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Hypoxia induced VEGF synthesis in visceral adipose depots of obese diabetic patients

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Abstract

VEGF is one the pro-inflammatory adipokines synthesized by the "adipose secretoma" of obese subjects as a response to hypoxic conditions; but the main function of VEGF is angiogenesis, being recognized as the most important factor increasing blood capillaries in the adipose tissue by stimulating endothelial cell growth. In this paper, we propose a comparative study of the vascular response to VEGF synthesis in the subcutaneous and central-peritoneal adipose depots in lean, obese and obese diabetic patients. We used CD31 to label the endothelial cells in order to evaluate the response of the vascular network to VEGF synthesis. Our results showed an increase of VEGF protein synthesis in obese and obese-diabetic patients compared to lean subjects where the protein was absent. The positivity for VEGF in obese diabetic samples was observed in numerous structures from the adipose depots, both in the stromal vascular fraction – blood vessels and stromal cells – as well as in the cytoplasm of adipocytes. Positivity in the vascular wall was observed more frequently in areas of perivascular and intralobular fibrosis. Obese and diabetic patients showed similar incidence of CD31 immunoreactivity with lean subjects in both subcutaneous and peritoneal depots. In conclusion, human adipose depots show a different incidence of VEGF positive cells in relation with their disposal and the metabolic status. VEGF synthesis in visceral adipose tissue is inefficient being not followed by angiogenesis to counterbalance tissue hypoxia. We suggest that may be a pathogenic link between the degrees of intralobular fibrosis in adipose depots and VEGF expression.

Keywords: human adipose tissue, obesity, diabetes, VEGF, angiogenesis, fibrosis.

⌂ Introduction

Adult adipose organ is composed by two types of adipose depots divided into adipose lobules of unilocular adipose tissue (WAT – White Adipose Tissue) composed of unilocular cells sustained by the stromal-vascular fraction well vascularized and innervated [1–3]. Anatomically, the adult adipose organ is divided in two great depots: subcutaneous/peripheral and visceral/ central, which differ through the composition of the stromal vascular fraction [3–5]. As the leptin gene was discovered [6], WAT was considered not only an organ for energy storage but also an endocrine organ and, recently, an immune organ [7–11].

Recent studies showed that WAT from different locations is characterized by a different structure of the "adipose secretoma" [8], term which referrers to the chemical substances – adipokines or adipocytokines – synthesized by the whole adipose tissue (adipocytes and cells from the stromal vascular fraction). These adipokines are humoral factors with numerous metabolic and vascular effects. In obesity, adipocytokines' synthesis is impaired [12]. For this reason, it is accepted a direct relationship between the levels of pro-inflammatory

adipokines and the emergence and development of some pathological conditions such as the metabolic syndrome or a series of abdominal and pelvic cancers associated with apple-shaped obesity that means visceral/central fat accumulation [13–16]. The metabolic syndrome cumulates all the factors which increase the risk for heart attack, stroke and type 2 diabetes mellitus [17].

Vascular Endothelial Growth Factor (VEGF) synthesized by adipocytes [18] acts indirectly in inflammation by increasing vascular permeability. The main function of VEGF is angiogenesis being recognized as the most important factor increasing blood capillaries in the adipose tissue by stimulating endothelial cell growth [6, 19]. Although adipose tissue is recognized as highly vascularized, almost every adipocytes surrounded by a capillary [20], some studies showed that as adipose tissue mass expands in obesity, clusters of enlarged adipocytes became distant from the vasculature, leading to local pockets of hypoxia [10, 21]. It is thus clear that further development of fat mass requires branching of parenchymal capillary network.

It is widely suggested that in obese patients WAT depots are hypoxic [10, 22, 23]. VEGF is strongly

induced in hypoxic condition by HIF (Hypoxia Inducible Factor) [24]. HIF-1α has been shown to be present in cultured adipocytes and the amount of protein increases as oxygen pressure decreases [25, 26].

In the literature, there are no reports on the expression of VEGF in cells from human adipose tissue in different nutritional and metabolic conditions, such studies being conducted only on cell cultures and animals models.

A number of genes encoding pro-inflammatory adipokines seems to have an increased expression in human and murine adipocytes cultured under hypoxic conditions: apelin, interleukin-6 (IL-6), leptin, macro-phage migration inhibitory factor (MIF), plasminogen activator inhibitor-1 (PAI-1), VEGF and visfatin [10, 25–28] while hypoxia inhibits adiponectin synthesis [26, 27].

In mice and rats, VEGF expression and synthesis are attributed mostly to adipocytes [29, 30] and appears to be dependent on considered depots, in rats being higher in the omental depot [29].

In this paper, we propose a comparative study of the vascular response to VEGF synthesis in the subcutaneous and central-peritoneal adipose depots in lean, obese and obese diabetic patients.

⊟ Materials and Methods

Subjects

Patients were recruited from the Emergency County Hospital of Craiova; males and females aged between 45–73 years were distributed into three groups:

▪ **Ist Group** – control, lean patients (*n*=5) with a normal body mass index (BMI) <30; 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.

▪ **IInd Group** – obese patients (n=5) 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 [31]; 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.

▪ **IIIrd Group** – obese and diabetic patients (*n*=5), clinically diagnosed with type 2 diabetes mellitus, not 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 were excluded 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. For each subject, two samples were obtained: (i) subcutaneous (peri-umbilical) and (ii) central (peritoneal).

Informed consent was obtained from all patients included in the study, which was approved by the Ethic Committee of the University of Medicine and Pharmacy of Craiova.

Histological staining

Immediately after sampling from each patient, adipose 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

Serial sections of 3 μm were dewaxed and rehydrated. Antigen retrieval was performed after microwave incubation of sections in the appropriate buffer. Endogenous peroxidase was blocked after incubation with hydrogen peroxide–methanol solution. After blocking unspecific binding, sections were incubated over night, at 4^0C, with one of the mouse monoclonal primary antibodies mentioned in Table 1.

Table 1 – *Panel of antibodies used for the immuno-histochemical study*

Antibody	Dilution	Source	Antigen retrieval	Method
VEGF	1:50	Abcam (clone VG1)	Citrate buffer, pH 6	LSAB+/HRP (Dako)
CD-31	1:40	Dako (clone JC70A)	Tris-EDTA, pH 9	EnVision+Dual Link System/HRP (Dako)

Then, sections were washed and processed for amplification of the immune signal using the appropriate method as mentioned in Table 1. 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 coupled to a digital camera. Images were finally processed using the Microsoft Office Picture Manager. For each antibody tested, was performed a negative control in which the primary antibody was replaced by 10 mM phosphate buffer saline, pH 7.4–7.6.

Evaluation

The evaluation of the immunohistochemical results was done 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.

⊟ Results

Fat pads from obese and obese diabetic samples displayed similar histological features: large adipose depots composed of lobules extremely expanded. Adipocytes from the peritoneal depots showed greater variability of their size than those subcutaneous which were composed of bigger cells, more homogenous in size. In both locations we noticed well represented connective septa between adipose lobules, fibrotic appearance being generally more evident in obese patients compared to those with normal weight (Figures 1 and 2). Trichrome stain revealed collagen accumulation, mainly in the periphery of lobules and also perivascular in the peritoneal depots (Figure 3). In one case, we noticed the presence of fibrotic intralobular septa within the subcutaneous depots (Figure 2a).
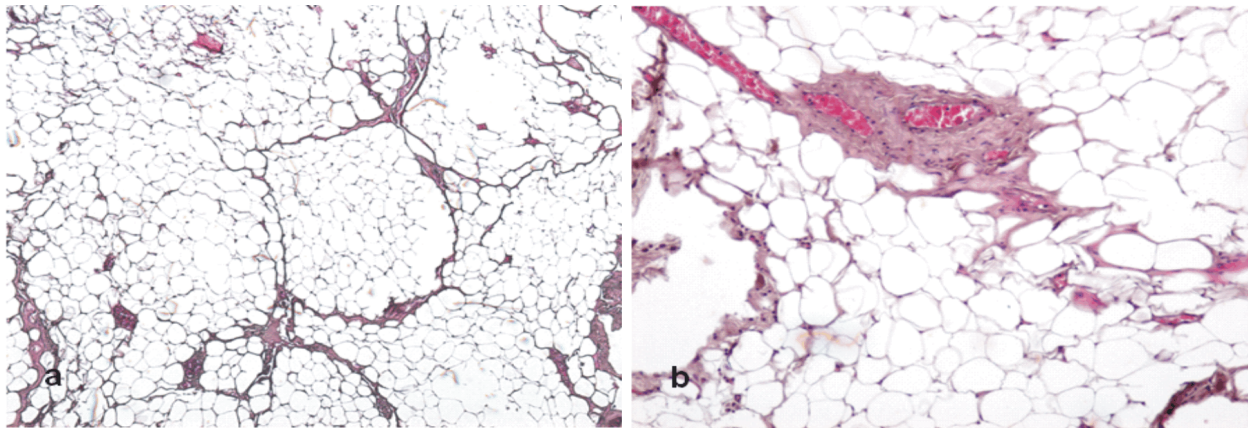
Figure 1 – *General view of the adipose depots from an obese patient: (a) Subcutaneous (HE stain, ob. ×4); (b) Peritoneal (HE stain, ob. ×10).*
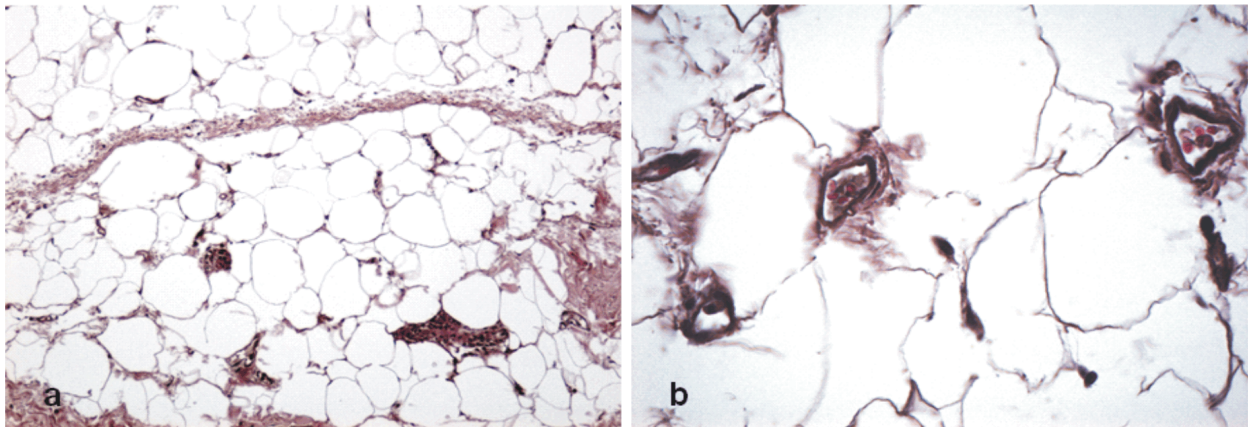

Figure 2 – *General histological view of the adipose depots from an obese-diabetic patient: (a) Subcutaneous (HE stain, ob. ×10); (b) Peritoneal (HE stain, ob. ×40).*
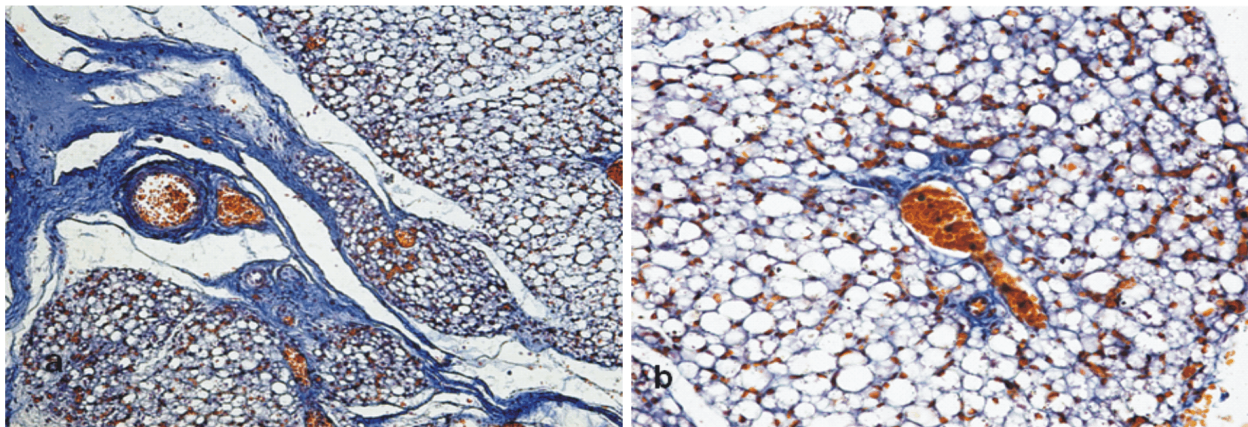

Figure 3 – *Fibrosis in the adipose depots from an obese-diabetic patient: (a) Interlobular (Masson's trichrome stain, ob. ×4); (b) Perivascular (Masson's trichrome stain, ob. ×10).*

Both the subcutaneous depots and those central-peritoneal obtained from the group consisting of normal weight patients revealed a normal number of capillaries among adipocytes – one or two CD31 positive capillaries for each adipocyte; capillaries were small, with a thin wall and without dilatations or the presence of immunocompetent cells in the capillary lumen. In these patients, the reaction for VEGF was negative in all lobular structures from both subcutaneous and visceral adipose depots (Figure 4). Obese and lean patients showed some differences regarding the vascular histology of adipose depots. The incidence of capillaries in the subcutaneous depots was almost similar while, in the

interstitial spaces between adipocytes from the visceral depots, blood vessels were fewer, but larger and more dilated in the vascular stroma and in fibrotic bundles between adipose lobules (Figure 5, a and b). In obese patients, VEGF showed a positive reaction in both subcutaneous and visceral depots, in the last location the presence of this growth factor being observed not only in the vessel wall but also in the cytoplasm of adipocytes (Figure 5, c and d).

Obese and obese-diabetic patients showed similar incidence of CD31 immunoreactivity with lean subjects in both subcutaneous and peritoneal depots.

The positivity for VEGF in obese diabetic adipose

samples was present in numerous structures both of the stromal vascular fraction – blood vessels and stromal cells – as well as in the cytoplasm of adipocytes (Figure 6).

Positivity in the vascular wall was observed more frequently in areas of fibrosis or in endothelial cells from the thickened vessels (Figure 6, a and c).
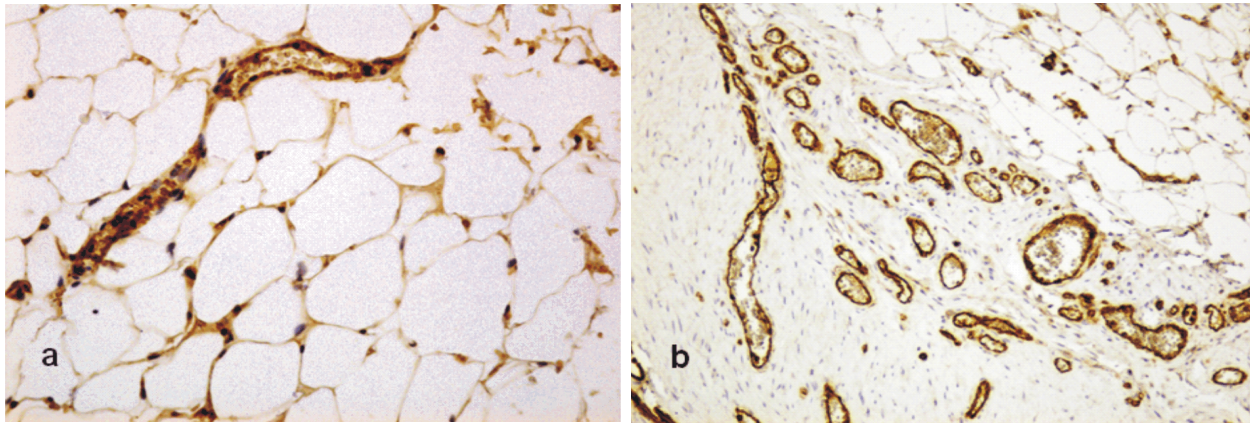

Figure 4 – CD31 and VEGF immunostaining in the adipose depots of a lean subject: (a) CD31 positive cells in a subcutaneous depot, ob. ×20; (b) CD31 positive cells in a peritoneal depot, ob. ×10; (c) VEGF immunoreaction in a visceral depot, ob. ×40.

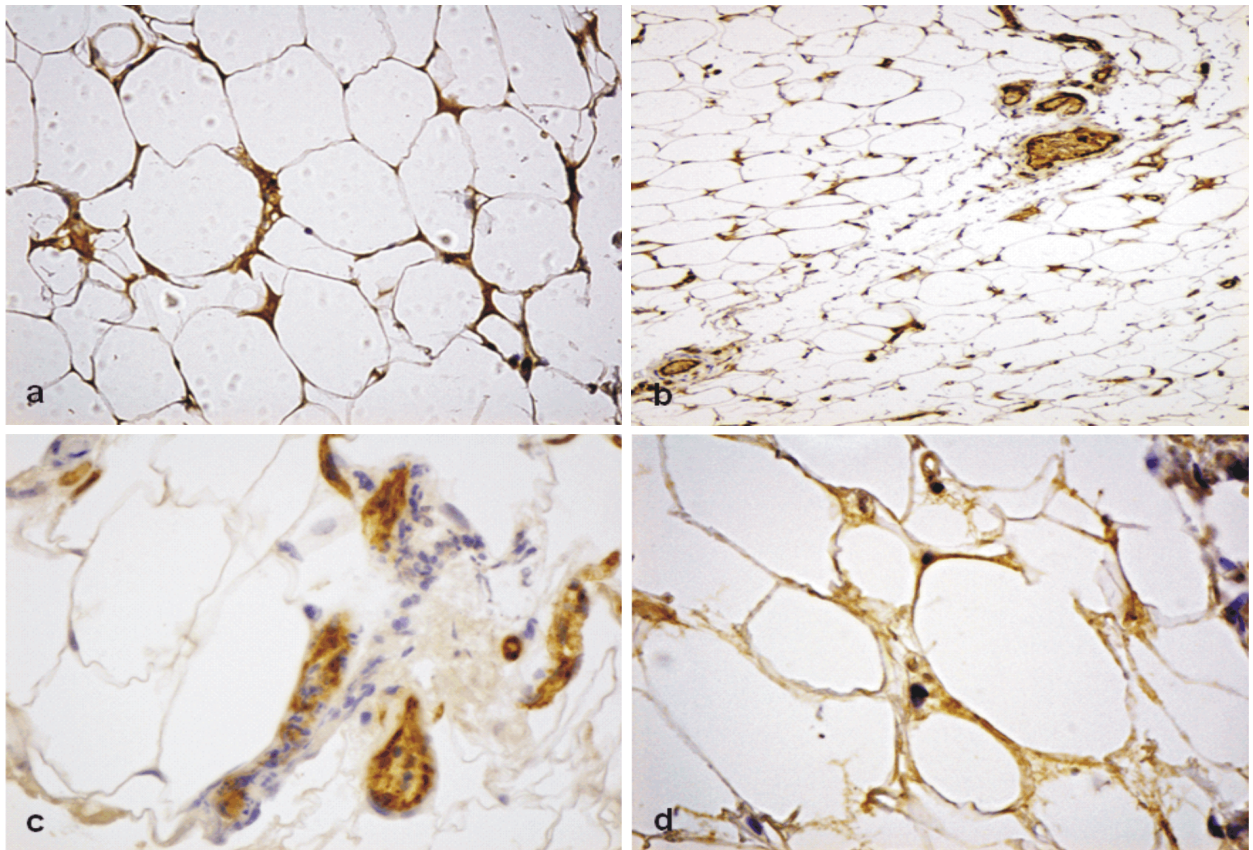**Figure 5 – CD31 and VEGF immunostaining in the adipose depots of an obese patient: (a) CD31 in a subcutaneous depot, ob. ×40; (b) Peritoneal expression of CD31, ob. ×40; (c) VEGF in a subcutaneous depot, ob. ×40; (d) VEGF in a peritoneal depot, ob. ×40.**
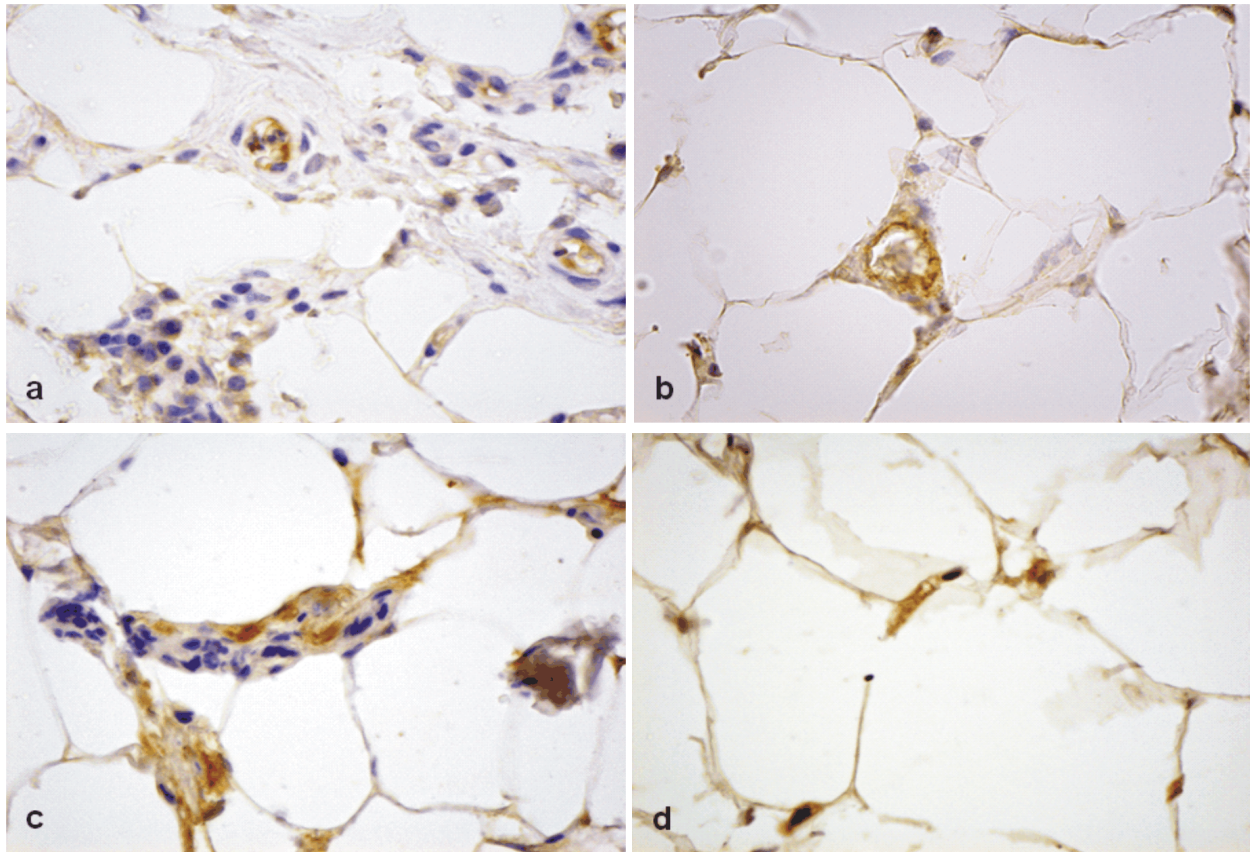
Figure 6 – *VEGF immunostaining in the adipose depots of an obese-diabetic patient subcutaneous depot: (a) ob. ×20; (b) ob. ×40; Peritoneal depot (c – ob. ×20; d – ob. ×40).*

Discussion

There are several theories explaining the change of cytokines/adipokines expression in obesity causing the permanent state of inflammation with metabolic consequences.

Trayhurn P *et al.* (2008) in a synthesis of these pathogenic pathways show three categories of changes responsible for the demodulation of pro-inflammatory cytokines in obesity: oxidative stress, endoplasmic reticulum stress and local hypoxia [10]. However, the authors showed that these pathways are not necessarily mutually exclusive, since endoplasmic reticulum stress and the generation of reactive oxygen species can be induced by hypoxia, so they incriminate hypoxia as the underlying cause in obesity. Hypoxia in turn induces the initiation of an inflammatory response so as to increase blood flow and stimulate angiogenesis. The main molecule responsible for angiogenesis is VEGF, which is synthesized as a response to HIF-1 action [10].

In this study we showed that there are differences regarding VEGF synthesis in the various cells from the adipose depots in relation to the nutritional and metabolic status of subjects (lean, obese and obese-diabetic) and also that this synthesis varies in different depots (subcutaneous or peritoneal).

These observations regarding the heterogeneity of the adipose secretoma were made also in connection with the increased synthesis of other molecules involved in the inflammatory response in obesity, such as TNF-α, TGF-β, interleukins or PAI-1 [32, 33] while others, such as adiponectin, recognized for its anti-inflammatory effect, has decreased expression in obesity [34] as well as in conditions of hypoxia [10, 27].

As we showed in our immunohistochemical study, VEGF positivity was higher in the adipose tissue of obese and obese-diabetic patients, especially in peritoneal depots. In normal weight subjects, both peripheral and central depots were VEGF negative.

Our results sustain an increasing trend of VEGF shared with other pro-inflammatory cytokines, which lead us to support the involvement of this growth factor in the metabolic changes of visceral obesity. How fat is normally highly vascularized, the increase of the adipose depots by increasing cell size and number of adipocytes requires additional vascular bed which is not the underlying rate of adipose depots resulting hypoxia. For this reason, angiogenesis has been considered to be a rate limiting step for fat tissue expansion [35].

Angiogenesis in the adipose depots of obese animals and in cell cultures seems to be a controversial event. Several authors argues inhibition of angiogenesis in the late stages of metabolic dysfunctions as confirmed by decreased adiponectin, a pro-angiogenic factor, in the visceral depots of patients with metabolic syndrome.

Another hypothesis to explain the persistence of hypoxia in obesity is that adipocytes frequently fail to mount a proper response to local hypoxia and do not produce sufficiently high levels of VEGF [36].

Our reactions showed an increase of VEGF protein synthesis in obese and obese-diabetic patients compared to lean subjects where the reaction was negative. In obese samples, we noticed VEGF positivity in endothelial cells and some cells from the stromal vascular fraction

while in obese-diabetic VEGF protein was present also in adipocytes.

Overall incidence of positive structures was increased in visceral depots compared to those subcutaneous, which is consistent with the results obtained by other researchers regarding other substances synthesized by adipocytes from central depots, particularly TNF-α, IL-6 and PAI-1 [3, 17].

There are authors who showed that angiogenic potential of adipose depots varies with respect to their location.

The vascular density and abundance of endothelial cells is higher in visceral adipose depots compared to subcutaneous fat pads and endothelial cells in central location exhibit more potent angiogenic and inflammatory properties [37].

Our results with CD31 labeling showed that VEGF synthesis is not accompanied by an increase in the number of capillaries; however, we noticed their expansion and an increased VEGF synthesis within these dilated vessels.

Blood vessels embedded in fibrotic areas showed also an increased synthesis of VEGF. In diabetic patients, fibrosis was more pronounced in central depots. Our results indicate that central adipose depots of subjects with metabolic syndrome present two types of changes: an inefficient synthesis of VEGF in the local structures as a response to hypoxia that do not cause a proper expansion of vascular bed and a relative grade of fibrosis which in turn maintains the hypoxia through a mechanical effect. There are several recent studies involving fibrosis of adipose depots in installing hypoxia and insulin resistance [36, 38].

Heterogeneity of unilocular adipose depots occurs also at the level of fibrosis, those visceral seeming to be not only less fibrotic than those peripheral in obese patients but also with a different distribution of collagen fibers, especially pericelular or intraparenchymatous [38]. This observation contradicts the known biological sequence of fibrosis development as a result of an abnormal activity of extracellular matrix synthesis and storage by fibroblasts activated in an inflammatory environment and, as we previously mentioned, adipose visceral depots are more inflamed than those sub-cutaneous [33].

It seems that not in all conditions, the expansion of adipose tissue is associated with inflammation. Subjects named "metabolically healthy obese" expend their adipocyte depots without inflammation consequences. This kind of expansion is associated with an enlargement of a given fat pad through recruitment of new adipocytes, along with the adequate development of the vasculature, minimal associated fibrosis, and the lack of hypoxia and inflammation [18, 35].

⊟ Conclusions

Human adipose depots show a different incidence of VEGF positive cells in relation with their disposal – subcutaneous or visceral – and the metabolic status. VEGF synthesis in visceral adipose tissue is inefficient being not followed by angiogenesis to counterbalance tissue hypoxia. We suggest that may be a pathogenic link between the degrees of intralobular fibrosis in adipose depots and VEGF expression.

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