Lymphatic vessels in human adipose tissue

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Abstract
Despite being considered present in most vascularised tissues, lymphatic vessels have not been properly shown in human adipose tissue (AT). Our goal in this study is to investigate an unanswered question in AT biology, regarding lymphatic network presence in tissue parenchyma. Using human subcutaneous (S-) and visceral (V-) AT samples with whole mount staining for lymphatic specific markers and three-dimensional imaging, we showed lymphatic capillaries and larger lymphatic vessels in the human V AT. Conversely, in the human SAT, microcirculatory lymphatic vascular structures were rarely detected and no initial lymphatics were found.

Keywords Lymphatics · Visceral · Subcutaneous · Adipose · Vessels

Introduction
The lymphatic system, an essential component of the immunological system, is responsible for draining the remaining 10% of interstitial fluid that does not return to blood veins through an extensive network of capillaries and vessels spread throughout the body (Jeltsch et al. 2003; Tammela and Alitalo 2010). Lymphatics are connected to lipid metabolism by lymphatic vessels in the small intestine and mesentery, which transport vitamins and lipids from diet (Kohan et al. 2011; Tammela and Alitalo 2010). As well as by the involvement in the removal of cholesterol from peripheral tissues (Nanjee et al. 2001; Martel et al. 2013; Randolph and Miller 2014). In spite of these associations and of being considered present in virtually all vascularised tissues of the human body, with the exception of bone (Tammela and Alitalo 2010), initial lymphatic vessels have been rarely described in adipose tissue. Previous studies have shown adipose tissue associated with collecting lymphatic vessels in lymph nodes (Wang and Oliver 2010), in ectopic deposits such as the epicardial adipose tissue (Montani et al. 2004) and in fat deposits adjacent to the aorta (Martel et al. 2013). In the few available descriptions, lymphatic capillaries were not found in...
the adipose subcutaneous tissue but only in its borders (Ryan 1989, 1995).

In mammals, white adipose tissue is divided into subcutaneous adipose tissue (SAT), mainly localised in the gluteus, thighs and abdomen and visceral adipose tissue (VAT), also known as intra-abdominal fat, mostly composed of mesenteric and omental fat. In healthy subjects, the SAT deposits correspond to 80% of total fat mass, whilst the VAT deposits correspond to 10–20% (Oka et al. 2010). The VAT compared with the SAT is more cellular, innervated and vascularised (Gealekman et al. 2011; Villaret et al. 2010); contains more leukocytes; and is also more metabolically active, having distinct adipokine secretion profiles (Ibrahim 2010). These divergences might be, at least in part, explained by different embryonic origins, as suggested by recent findings (Chau et al. 2014). Furthermore, an increase in VAT relative to SAT, also called visceral obesity, is a risk factor for the development of cardiovascular diseases, type 2 diabetes, insulin resistance, inflammatory diseases and metabolic syndrome in obese patients (Bergman et al. 2007; Matsuwa et al. 2011).

The lack of adequate lymphatic drainage in lymphedema is considered one of the main causes of SAT expansion observed in the affected areas (Warren et al. 2007). Additionally, it was observed that a high-fat diet induces lymphatic dysfunction accompanied by capillary dilation in the dermis and reduction of the contractile function of collecting vessels (Aschen et al. 2012; Blum et al. 2014; Sawane et al. 2013; Weitman et al. 2013). Despite these elusive connections between adipose tissue and lymphatic vasculature and the functional requirement of efficient interactions between them, the SAT and VAT lymphatic vascularisation remains incompletely described in humans.

In this study, we investigate lymph vessels in the human SAT and VAT, showing for the first time the lymphatic microvascular structure in human VAT.

Materials and methods

Patients and samples

Tissue samples were collected from non-obese patients (14 females and 1 male; mean age 46.3 ± 2.4 years) submitted to surgery for hernia corrections in the abdominal region, performed at the Clementino Fraga Filho University Hospital from the Federal University of Rio de Janeiro, after obtaining informed consent. This study was approved by the Clementino Fraga Filho University Hospital Ethics Committee (number 36864514.8.0000.5257). Fragments measuring approximately 125 mm³ were obtained from abdominal SAT and omental VAT. Visceral adipose tissue samples were only collected when surgeries involved access to visceral fat. Exclusion criteria for this study were a body mass index > 30 and the presence of clinically relevant conditions such as cancer or hepatic, neurological, cardiovascular, infectious and inflammatory diseases.

Antibodies and dyes

Antibodies used for lymphatic vessel characterisation by immunohistochemistry and immunofluorescence were lymphatic endothelial cell (LEC) markers such as VEGFR3 (1:100, mouse IgG, Millipore; 1:100, goat IgG, R&D Systems), Lyve1 (1:100, rabbit IgG, Novus Biologicals; 1:100, rabbit IgG R&D Systems), podoplanin (1:100 or 1:50, mouse IgG, Dako) and Prox1 (1:100, rabbit IgG, Novus Biologicals); CD31 (1:200, mouse IgG, Dako) for blood vessels; the pericyte marker CD146 (1:100, rabbit IgG, Abcam); the pan-leukocyte marker CD45 (1:200, mouse IgG, Abcam) for the identification of haematopoietic cells; and the macrophage marker CD68 (1:50, mouse IgG, Abcam). Secondary goat-anti-mouse and goat-anti-rabbit antibodies conjugated to Alexa488 or Cy3 (1:1000, Jackson ImmunoResearch), donkey-anti-mouse, donkey-anti-rabbit and donkey-anti-goat conjugated to Alexa488 or Cy3 (1:1000, Invitrogen) were used in appropriate combinations. Bodipy 493/503 (1:200, Invitrogen, USA) was used for adipocyte staining. To-Pro-3 (1:1000, Molecular Probes, USA) or DAPI (2.7 mg/ml, Sigma-Aldrich, USA) was used for nucleus labelling.

Immunohistochemistry

Samples from 9 (SAT, n = 9; VAT, n = 7) out of the 15 patients used in this study were fixed for 48 h in buffered formalin and subsequently processed for routine paraffin inclusion. Briefly, sections of 5 μm were collected on sylanised sides (Sakura, Netherlands) and used for immunohistochemistry. Following removal of paraffin and rehydration, slides were treated with 50 mM ammonium chloride for 15 min (Ramos-Vara 2005), permeabilised with PBS-Triton 0.5% and incubated for 15 min with 30% hydrogen peroxide. Heated antigen retrieval was performed with citrate buffer (pH 6.0) for 15 min (Pusztaszeri et al. 2006), followed by PBS washing and 60-min incubation in blocking buffer containing 5% of BSA and/or 5% of normal goat or

Fig. 1 Immunohistochemical identification of lymphatic vessels in human SAT and VAT. Endothelial-shaped Lyve1+ (a, b, e) or podoplanin+ (c, d, f) cells in close vicinity of adipocytes, perfusing the VAT parenchyma (arrows). (a’) Higher magnification of the image shown in a. (d) Structure presenting lymphatic capillary morphology in VAT (arrow). (d, f) Lymphatic vessels in VAT border areas (thick arrows). (h) Lyve1+ vessels in the dermis/hypodermis interface (asterisk) and interlobular fibrous septa (arrow) of SAT. Fusiform Lyve1+ cells scattered in SAT (g, h) and VAT (a’, c) parenchyma (stars). Bars = 100 μm (a, b, h); 50 μm (c–g).
donkey serum, before overnight incubation with primary antibodies. LSAB kit (Dako, USA) was used for secondary labelling and DAB for chromogenic revelation. At least 2 sections per patient/stain were entirely observed under a microscope. Slides were counterstained with Harris haematoxylin, mounted with Entellan.
(Merck-Millipore, USA) and photographed with an Eclipse E400 microscope (Nikon, Japan) equipped with QCapture software (SpectraServices, USA) or, with a Zeiss Axiovert 200M microscope (Carl Zeiss, Germany) equipped with Axiocam HRc and ZEN software (Carl Zeiss, Germany).

Whole mount staining

Tissue samples from 11 (SAT, n = 11; VAT, n = 7) out of the 15 patients used in this study were fixed in 4% freshly prepared paraformaldehyde for 2 h immediately after surgical resection. Whole mount processing protocol was performed as previously described (Xue et al. 2010; Daquinag et al. 2013). A minimum of 3 fragments measuring approximately 9 mm$^3$ were separated, washed in PBS-Triton 0.1% and incubated with 5% goat or donkey serum for 1 h. Samples were incubated for 48 h at 4 °C with primary antibodies under rotation, followed by PBS-Triton 0.1% washing and incubation with secondary antibodies conjugated to fluorochromes and the nuclear marker To-Pro or DAPI. Bodipy staining (1:200) was performed under rotation for 30 min. After washing with PBS, fragments were mounted with Vectashield (Vector Laboratories, UK) and sealed with coverslips. Optical slice images were obtained serially using a Zeiss LSM510 Meta laser scanning confocal microscope (Carl Zeiss, Germany) covering 30 to 300 μm of thickness in the z axis, or with an Axio Observer.Z1 inverted microscope equipped with a CSU-X1A 5000 Yokogawa Spinning Disk confocal unit (Carl Zeiss, Germany) with an EMCCD Camera QImaging Rolera em-c2 (Teledyne Technologies, Netherlands) run by Zen 2011 software (Carl Zeiss Microscopy, Germany). Zen software was used to process z-stacked images and for 3D reconstruction. Photoshop CC 2013 (Adobe, USA) was used to merge the fields. At least 3 fragments measuring approximately 9 mm$^3$ were entirely observed in all the focal plans with a ×10 objective. Lymphatic vessel counting was performed in 5 fields of z-stack projections per patient stained with Lyve1 or VEGFR3. Each branch of the vessels was counted as one. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, USA). An unpaired two-tailed t test was used to compare both groups.

Results

Lymphatic vessels are abundant in human VAT but are rare in SAT

Immunohistochemistry performed in samples from 9 patients (SAT, n = 9; VAT, n = 7) revealed the presence of small- and medium-calibre vessels lined by Lyve1+ or podoplanin cells in the borders and within the lobes of VAT samples in all 7 patients (Fig. 1a–f). In the SAT samples, lymphatic vessels were only detected in 2 out of 9 patients. When observed, they were found in the subcutaneous vascular plexus or in the fibrous septa crossing the fat pads (Fig. 1h). In both VAT and SAT, isolated Lyve1+ cells were observed in close contact with adipocytes, without forming vascular structures (Fig. 1a’, e, g, h).

Lymphatic vessel distribution was investigated more thoroughly by the whole mount technique (Xue et al. 2010; Daquinag et al. 2013) using confocal microscopy and 3D reconstruction. In the VAT, Lyve1+ lymphatic vessels with different calibres were observed (Fig. 2). Wider vessels juxtaposed to CD31+ blood vessels were identified (Fig. 2a). CD31+ blood capillaries were detected in close proximity with adipocytes, as previously described (Gao et al. 2015). Lymphatic capillaries characterised by the detection of Lyve1 (Fig. 2b) or by the detection of VEGFR3 and the LEC nuclear marker Prox1 (Fig. 2c) exhibited classical morphology, presenting blunt ends and apparent loose intercellular spaces (Baluk et al. 2007). Their distribution pattern was distinct from that of blood capillaries. The lymphatic vessels were observed in between unilocular adipocytes, within the VAT parenchyma (Fig. 2d–f).

As opposed to the VAT, where Lyve1+ or VEGFR3+ vessels were readily observed in all 7 patients (7/7), in the SAT, albeit extensive and arduous observation of samples from 11 patients, Lyve1+ or VEGFR3+ lymphatic vessels were only found in 3 of them (3/11). Importantly, lymphatic vessels were detected in the adjacent dermis of all patients (Fig. 3a, d), nevertheless, when the interface between the dermis and adipose tissue was analysed, there was no continuity of the vessels to the SAT (Fig. 3b, e). The Lyve1+ (Fig. 3c) or VEGFR3+ (Fig. 3f) vessels were found predominantly in the fibrous connective interlobular tissue within the adipose tissue, presenting a diameter.
Additionally, round VEGFR3+ cells were associated with lymphatic vessels. These were clearly distinguished from the LECs by their morphology, localisation and labelling intensity (Fig. 3f). A basic scoring method to quantify differences observed between SAT and VAT vessel numbers was used. Individual branches of Lyve1+ or VEGFR3+ lymphatic vessels were scored as 1 vessel. Whilst the mean vessel count in the SAT group was 2.727 (± 1.526), the VAT group showed a 4.96 times higher vessel count (13.43 ± 2.202), significantly different from the amount found in SAT (p = 0.0008) (Fig. 4).

Numerous Lyve1+ cells are observed in association with blood vessels from the adipose tissue

Three-dimensional reconstructions and z-stack projections showed Lyve1+ cells surrounding adipocytes and neighbouring adipocyte-associated CD31+ capillaries. These cells were forming neither a lymphatic continuum nor vascular structures (Fig. 5a, b). Aiming to address whether these cells were pericytes, as their morphology was compatible with that of pericytes associated to capillaries, double labelling with CD146 was performed. Most of the cells were Lyve1+CD146−.
Discussion

In the present study, we could not identify initial lymphatic capillaries within the human SAT. However, lymph vessels were clearly observed in the interlobular fibrous septa, in the fibrous septa crossing the adipose parenchyma, as well as in association with the subcutaneous vascular plexus. These results are in accordance with morphological studies from Ryan (1989, 1995), who described lymphatic vessels only in the SAT borders, as well as with updated studies on dermal microcirculation, in which lymphatic capillaries remain mostly unreported (Breslin et al. 2019), despite eventual imaging of collecting lymphatics in the SAT (Tashiro et al. 2017). Regardless of extensive analysis of samples using immunohistochemistry and/or whole mount, we could not identify a continuity of Lyve1+ or VEGFR3+ lymph vessels from the dermis into the adjacent adipose tissue parenchyma. Lymph vessels usually collapse in tissue sections due to their delicate structure, which hampers their visualisation (Jackson 2003). Additionally, the inherent technical difficulties from adipose tissue preparation could impair proper microscopic observation of its structural details (Xue et al. 2010). However, it is unlikely that the lymphatic capillaries are abundant in the SAT but undetected due to methodological issues, since their observation in the VAT samples was possible. Furthermore, previous studies based on colloid technetium injection in fat pads indicated poor lymphatic drainage in the SAT parenchyma, showing slow or absent depuration (Mortimer et al. 1990; Ryan 1989) even after vigorous massage (Lubach et al. 1996; Ryan 1997; Ryan and De Berker 1995). Hence, taken together with the literature, our data indicate that human SAT, in contrast to most vascularised human tissues, including VAT, does not present a lymphatic capillary network intimately surrounding adipocytes, accompanying the blood capillaries, as could be expected.

It should be noted though, that the dense blood capillary network in SAT lobes suggests lymph formation in the interstitial space (Ikomi and Schmidt-Schönbein 1995; Ikomi and Schmidt-Schönbein 1996; Lubach et al. 1996; Ryan and De Berker 1995). For this reason, the possibility of SAT interstitial fluid drainage via lymphatics from the dermis/hypodermis interface, from the interlobar septa or by mechanisms that are independent of classical initial vessels in proximity to blood capillaries should not be discarded. Further investigations on SAT lymphatic drainage and flow are needed to clarify these questions.

Conversely to the observations in SAT, blunt-ended capillaries converging to higher calibre lymphatic vessels presenting classical morphology (Oliver and Alitalo 2005; Schulte-Merker et al. 2011) were observed in human VAT. Vessels were detected surrounding fat lobes, in the fibrous septa, as well as perfusing the tissue parenchyma. To our knowledge, this is the first demonstration of lymphatic capillaries and vessels in human VAT using specific LEC molecular markers.

In the present study, we also showed fibroblastoid Lyve1+ cells in between adipocytes and with perivascular localisation. Although occasionally expressing the pericyte maker CD146, Lyve1+CD45+ cells with such morphology were abundant, indicating a haematopoietic phenotype. Round-shaped Lyve1+CD45+ and VEGFR3+CD45+ cells were also detected. Most of the fusiform Lyve1+ cells scattered in between adipocytes were also CD68+, confirming the macrophage phenotype, in accordance with studies reporting Lyve1+ or VEGFR3+ macrophages based on their morphology and on the lack of a lymphatic continuum formation (Haiko et al. 2008; Martinez-Santibañez et al. 2014; Sawane et al. 2013; Wang et al. 2014).

The SAT and VAT lymphatic architectural particularities described in the present work open a new front of
investigation in adipose tissue biology, with important implications in the understanding of lymphedema, as well as in elucidating the different roles of these depots in obesity, which is under investigation in our group.
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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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