophages to M1 pro-inflammatory phenotype. These M1 macrophages produce inflammatory cytokines and chemokines, such as TNFα, IL1, IL6 and MCP-1, that, in turn, induce insulin resistance in adipocytes[7-10].

In white adipose tissue in obese individuals, expression of many markers of M2 macrophages correlate with pro-inflammatory cytokine secretion[11].

Macrophages of obese individuals do not always display surface markers that resemble a state of metabolic activation induced by diverse metabolic stimuli (e.g. free fatty acids, high insulin, high glucose) rather than the classically activated M1 or alternatively activated M2 macrophage types[12].

Decreased inflammatory gene expression (monocyte chemotactic protein [MCP-1], plasminogen activator urokinase receptor [PLAUR], colony-stimulating factor [CSF-3]) in white adipose tissue has been observed following the loss of macrophages resulting from bariatric weight reduction surgery, which is carried out in morbidly obese individuals. Similarly, a decrease in the number of macrophages in adipose tissue, was observed in obese individuals after modifying their lifestyle (diet, exercise) (ref.[13,14]).

Healthy adipose tissue of non-obese individuals contains anti-inflammatory M2 macrophages, which maintain tissue homeostasis and insulin sensitivity by secreting the anti-inflammatory cytokine IL-10. We found few M2 macrophages in our material from non-obese persons. Based on kinetic studies performed in obese mice, other cells including neutrophils, CD8+ T cells and mast cells are likely present prior to the infiltration of adipose tissue by M1 macrophages[15]. Obesity is associated with a loss of balance between the M1 and M2 macrophage populations, where M1 pro-inflammatory macrophages significantly outweigh the number of M2 anti-inflammatory macrophages[16].

Based on the most recent, mainly immunological studies (for review see Russo 2018) (ref.[18]), we can conclude that both CD68+ and CD204+ macrophages evaluated in our specimens are so called a resident macrophage type[17]. In obesity, these cells proliferate and additionally, metabolic signals (such as high insulin, free acids, high glucose, oxidized low-density lipoprotein, oxidized phospholipids) can change the phenotype of these macrophages and they can become metabolically activate[1,18].

CONCLUSION

The straightforward macrophage typing, carried out by immunohistochemistry in samples taken from obese and non-obese patients in this study, confirms a positive correlation between T2D and the total number of adipose tissue macrophages.

Significantly more macrophage infiltration was found in visceral adipose tissue in obesity, and especially in patients with T2D. This finding of a virtually identical quantitative representation of CD68+ and CD204+ macrophages in all studied groups concurs with the functional concept of "resident macrophages" defined by Russo[18]. Our study offers a new evaluation of macrophages and their polarization status, the complexity and various metabolic impacts on the activation of macrophages.

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