

Expanded Materials and Methods

Living tissue imaging

Eight-week-old diabetic model (db/db) and control (db/+) mice were obtained from Charles River Inc. The db/db mice were separated into two groups: the db/db+antiVEGF group was subcutaneously administered a monoclonal anti-VEGF antibody (100 µg/mouse; Genentech) three times a week for 2 weeks prior to experimentation, while the control db/db group received non-specific IgG in a similar manner. Alternatively, some db/db mice were examined after fasting for 24 hours.

Mice were sacrificed by cervical dislocation, after which the epididymal fat was removed using sterile techniques and minced into small pieces (~2-3 mm) using a scalpel. The tissue pieces were then washed and incubated with FM1-43, BODIPY, acetylated low-density lipoprotein (AcLDL) conjugated with Alexa Fluor, or *Griffonia simplicifolia* isolectin GS-IB4 conjugated with Alexa Fluor (Molecular Probes) before observation. Nuclei were counterstained with Hoechst 33342 (Molecular Probes). Dyes, vendors, incubation time, and concentration used in the present study were summarized in supplemental table.

Griffonia simplicifolia IB₄ isolectin is reportedly a useful histochemical probe that specifically labels endothelial cells in many species and tissues, including adipose tissue (1-5). We confirmed those findings by double-staining adipose tissue with anti-CD31 (PECAM) and lectin, which produced identical staining patterns (Supplemental Figure S1). We also performed the immunohistochemical study of smooth muscle α -actin, but in the capillary level of adipose tissue, significant staining could not be obtained (data not shown).

All experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments and strictly adhered to the guidelines for animal experiments of the University of Tokyo.

Confocal microscopy

A confocal laser scanning microscope (CSU22; Yokogawa-denki, and LSM510 Meta; Carl Zeiss) equipped with 10x dry, 40x dry, and 63x oil objectives was used to collect stacks of images at regular intervals along the optical axis. The tissue was excited using multiple color laser lines, and the emission was collected through appropriate narrow band-pass filters. Each image was produced from an average of 8 frames after which the acquired images were processed to produce a surface-rendered 3-dimensional model.

In preliminary experiments, adipose tissue from control mice labeled with BODIPY and lectin was observed using 5- μm -thick stacks of images obtained at 0.5- μm intervals and 50- μm -thick stacks obtained at 2- μm intervals (Supplemental Figure S2). Using the latter, we confirmed that every adipocyte within the adipose tissue was surrounded by microvessels, but we also found that it was difficult to visualize the precise details of structures because of insufficient focus in the deep slices. We therefore chose to use mainly stacks of $< 10 \mu\text{m}$ for subsequent imaging.

Determination of adipocyte size and numbers and the numbers of adipo-/angiogenic cell clusters

Adipocyte diameters and numbers were determined using IpLab software. Five low-power field images were acquired at regular spatial intervals from four animals in each group, after which the diameters of 50 cells in each field were measured by an observer blinded to the conditions. Adipocyte was defined by regularly round BODIPY⁺ cells without plasma membrane disruption. The histogram shown was constructed from 1000 cells from each group. In preliminary experiments, we confirmed that the adipose tissue was structurally homogenous by examining four slices taken from epididymal fat pads at regular spatial intervals (Supplemental Figure S3). To calculate the numbers adipocyte within the epididymal fat pads, the fat volume was divided by the determined cell number per unit volume.

To quantify the numbers of adipogenic cell clusters, four slices were taken at regular

spatial intervals from each epididymal fat pad, and images of five low-power fields stained with BODIPY and lectin were examined for each slice. Four animals were analyzed in each group (a total of 80 low-power fields for each group). The number of adipo-/angiogenic cell clusters (defined by vessel sprouting, and the presence of small adipocytes surrounded by lectin-binding cells) were determined by an observer blinded to the conditions using IpLab software. The preliminary experiments showed that the adipogenic cell clusters also were homogeneously distributed throughout all of the sections taken from different parts of the epididymal fat pads of db/db mice (Supplemental Figure S3). We analyzed at least five fields per slice and four slices per fat mass to obtain representative images.

Organ culture and time-lapse imaging

For time-lapse imaging, the endothelial cells in living tissue were prelabeled with lectin and then cultured in Dulbecco's Modified Eagle's Medium (Sigma) containing 10% fetal bovine serum (Sigma) on a microscope CO₂ incubator stage (Olympus) with or without VEGF (10 ng/ml; Sigma). The specimen was observed using time-lapse imaging, during which with the preparation was illuminated for 500 ms at intervals of 30 s. The cell migration speed was determined using IPLab software by tracing the cells in acquired movies.

Immunofluorescent staining of incorporated bromodeoxyuridine (BrdU)

Mice were intraperitoneally administered BrdU (30 mg/kg body weight) two days before sacrifice. Thereafter, resected adipose tissue or isolated adipocytes by collagenase digestion were fixed, permeabilized, treated with DNase, incubated with FITC-conjugated anti-BrdU antibody (all chemicals provided as BrdU flow kit, Becton Dickinson), and counterstained with BODIPY and Hoechst 33342.

Detection of reactive oxygen species (ROS)

To detect ROS production, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA, Molecular Probes) was injected to mice before sacrifice.

Immunohistochemistry

For immunohistochemical analyses, isolated tissue pieces were fixed in 4% formaldehyde for 45 min and then permeabilized with 1% Triton X-100 (CalBiochem) for 10 min. The specimens were then blocked with 1% bovine serum albumin and incubated with a pair of primary and secondary antibodies. Antibodies, dyes, vendors, incubation time, and concentration used in the present study were summarized in supplemental table.

Isolation of the stromal vesicular fraction (SVF) and flow cytometry

To isolate the SVF, minced adipose tissue was incubated in a collagenase solution (2 mg/ml, collagenase type 2; Worthington) for 30 min and then centrifuged. The resultant pellet containing the SVF was filtered through 70- μ m mesh, incubated for 10 min in erythrocyte-lysing buffer, and finally resuspended in phosphate buffered saline. The cells were then incubated with labeled monoclonal antibody or isotype control antibody [Alexa Fluor 488-conjugated anti-CD68 (Serotec) and PE-conjugated anti-CD34 (eBioscience)] and analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (Becton Dickinson).

Statistics

The results are expressed as means \pm S.E.M. The statistical significance of differences between two groups was determined using Student's *t*-tests; differences among three groups were evaluated using ANOVA and post hoc Bonferroni tests. Values of $p < 0.05$ were considered significant.

References

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