Abstract

White adipose tissue from different locations is characterized by significant differences in the structure of adipocyte “secretoma”. Fat accumulation in the central-visceral depots is usually associated with a chronic inflammatory state, which is complicated by the metabolic syndrome. Recently, the adipose tissue was emerged to have an essential role in the innate immunity, adipocytes being considered effector cells due to the presence of the Toll-like receptors (TLRs). In this study, we compared the expression of TNF-α, TLR2 and TLR4 in peripheral-subcutaneous and central-peritoneal adipose depots in three different conditions – lean, obese and obese diabetic – using immunohistochemistry. Our results suggest a correlation between the incidence of the stromal vascular cells and adipocytes TNF-α and TLR4 in the visceral depots in strong correlation with adipose tissue expansion. TLR2 positive cells were seen in the peripheral depots from all groups without any association with fat accumulation. These results focus on the existence of a new pathogenic pathway, the activation of TLR4, for the involvement of visceral adipose tissue in the activation and maintenance of the inflammatory cascade in obesity.

Keywords: human adipose tissue, inflammation, macrophages, TNF-α, TLR2, TLR4.

Introduction

The adult adipose organ is divided in two great depots – subcutaneous/peripheral and visceral/central, having two main locations, mediastinal and abdominal, subdivided into retroperitoneal, intraperitoneal and perirenal depots [1–3].

Recent studies showed that WAT from different locations is characterized not only by discrete structural variations of adipose lobules but also by significant differences in gene expression [7] and hence the biochemical structure of WAT “secretoma” [8] with significant impacts on individual health. A direct relationship between abdominal visceral fat accumulation – apple-shaped obesity, and the emergence and development of some pathological conditions such as the metabolic syndrome or abdominal and pelvic cancers is accepted [9–12].

Pear-shaped obesity – subcutaneous fat accumulation – has a minimal risk for the development of such pathologies even at the same body mass index (BMI) greater than 30 [3].

Numerous studies have been conducted to highlight
the cells residing in the adipose tissue responsible for the synthesis and release of the proinflammatory factors that trigger the metabolic syndrome. TNF-α represents one of the most potent proinflammatory cytokine with proved effect on insulin resistance onset in diabetes [13–16] whose main endogenous source is the adipocytes themselves [17, 18], its level is significantly increased in obese adipose tissue [14] and in the case of metabolic syndrome seems to be synthesized also by the proinflammatory cells [19, 20].

In the adipose tissue, inflammation is triggered and maintained by increased macrophage infiltration of the stromal vascular fraction, more obvious as the adipose tissue develops. The main factors, which attract the macrophages are MCP-1 (monocytes chemoattractant protein-1) [18], MIF-1 (macrophage migration inhibitory factor) secreted by the adipocytes [21], but also TNF-α which upregulates MIF production by local adipocytes [8, 22]. A recent study revealed that the visceral adipose tissue of obese patients is strongly infiltrated with T-lymphocytes, which could initiate the afflux of macrophages through IFN-γ activation [18].

All these studies showed the strong involvement of WAT in the immune function by the infiltration with immunocompetent cells and also by direct secretion of adipocytes.

Isakson P et al. show that TNF-α impairs preadipocyte differentiation forming macrophage-like preadipocytes and human preadipocytes assume a macrophage-like phenotype in the presence of TNF-α or lipopolysaccharide [23], while Chazenbalk G et al. suggest a reverse differentiation pathway of the macrophages from the adipose tissue in preadipocytes [24].

All these studies argue in favor of cell plasticity in the adipose depots and support the important role of a cross-talk between adipocytes and inflammatory cells in obesity.

The presence of toll-like receptors (TLRs) in the adipocytes gave to the adipose tissue a key role in the innate immunity [25–27] such cells being involved in activation and maintaining the inflammatory process by a new pathogenic pathway – TLRs activation.

TLRs, first described by Medzhitov R et al., in 1997 [28], are members of a larger superfamily of interleukine-1 transmembrane receptors that play a critical role in recognition and defense against all types of infectious pathogens, acting in innate, non specific immunity [25]. All the ten types of TLRs described in humans are directed against a lot of pathogen-associated molecular patterns (PAMPS) such as constituent of microbial cell walls – bacterial lipoproteins, pathogen specific nucleic acids – double stranded RNA viruses [29, 30] and therefore are referred as pattern recognition receptors (PRR) [25].

Recently it has been shown that TLRs can also recognize endogenous proteins that have been modified by oxidation or nitration [31] as well as heat shock proteins that are released during acute inflammation and from damaged or dying cells [32]. Both TLR2 and TLR4 were incubated in atherosclerosis and insulin resistance [26, 33], with a reported increased expression and activity of TLR2 and TLR4 in circulating monocytes [34]. The activation of TLR promotes the rapid induction of intracellular proinflammatory cascade and the up-regulation of cytokines, chemokines and adhesion molecules [26, 33].

Despite the synergism of biological mechanisms triggered by the activation of TNF-α synthesis and TLRs receptors in the same cells from the adipose tissue which complete by the onset of the metabolic syndrome and insulin resistance, there is no data in the literature on the correlation between their synthesis and expression in adipose tissue in relation to the distribution of adipose depots.

In this paper, we proposed a study of TLR2 and TLR4 expression in peripheral-subcutaneous and central-peritoneal adipose depots in both obese and diabetic patients compared with obese but normoglycemic patients, and the correlation of their expression with the incidence of TNF-α-positive cells within the same adipose depots.

Materials and Methods

Subjects

All subjects were recruited from the Emergency County Hospital of Craiova. The subjects, males and females aged between 45 and 73 years, were distributed into three groups:

• 1st Group – control, lean patients (n=7) with a body mass index (BMI) <30. Other exclusion criteria for lean subjects were: fasting plasma glucose level >100 mg/dL, taking no medication for lowering blood pressure, and LDL-cholesterol level >240 mg/dL.

• 2nd Group – obese patients (n=4) with a BMI >30, with abdominal obesity according the criteria of the International Federation of Diabetes >94 cm in men and >80 cm in women [35]. Exclusion criteria for obese subjects were: fasting plasma glucose level >100 mg/dL, taking no medication for lowering blood pressure, and LDL-cholesterol level >240 mg/dL.

• 3rd Group – obese and diabetic patients (n=4), patients clinically diagnosed with type 2 diabetes mellitus, no exclusion criteria for LDL-cholesterol or blood pressure values.

Human adipose tissue samples were obtained after abdominal surgery for gastric ulcer, gallbladder stones or herniotomy.

For all groups’ patients with abdominal or pelvic surgical pathologies with any inflammatory response on tissues nearby (malignant tumors or peritoneal reactions, which are the result of local pathology) and those with metabolic disorders, autoimmune and infectious diseases were excluded. For each patient two samples were obtained: (1) subcutaneous periumbilical and (2) peritoneal. Informed consent was obtained from all patients included in the study, which was approved.
Role of innate immune receptors TLR2 and TLR4 as mediators of the inflammatory reaction in human visceral...

by the Ethics Committee of the University of Medicine and Pharmacy of Craiova.

**Histological staining**

For each patient, immediately after sampling, tissue fragments were fixed in 10% buffered formalin for 24–48 hours at room temperature and then processed for paraffin embedding. Sections of 3–4 μm were obtained with a rotary microtome and routinely stained with Hematoxylin–Eosin and Masson’s trichrome.

**Immunohistochemistry**

Sections of 3-μm were dewaxed and rehydrated. Antigen retrieval was performed after microwave incubation of sections in 10 mM citrate buffer, pH 6. Endogenous peroxidase was blocked after incubation with normal swine serum, and then the slides were incubated over night, at 4°C, with one of the primary antibodies mentioned in Table 2.

**Table 2 – Antibodies used for the immunohistochemical study**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>Mouse monoclonal anti-human CD68</td>
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<td>Abcam, KP1ab955</td>
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<tr>
<td>Rabbit polyclonal anti-human TNF-α</td>
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<td>Abcam, ab6671</td>
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<tr>
<td>Rabbit polyclonal anti human TLR2</td>
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<td>Abcam, ab24192</td>
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<tr>
<td>Rabbit polyclonal anti human TLR4</td>
<td>2 μg/mL</td>
<td>Abcam, ab13556</td>
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Sections were then washed and processed for amplification of the immune signal using the polyclonal swine anti-mouse, anti-rabbit biotinylated IgG Multi-Link (Dako) and the Avidin–Biotin complex (Vector Laboratories). 3,3′-Diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide (Merck) were used for color development and Mayer’s Hematoxylin for nuclear counterstaining. Slides were observed and registered with a Nikon Eclipse microscope connected to a digital camera. Images were finally processed using the Microsoft Office Picture Manager. For each antibody tested, a negative control in which the primary antibody was replaced by 10 mM phosphate buffer saline, pH 7.4–7.6 was performed.

**Evaluation**

The evaluation of the immunohistochemical results was performed by two different observers, according to the following: immunohistochemical reactions (brown deposits in labeled structures) were graded as absent (negative signal) or present (moderate or strong intensity of the signal) in all microscopic fields from the whole slide. In order to compare the immunohistochemical data, we randomly selected five different microscopic fields at ×20 magnification from three sections of each individual depot. The total number of cells and positive cells for every antibody was counted for each field. The fraction of positive cells for each sample was reported as: (-) – less than five positive cells; (+/-) – 5–10 positive cells; (+) – 10–50 positive cells; (++) – more than 50 positive cells.

Results

Both sites fat – peripheral and central – showed lobular organization of the adipose tissue with a stromal vascular fraction better represented in the central depots where the lobules were smaller and even absent in lean subjects when mainly isolated adipocytes were noticed. Subcutaneous depots showed bigger adipocytes and, generally, more uniform in size compared to those localized in the peritoneal adipose lobules (Figure 1, a and b).

**CD68 immunoexpression**

In lean patients, both types of depots showed a very low number of proinflammatory CD68-positive cells in the interstitial space between adipocytes, not more than two cells for each microscopic field (Figure 2, a and b).

An increased vascularization was observed in the peritoneal depots without an increase of the number of CD68-positive immunocompetent cells. The subcutaneous abdominal adipose tissue of obese and lean patients showed similarity for the expression of CD68.
In the visceral adipose depots, we observed an increased number of small and medium dilated vessels with many CD68-positive cells (Figure 2, c and d).

Both types of depots obtained from diabetic obese patients presented an increased number of blood vessels not only in the vascular stroma but also between the adipocytes. In the peritoneal connective tissue, we observed leukocyte margination with CD68-positive cells but we did not notice the presence of macrophage crowns in any of the samples analyzed (Figure 2, e and f).

**Figure 2 – CD68 immunoexpression in lean subcutaneous (a, ×400) and peritoneal depots (b, ×400) vs. obese subcutaneous (c, ×400) and peritoneal (d, ×400) or obese diabetic peripheral (e, ×200) and central peritoneal depots (f, ×200) (CD68 immunohistochemistry).**

**TNF-α immunoexpression**

All the samples obtained from lean subjects displayed negative reaction in adipose depots from both peripheral and central location (data not show). In the peripheral subcutaneous depots from obese patients, the positive reaction for TNF-α was present not only in the proinflammatory cells, but also in some endothelial cells (Figure 3a) and absent in adipocytes.

In central peritoneal depots from the obese patients, the positivity for TNF-α was present in macrophages and monocytes inside the vessels and also in few adipocytes. As a common feature of the central depots in obese patients, we mention the presence of some cells with one or few extremely small vacuoles, which could be delipidated cells or precursors beginning to accumulate fat (Figure 3b).

Subcutaneous depots from obese diabetic patients showed the same expression of TNF-α. Visceral adipose depots presented an increased number of TNF-α-positive intravascular leukocytes but also its presence in the cytoplasm of adipocytes and endothelial cells (Figure 3, c and d).
**TLR immunoexpression**

Both the subcutaneous and visceral depots form lean subjects showed an inconstant presence of the receptors TLR2 and TLR4 in immunocompetent cells from the stromal vascular fraction but the absence of those in other cells (endothelial cells and adipocytes) (Figure 4, a and b).

In obese and obese diabetic patients, TLR2 showed a similar distribution in the central depots, being present in immunocompetent cells and inconstantly present in the endothelial cells and some adipocyte membranes (Figure 4d).

In the peripheral subcutaneous depots, the immuno-reaction for TLR2 was more heterogeneous between samples – sometimes absent sometimes present in pro-inflammatory cells (Figure 4c) without relation to the presence of diabetes or obesity.

The immune reaction for TLR4 in peripheral depots was different to that of TLR2, variable in intensity in the stromal vascular fraction (immunocompetent and endothelial cells) from both obese and obese diabetic patients (Figure 4, e and g), and a more frequent positive reaction in the peritoneal depots, not only in the stromal vascular fraction but also in the majority of adipose cells (Figure 4, f and h).

Table 3 summarizes the results of the immunohistochemical reactions.

![Figure 3 – TNF-α immunexpression in obese subcutaneous (a, ×400) and visceral depots (b, ×400) vs. obese diabetic visceral depots (c and d, ×400) (TNF-α immunohistochemistry).](image)

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Obese</th>
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<tr>
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<td>sc</td>
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sc – Subcutaneous depot; per – Peritoneal depot; icc – Immunocompetent cell; ves – Vessels; adc – Adipose cells.
**Discussion**

Fat accumulation, especially in visceral and less in subcutaneous depots, is linked to the onset and maintenance of a state of chronic inflammation associated with metabolic disorders and cardiovascular events. Increasing of BMI>30, so accumulation of fat, is not a sufficient condition for the onset of metabolic syndrome, being necessary the simultaneous storage of adipose tissue in special locations – visceral/central, causing apple-shaped obesity [3].
This condition points to the difference of biologically active substances, especially cytokines such as IL6 and TNF-α, synthesized by the adipose tissue in relation to its various locations. Susceptibility of fat accumulation with specific topography is determined by genetic factors, and regional variation at metabolic level is associated with variation in adipose gene expression [7]. Metabolic dysfunctions occurring consecutively to the central fat accumulation may be due to blood drainage coming from the abdominal viscera to the liver via the portal vein, but also to a close anatomical and functional association between visceral adipose tissue, especially the peritoneal fat, and the immune/inflammatory response of the body. This connection became more apparent with emphasis of the presence of T-lymphocytes infiltration in visceral adipose tissue [18] or functional TLR2 and TLR4 on human adipocytes [25].

In this paper, we correlated the expression of TNF-α, the main pro-inflammatory cytokine, with that of the innate immunity receptors TLR2 and TLR4 in cells of adipose tissue with different locations, peripheral and central peritoneal, so with a different genetic pattern of activation of the adipose secretoma.

The expansion of the adipose tissue in obesity determines an increase of the number of proinflammatory cells through the concomitant enrichment of the stromal vascular fraction. It is accepted that the intersistium of the adipose tissue is invaded by a great number of macrophages, mainly in the central adipose depots [36, 37]. Cinti S et al. explain this infiltration of monocytes-macrophages by the chemoattractant action of necrotic adipocytes on blood macrophages [38]. Necrosis favors the elimination of cell content in the surrounding medium where it induces the onset of the anti-inflammatory cascade [39].

Our results suggest a more increased number of CD68-positive cells in the blood vessels and in the interstitial space between adipocytes in the central-peritoneal depots of obese patients and in the central and subcutaneous depots in obese diabetic patients.

Many studies sustain that the cells of the stromal vascular fraction of the adipose tissue represent the main source of proinflammatory cytokines, including TNF-α and IL6 [19, 20, 36]. We consider that the results regarding the levels of positivity for the inflammatory cytokines, including TNF-α, must be analyzed in the context of all the cells which reside in the adipose depots – the adipocytes and the stromal vascular fraction.

TNF-α synthesis in the adipose tissue is still controversial. Fontana L et al. sustain that its secretion has the same intensity in the subcutaneous and visceral depots [40] while Winckler G et al. report higher blood levels of TNF-α related only to the volume of adipose cells [41] and in this way, somewhat surprising, they have observed a slightly higher expression of TNF-α protein in the subcutaneous depot, both for lean an obese patients.

Studies performed by Mohamed-Ali’s group mention a release of IL6 but not of TNF-α by the subcutaneous adipose tissue in vitro [42].

In accordance with the last report, our results suggest the absence of TNF-α positive cells in all the adipose depots in lean subjects, while the stromal vascular cells and adipocytes from obese and obese diabetic patients showed the presence of TNF-α protein.

We can sustain the observation that the size of adipocytes has an indirect effect on TNF-α synthesis because of the lysis of triglyceride-overloaded adipocytes followed by monocytes affluence and their activation, which is in accordance with the observations of other researchers [36, 38]. Also, on our preparations the visceral depots presented adipocytes of various sizes, some of them containing small fat droplets coexisting with CD68-positive cells.

The incidence and intensity of positivity for TNF-α was positively correlated to that of TLR4 but not of TLR2.

Recently, Bès-Houtmann S et al. proved that TLR2 and TLR4 were expressed on the surface of human adipocytes in higher levels compared with monocytes and that adipocyte stimulation with LPS or with lipoteichoic acid, ligands for TLR4 and respectively for TLR2, raises the TNF-α production in cultures of mature human adipocytes [25].

The feature that activation of TLR’s leads to production of proinflammatory cytokines, such as TNF-α [43, 44], IL6 [45] was proved, thus TLR4 activation contributes to the inflammatory process in obesity and the onset of the metabolic syndrome.

Our results suggest that the same cells, mainly macrophages and endothelial cells, showed positive immune reaction for both TNF-α and TLR4, the positive reaction being noticed only in the peritoneal adipose depots of obese and obese diabetic patients.

We did not observe a significant correlation between the positive reaction for TLR2 and TLR4 in the subcutaneous adipose tissue, TLR2 showing an inconstant presence in the subcutaneous depots of all groups. The small number of cases studied and the existence of an abdominal pathology does not exclude that this positivity is induced by TLR activation by their specific ligands. Positive reaction for TLR4 was found not only in the immunocompetent cells but also in cells from the vessel walls and visceral adipocytes from obese and obese diabetic patients.

Excluding the activation similar to that proposed for TLR2, we assume that TLR4 could be activated by the release of free fatty acids by necrotic adipocytes. But, the activation of TLR2 and TLR4 by saturated fatty acids is only a partially accepted mechanism [26, 33, 46, 47]. Recently, an alternative role of TLR4 activation has emerged, attributing an important role to the bacterial environment of the digestive tract as well as to that of saturated lipids in food [44]. But, these studies do not explain the individual differences in susceptibility toward the development of the metabolic syndrome despite the colonization of bowel with ligands for TLRs.

**Conclusions**

We sustain the correlation between the incidence of
TNF-α and TLR4-positive stromal vascular cells and adipocytes in abdominal obesity and obesity associated with type 2-diabetes mellitus.

Evaluation of the proinflammatory effect and its metabolic consequences, which succeed the expansion of adipose tissue needs the study of the involvement of all types of cells – adipocytes, macrophages, and endothelial cells – from the adipose organ. The adipocytes close the vicious circle of inflammation in obesity, representing both the source and the effectors of proinflammatory factor synthesis. Following studies are necessary to understand the role of TLR in local homeostasis of the adipose tissue.

References


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