Review Article

Obese adipose tissue remodeling, malfunctioning, and chronic inflammation visualized by *in vivo* molecular imaging

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Metabolic syndrome is a major risk factor of cardiovascular events, and obese visceral adipose tissue remodeling and malfunctioning based on chronic inflammation play a central role. To assess dynamic multicellular interplay, a novel *ex vivo* and *in vivo* adipose tissue imaging method was developed. We found close spatial and temporal interrelationships between angiogenesis and adipogenesis, and both were augmented in obese adipose. In addition, we found increased leukocyte-platelet-endothelial cell interactions in the microcirculation of obese visceral adipose that were indicative of activation of the leukocyte adhesion cascade, a hallmark of inflammation. Both macrophages and endothelial cells showed increased adhesion molecules, and platelets were also activated locally in obese adipose. Up-regulated expression of adhesion molecules on multiple cell types suggests that their increased interactions contribute to local activation of inflammatory processes within visceral obese adipose tissue. Interestingly, the heightened leukocyte-platelet-endothelial interactions were not observed in obese subcutaneous fat pads. Our results demonstrated the power of our imaging technique to analyze complex cellular interplays *in vivo* and to evaluate new therapeutic interventions against them. Results also indicate that visceral obese adipose tissue is an inflammatory site itself.

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Introduction

Inflammation is now considered to play a pivotal role in the development of metabolic diseases[1]. In particular, obese adipose tissue exhibits the hallmarks of chronic inflammation, including macrophage infiltration, adipogenesis, angiogenesis and tissue remodeling[2,3]. Moreover, the inflammation is thought to alter adipose tissue function, leading to systemic insulin resistance[4]. Adipose tissue contains multiple cell types including stromal cells: adipocytes, macrophages, and endothelial cells, and their interaction is important in obese adipose tissue remodeling including angiogenesis and adipogenesis, thus leading to a dysfunction at the tissue level. However, little is known about the detailed mechanisms of these cell-cell interactions, because much of the structural and functional integrity of the tissue is lost when it is fixed, processed and sectioned. To elucidate the significance and mechanism of the interactions between stromal cells, vascular cells and adipocytes, a novel confocal microscopy-based visualization technique was recently developed that enables the observation of living adipose tissue while still maintaining its 3-dimensional structural integrity. The contribution of chronic inflammation is also revealed by in vivo molecular imaging method.

Living adipose tissue imaging method

A novel visualization technique for living adipose tissue based on laser confocal microscope has been developed by reforming the technique originally developed to obtain cytoskeletal images in single cardiomyocytes[5-7]. Endothelial cells were distinctively visualized using fluorescent Iso-lectin dyes (red), nuclei stained by Hoechst 33342 (green), and adipocytes were stained using fluorescent free acid (BODIPY, blue) in intact living adipose tissue (Fig.1). Lectin is reportedly a useful histochemical probe that specifically labels endothelial cells in many species.

A confocal microscope (CSU22, X1; Yokogawa-denki) equipped with objectives and a CCD camera (Impactron CCD; Nihon TI), (iXon; Andor) was used to obtain the images. The tissue was excited using multiple color laser lines (405 or 408, 488, and 568nm), and the emission was collected through appropriate narrow band-pass filters.

In conventionally used paraffin embedded specimens (Fig.1c), it was impossible to identify and discriminate the stromal cells, adipocytes, and vasculature. However, this novel living tissue imaging method yielded three-dimensional precise images without any artifacts, which therefore enabled the visualization of three-dimensional structures in adipose tissue in detail (Fig. 1a,b).

Adipose tissue remodeling in obesity

Using this novel imaging methods, we found that there were close spatial and temporal interrelationships between the blood vessel development and adipogenesis, and both were consistent with visceral adipose tissue from animal models of obesity[8]. The epididymal adipose tissue specimens from 8-weeks-old obese db/db mice were compared with the control animals (Fig.2). We mainly use db/db mice, which is established animal model of severe diabetes and obesity, but the same phenomena were observed in other diabetic and obese models including diet-induced-obese mice. The average size of the adipocytes was significantly larger for obese mice, and a distinct population of adipose cells with small diameters (<50 μm) was also found in the db/db mice (Fig.2b,c). This indicated that bimodal cell populations existed in the obese adipose tissue.

The appearance of new small adipocytes could be indicative of either adipogenesis or lipolysis. The following observations strongly suggest that those cells had undergone adipogenesis. 1) The appearance of small BODIPY-positive cells coincided with hyperplasia of the epididymal fat. 2) The small BODIPY-positive cells also were positive for incorporation of BrdU, which was observed both *in situ* and in isolated adipocytes, indicating that they had recently undergone cell division. 3) The small BODIPY-positive cells surrounded by lectin positive cells strongly stained for perilipin, which is an adipocyte-specific lipid droplet-associated protein whose expression is induced during adipocyte differentiation from preadipocytes[9]. Cinti et al. showed that dying adipocytes were negative for perilipin[10]. 4) We observed no staining of dead cell markers in the small BODIPY-positive cells.

Within the adipose tissue from control mice, the capillaries characterized by tightly aligned, lectin-stained endothelial cells were interspersed among the adipocytes. Although similar networks of capillaries also were found in obese adipose tissues, a striking finding was that the smaller adipocytes were always provided with a supply of blood vessels and they were also surrounded by small lectin-binding cells. Moreover, 3-dimensional reconstruction of the images confirmed that the vessels supplying the clusters had dead ends, thus suggesting they had the characteristics of vessels sprouting from the existing vasculature.

To examine the extent of the coupling between adipogenesis and angiogenesis within the angiogenic cell clusters, the extent to which perturbation of angiogenesis would affect adipogenesis was assessed by treating db/db mice for 2 weeks with an anti-VEGF antibody. The results showed that anti-VEGF-treated mice tended to show a smaller increase in body weight. At the

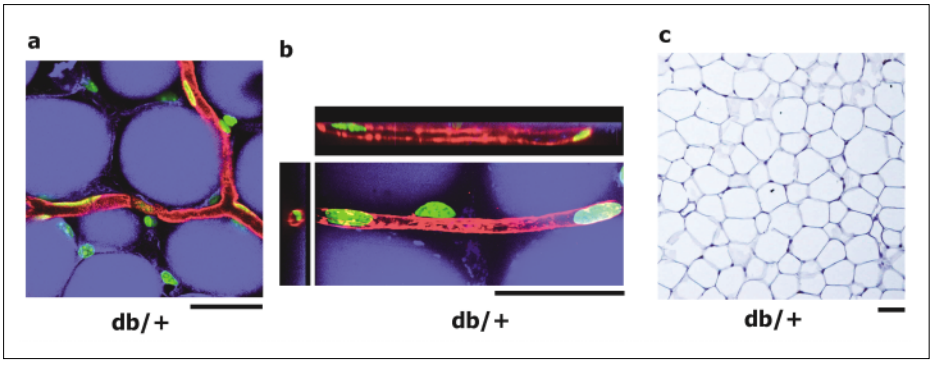

Fig.1 Living adipose tissue imaging method

Lean (db/+) adipose tissue visualized by living tissue imaging method (a,b), and conventional one using paraffin embedded section (c). The adipose tissue was stained with BODIPY (blue), lectin (red), and Hoechst (green). Scale bars represent 50 μm.

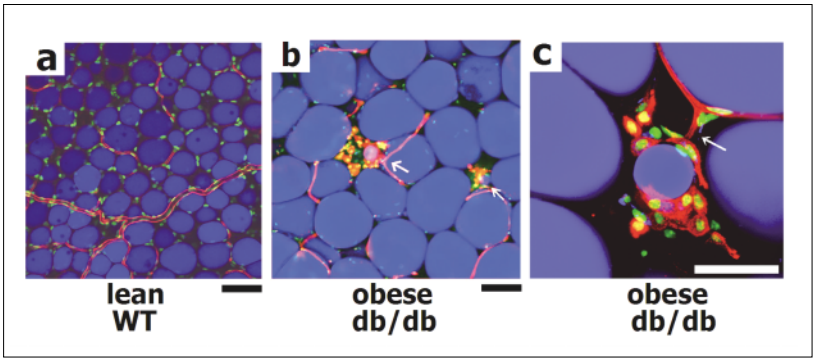

Fig.2 Coupled adipogenesis and angiogenesis in obese adipose tissue revealed by living tissue imaging

Unfixed living adipose tissue from 8-week-old wild type mice (a), and obese db/db mice (b,c) were visualized as in Figure 1. In db/db mice, small BODIPY+ adipocytes appeared with surrounding lectin positive cells and vessel sprouts (c). Arrows shows adipo-/ angiogenic cell clusters in b, and vascular sprouting indicating angiogenesis in c. Scale bars represent 100 μm in a-b, and 50 μm in c.

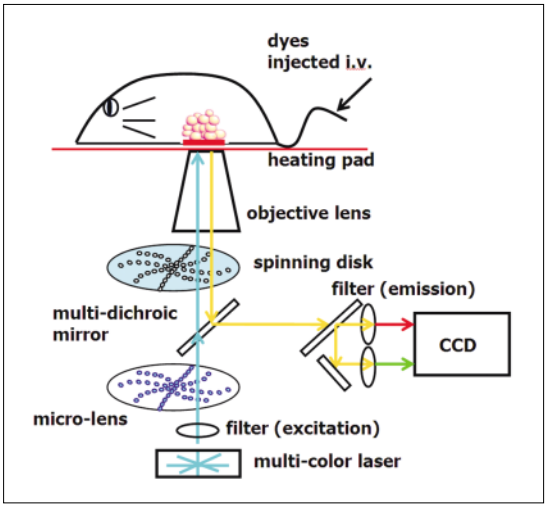

Fig.3 Diagram of the confocal microscope for real time *in vivo* imaging

To visualize cell dynamics in vivo, fluorescent dyes were injected into the tail veins of anesthetized mice. A small incision was then made for an observation window, and an inverted microscope equipped for Nipkow-spinning disk confocal laser microscopy, which enabled scanning at up to 1,000 frames/s, was used to visualize the tissue.

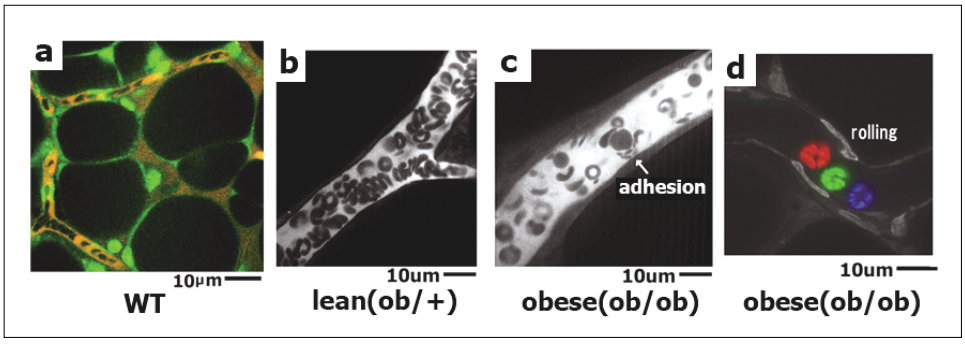

Fig.4 Inflammatory cell dynamics in visceral obesity visualized *in vivo* imaging

The adipocyte, erythrocyte, leukocyte and platelet cell dynamics can be visualized with high time and spatial resolutions in wildtype (WT, a) adipose tissue by *in vivo* molecular imaging method (Red: TMRE-dextran, Green: Acridine Orange). Cellular dynamics in adipose microcirculation in lean (ob/+, b), and obese (ob/ob, c,d) was visualized by FITC-dextran (b-c), and Acridine Orange (d). Note the firmly adherent leukocyte and platelets in vascular wall in obese mice post capillary venules (c, arrow), and rolling leukocytes (d) thus suggesting the inflammatory status in obese adipose tissue. Scale bars represent 10 μm.

cellular level, anti-VEGF markedly inhibited formation of the smaller differentiating adipocytes, as well as the formation of blood vessel sprouts and adipo-/angiogenic cell clusters. This indicated that the coupling of adipogenesis and angiogenesis is essential for the differentiation of adipocytes in obesity, and that VEGF is a key mediator of that process.

In vivo molecular imaging method

Visceral obesity and metabolic syndrome with chronic inflammation have been recognized to be a leading cause of cardiovascular disease. But, little is known about the detailed mechanisms of multi-type cell-cell interactions in obese adipose *in vivo*. A visualization technique, based on laser confocal microscopy was therefore developed that made it possible to precisely evaluate the three-dimensional structures in living tissue, and the cell dynamics *in vivo* with a high time and spatial resolution.

Briefly, to obtain *in vivo* molecular images, the dyes were injected intravenously, and then the anesthetized mice were set into the custom-made chamber on an inverted microscope. Imaging was performed through a small incision of abdomen with a spinning disk confocal microscope, and CCD camera (Fig.3). This yielded a high spatial and time resolution almost free from motion-artifacts from body movement and heartbeat.

The blood flow and cell dynamics can be visualized using FITC- or TMRE- dextran. The nuclei were stained with Acridine Orange dyes to identify leukocyte in microcirculation. Platelets were specifically visualized using fluorescent-tagged anti-CD41 antibody.

Inflammatory cellular dynamics in obese adipose

Our novel *in vivo* imaging methods revealed inflammatory cellular dynamics in obese adipose[3]. We found increased leukocyte-platelet-endothelial cell interactions in the microcirculation of obese visceral adipose tissue in ob/ob and high-fat-diet-induced obese mice, which were indicative of activation of the leukocyte adhesion cascade, a hallmark of inflammation. Local platelet activation in obese adipose tissue was indicated by increased P-selectin expression and formation of monocyte-platelet conjugates. Upregulated expression of adhesion molecules on macrophages and endothelial cells suggests their interactions contribute to local activation of inflammatory processes within obese visceral adipose tissue.

Interestingly, the heightened leukocyte-endothelial interactions were not observed in subcutaneous fat in the same mice, and administration of anti-ICAM1 antibody normalized the cell dynamics seen in visceral fat. Using our new imaging technique to analyze the complex cellular interplay within obese adipose tissue, we have been able to show that visceral adipose tissue obesity is an inflammatory disease and to evaluate potential therapeutic interventions against it.

The blood flow could also be visualized and the flow velocity could thus be directly determined. While the blood flow in lean adipose tissue was largely continuous even at the capillary level, the blood flow at the capillary level in obese adipose tissue was quite varied and often discontinuous due to transient leukocyte plugging. As a result, the average blood flow in relatively small capillaries was slower in obese adipose tissue than that in lean adipose tissue. Tissue hypoxia examined by pimonidazole treatment was also remarkable in obese adipose tissue possibly reflecting the low circulation.

Inflammation and obesity

Previous studies have shown that obese adipose tissue secretes various inflammatory mediators (e.g., TNF-α and IL-6) as well as adipokines such as adiponectin suggest that adipose tissue plays a crucial and integral role in systemic inflammation in obese subjects. Subsequent findings of augmented infiltration of macrophages into obese adipose tissue and the vicious interactions between adipocytes and macrophages via inflammatory cytokines and lipids suggested the inflammatory changes seen in obese adipose tissue may be the key pathology that promotes systemic inflammatory states in obese subjects. Still, little was known about how inflammation is initiated and what inflammatory changes take place within obese adipose tissue. In this review, our results clearly demonstrated the power of our imaging technique to analyze complex cellular interplays, and inflammatory cellular status in obese adipose tissue. However, there remained to be clarified about the most primary and key trigger in inflammatory vicious cellular interactions.

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