Original Article

Adipogenesis in Obesity Requires Close Interplay Between Differentiating Adipocytes, Stromal Cells, and Blood Vessels

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OBJECTIVE—The expansion of adipose tissue mass seen in obesity involves both hyperplasia and hypertrophy of adipocytes. However, little is known about how adipocytes, adipocyte precursors, blood vessels, and stromal cells interact with one another to achieve adipogenesis.

RESEARCH DESIGN AND METHODS—We have developed a confocal microscopy-based method of three-dimensional visualization of intact living adipose tissue that enabled us to simultaneously evaluate angiogenesis and adipogenesis in *db/db* mice.

RESULTS—We found that adipocyte differentiation takes place within cell clusters (which we designated adipogenic/angiogenic cell clusters) that contain multiple cell types, including endothelial cells and stromal cells that express CD34 and CD68 and bind lectin. There were close spatial and temporal interrelationships between blood vessel formation and adipogenesis, and the sprouting of new blood vessels from preexisting vasculature was coupled to adipocyte differentiation. $CD34^+$ $CD68^+$ lectin-binding cells could clearly be distinguished from $CD34^-$ $CD68^+$ macrophages, which were scattered in the stroma and did not bind lectin. Adipogenic/angiogenic cell clusters can morphologically and immunohistochemically be distinguished from crown-like structures frequently seen in the late stages of adipose tissue obesity. Administration of anti–vascular endothelial growth factor (VEGF) antibodies inhibited not only angiogenesis but also the formation of adipogenic/angiogenic cell clusters, indicating that the coupling of adipogenesis and angiogenesis is essential for differentiation of adipocytes in obesity and that VEGF is a key mediator of that process.

CONCLUSIONS—Living tissue imaging techniques provide novel evidence of the dynamic interactions between differentiating adipocytes, stromal cells, and angiogenesis in living obese adipose tissue. ***Diabetes*** **56:1517–1526, 2007**

Adipose tissue is an active endocrine organ that produces a variety of humoral factors, known as adipocytokines, which in turn exert numerous metabolic and vascular effects. Notably, obesity alters production of adipocytokines such that obese adipose tissue produces the cytokines involved in the initiation and development of metabolic and cardiovascular diseases (1,2). Thus, adipose tissue obesity is critically involved in enhancing the clinical risk of cardiovascular disease. Extensive research using in vitro adipocyte differentiation models has provided us with detailed information on the molecular programs involved in adipocyte differentiation (3,4). It is known, for example, that differentiation from adipocyte precursors (preadipocytes) to adipocytes is governed by a network of transcription factors, including peroxisome proliferator–activated receptor γ. However, much less is known about how adipose tissue becomes obese in adult animals. Because the number of adipocytes in a given fat mass increases in obesity (5), adipose tissue obesity is thought to depend on both hypertrophy of preexisting adipocytes and hyperplasia due to formation of new adipocytes from precursor cells (adipogenesis) (5–7). The adipose tissue stroma contains blood vessels and other cell types. It is well documented that among the stromal cells, there are preadipocytes that can be induced to differentiate into adipocytes in vitro (8–10). Recent studies have also demonstrated that obesity induces macrophage infiltration of the stroma of adipose tissue (11,12) and that inhibition of angiogenesis reduces adipose tissue mass (13–16). These findings strongly suggest that stromal cells and blood vessels play key roles in adipogenesis and obesity. However, little is known about how adipogenesis proceeds in vivo or the significance and mechanism of the interactions between stromal cells, vascular cells, and adipocytes (17,18). This is in part because adipose tissue is largely comprised of adipocytes containing large lipid droplets. Much of the structural integrity of adipose tissue is lost when it is processed and sectioned, which has hindered our understanding of the histological basis of obesity. Here, we show that adipocyte differentiation depends on

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BrdU, bromodeoxyuridine; HIF, hypoxia inducible factor; ROS, reactive oxygen species; VGEF, vascular endothelial growth factor.

dynamic interplay between adipocyte precursors, stromal cells, and vascular endothelial cells using a novel visualization method of intact living adipose tissue.

RESEARCH DESIGN AND METHODS

An expanded version of the RESEARCH DESIGN AND METHODS section is available in the online appendix "Materials and Methods" (available at http://dx.doi.org/10.2337/db06-1749).

Living tissue imaging. The 8-week-old *db/db* mice were separated into two groups: a *db/db* + anti–vascular endothelial growth factor (VEGF) group (that was subcutaneously administered a monoclonal anti-VEGF antibody [100 μg/mouse] for 2 weeks before experimentation) and a control *db/db* group (that received nonspecific IgG).

Mice were killed by cervical dislocation, after which 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 LDL conjugated with Alexa Fluor, or *Griffonia simplicifolia* isolectin GS-IB4 conjugated with Alexa Fluor. Nuclei were counterstained with Hoechst 33342.

Griffonia simplicifolia IB_4 isolectin is reportedly a useful histochemical probe that specifically labels endothelial cells in many species and tissues, including adipose tissue (19). We confirmed those findings by double staining adipose tissue with anti-CD31 (platelet/endothelial cell adhesion molecule) and lectin, which produced identical staining patterns (online appendix Fig. 1).

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) was used. 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 eight frames, after which the acquired images were processed to produce a surface-rendered three-dimensional model.

Using the latter, we confirmed that every adipocyte in the adipose tissue is surrounded by microvessels using 50–μm–thick stacks, but we found it difficult to visualize the precise details of the structures due to insufficient focus in the deep slices. We therefore chose to use mainly stacks of <10 μm for subsequent imaging (online appendix Fig. 2).

Determination of adipocyte size and numbers and the numbers of adipogenic/angiogenic cell clusters. 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 1,000 cells from each group. To calculate the number of adipocytes 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 adipogenic/angiogenic cell clusters (defined by the presence of small adipocytes surrounded by lectin-binding cells and blood vessels) was determined. We analyzed at least five fields per slice and four slices per fat mass to obtain representative images.

In preliminary experiments, we confirmed that the adipose tissue was structurally homogenous by examining fat pads at regular spatial intervals in *db/+*, and 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 (Online appendix Fig. 3).

Immunofluorescent staining of incorporated bromodeoxyuridine. Mice were intraperitoneally administered bromodeoxyuridine (BrdU) (30 mg/kg body wt) 2 days before being killed. Thereafter, resected adipose tissue was fixed, permeabilized, treated with deoxyribonuclease, and incubated with fluorescein isothiocyanate–conjugated anti-BrdU antibodies.

Immunohistochemistry. For immunohistochemical analysis, isolated tissue pieces were fixed in 4% formaldehyde and permeabilized with 1% Triton X-100. The specimens were then blocked with 1% BSA and incubated with a pair of primary antibodies and then with a secondary antibody. Antibodies, dyes, vendors, incubation time, and concentration used in the present study are summarized in an online appendix table.

Statistics. The results are expressed as means ± SEM. 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.

RESULTS

Adipogenesis takes place within adipogenic/angiogenic cell clusters containing multiple cell types and blood vessel sprouts. To better analyze adipogenesis in vivo, we developed a confocal microscopy–based method to visualize intact living adipose tissue. We compared epididymal adipose tissue from 8-week-old obese *db/db* mice with that from control heterozygous db/+ mice (body weight 40.8 ± 0.6 and 26.0 ± 0.4 g, respectively; $n = 6$ for each group, $P < 0.05$). We first examined the structure of the tissue by visualizing the cell membranes using FM1–43 (Fig. 1*A*). We found that the adipose tissue consists of round adipocytes and other smaller cell types along with fine networks of capillaries running between the adipocytes. To examine the spatial relationship between the blood vessels and the adipocytes in more detail, endothelial cells were visualized using lectin (red) (20), while the adipocytes were stained with BODIPY (blue). Adipose tissue from both control (Fig. 1*B*) and obese (Fig. 1*C*) mice contained large BODIPY-stained adipocytes, but the average size of the adipocytes was significantly larger in obese mice than in control mice (90.4 ± 1.9 and 68.2 ± 0.5 μm, respectively; $n = 1{,}000$ cells in each group, $P < 0.05$). In addition, we also observed a distinct population (21.8%, $n = 1{,}000$ cells) of BODIPY-stained (i.e., lipid-containing) cells with smaller diameters (<50 μm) in *db/db* mice, suggesting that obese adipose tissue may contain a bimodal population of adipocytes (Fig. 2*A*). These smaller BODIPY-stained cells were rarely found in db/+ mice (5.4%, $n = 1{,}000$ cells).

Within the adipose tissue from control mice, capillaries characterized by tightly aligned, lectin-stained (red) endothelial cells were interspersed among the adipocytes (Fig. 1*B*). Although similar networks of capillaries also were found in obese adipose tissues, a striking finding was that the smaller BODIPY-stained cells were always provided with a supply of blood vessels, suggesting that angiogenesis was taking place. The small BODIPY-staining cells were also surrounded by small lectin-binding cells, which did not form the capillary-like structure (Figs. 1*C* and 3).

To further confirm the idea that angiogenesis was ongoing with these cell clusters and to visualize the clusters in more detail, we obtained higher magnification images of tissues stained with acetylated LDL, lectin, and Hoechst 33342 (Fig. 3). Lectin-stained capillary endothelial cells were clearly visible surrounding clusters. Moreover, three-dimensional reconstruction of the images confirmed that the vessels supplying the clusters had dead ends (Fig. 3*E* and *F*); i.e., they had the characteristics of vessels sprouting from the existing vasculature. It thus appears that these cell clusters are indeed sites of active angiogenesis.

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. The number of adipocytes markedly increased in *db/db* animals during the 3-week period over which they went from 5 to 8 weeks of age ($1.02 \pm 0.13 \times 10^6$ vs. $1.71 \pm 0.10 \times 10^6$ cells/fat pad, respectively; $n = 5$ animals, $P < 0.05$). *2*) The small BODIPY-positive cells also were positive for the incorporation of BrdU, which

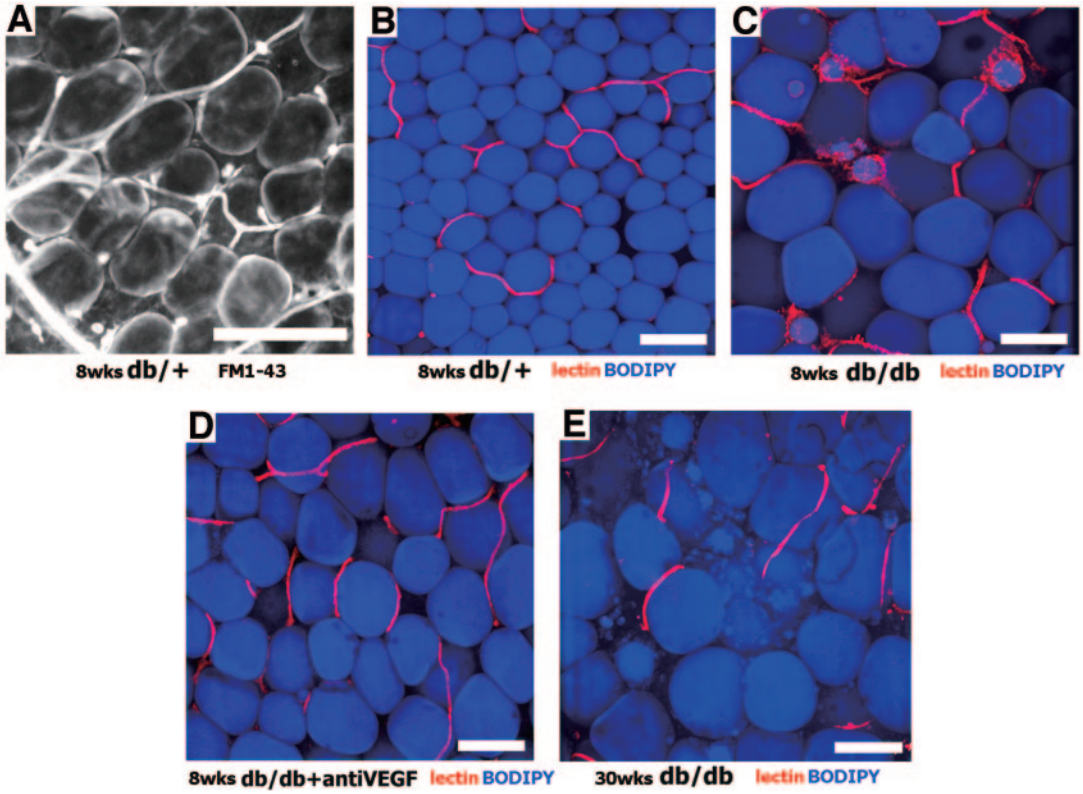

FIG. 1. Coupled adipogenesis and angiogenesis in living adipose tissue are represented. Unfixed living adipose tissue from 8-week-old control db/+ mice (*A* and *B*), IgG-treated control *db/db* mice (*C*), anti-VEGF–treated *db/db* mice (*D*), and 30-week-old *db/db* mice (*E*) were labeled with either FM1-43 (*A*) or lectin (red) for endothelial cells and BODIPY (blue) for fatty acid (*B–E*). In the adipose tissue of 8-week-old *db/db* mice, a number of small BODIPY$^+$ cells appeared surrounded by lectin$^+$ cells that did not form the capillary-like structure. Note that in the adipose tissue of 30-week-old *db/db* mice, crown-like structures were frequently observed (*E*; also refer to Fig. 6), while very few adipogenic/angiogenic cell clusters were found. In double staining for BODIPY and lectin, crown-like structures were characterized by the clusters of lectin$^-$ macrophages uptaking BODIPY, which appeared unrelated to blood vessel sprouting (*C* vs. *E*). Following administration of the anti-VEGF antibody, angiogenesis was inhibited, and the number of the smaller adipocytes was markedly reduced (*C* vs. *D*). Bars represent 100-μm Z stacks; 10 μm (*A*) and 5 μm (*B–E*).

was observed both in situ and in isolated adipocytes, indicating that they had recently undergone cell division (Fig. 4 and online appendix Fig. 4). *3*) The small BODIPY-positive cells surrounded by lectin$^+$ cells strongly stained for perilipin (Figs. 4*B* and 5), which is an adipocyte-specific lipid droplet-associated protein whose expression is induced during adipocyte differentiation from preadipocytes (21). Cinti et al. (22) showed that dying adipocytes were negative for perilipin. *4*) We observed no staining of dead cell markers in the small BODIPY-positive cells (online appendix Fig. 5). *5*) Fasting for 24 h did not induce the cell clusters containing small adipocytes and surrounding lectin$^+$ cells (online appendix Fig. 6).

Rodent studies have shown that the contribution made by adipocyte hyperplasia to the growth of epididymal fat mass, relative to hypertrophy, is much higher in young

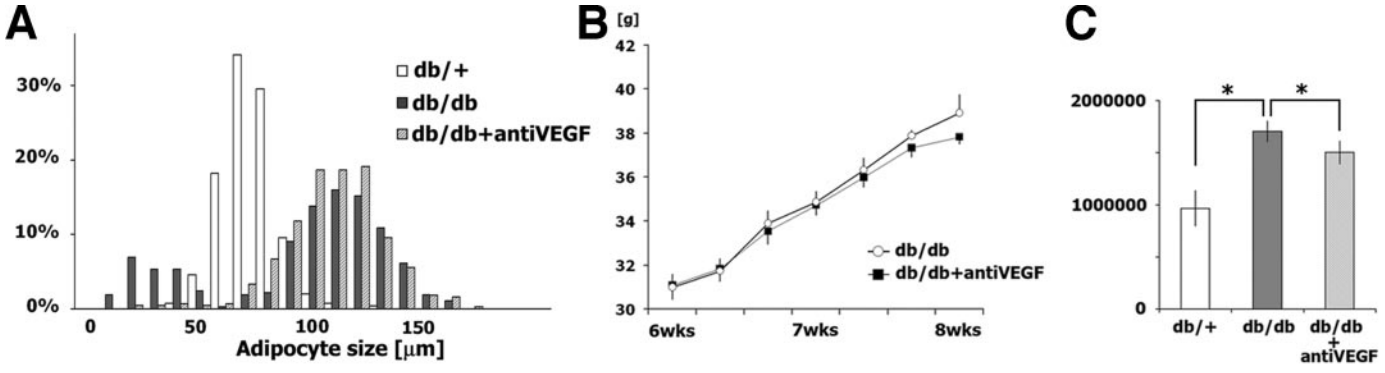

FIG. 2. Adipocyte size, body weight changes, and cell numbers within fat pads. Adipocyte diameters and numbers were determined from 8-week-old control db/+ mice, IgG-treated control *db/db* mice, and anti-VEGF–treated *db/db* mice as shown in the histogram (*A*) (n = 1,000 cells from four animals in each group). The mean diameters of the adipocytes are larger in obese *db/db* mice than in control mice, and in *db/db* adipose tissue, a population of very small adipocytes (<50 μm) coexists with the larger adipocytes, indicating a bimodal distribution of adipocyte cell size. *B*: Mice treated with anti-VEGF tended to gain less weight than control animals, but the difference did not reach statistical significance within the 2-week experimental period (n = 10 animals in each group). *C*: The numbers of adipocyte within the epididymal fat pads were calculated by dividing the fat volume by the determined cell number per unit volume (n = 5 animals in each group). *P < 0.05.

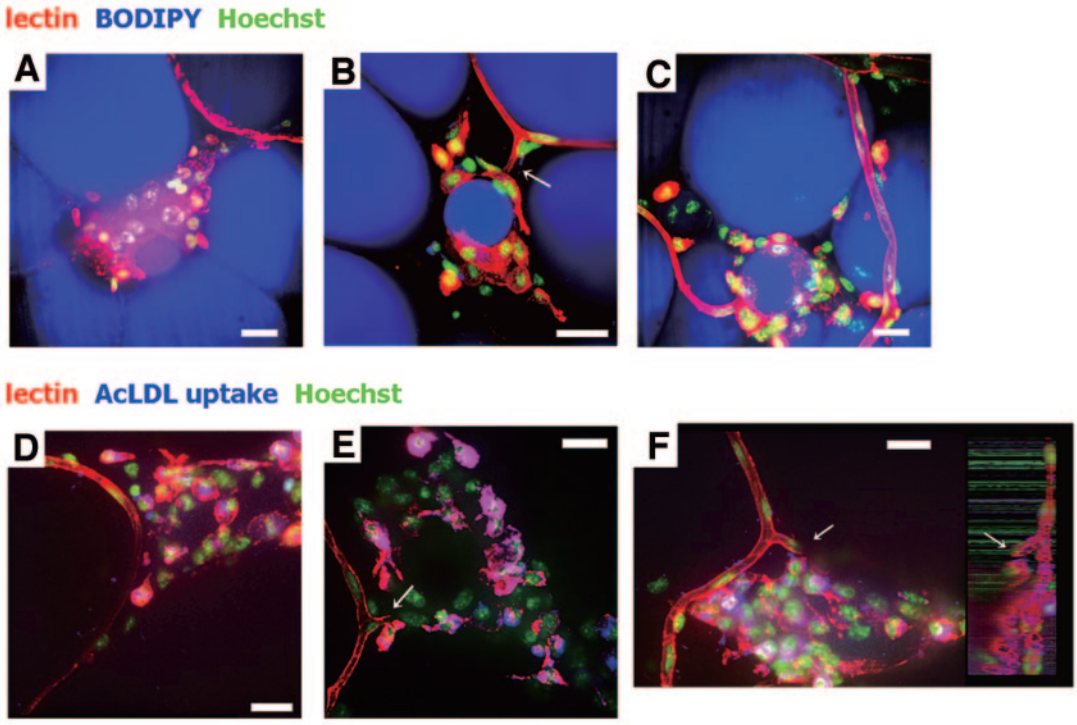

FIG. 3. Adipogenic/angiogenic cell clusters visualized by living tissue imaging. Unfixed living adipose tissue from 8-week-old *db/db* mice was labeled with a combination of lectin (red) (*A–F*), BODIPY (blue) (*A–C*), acetylated LDL (blue) (*D–F*), and Hoechst 33342 (green) (*A–F*). Images represent different stages from perivascular lectin$^+$ cells migration surrounding small adipocytes (*A* and *D*) to blood vessel sprouting (*B*, *C*, *E*, and *F*) from the existing vasculature. The tip of the sprouting vessel is clearly visible (arrows in *B*, *E*, and *F*). The side view of a three-dimensional reconstructed image also demonstrates the dead end of the vessel sprout (*F*). Scale bars represent 20-μm, 30-μm (*A* and *C–E*), 4-μm (*B*), and 40-μm Z stacks (*F*).

animals whose body weights are exponentially rising than in older animals (5,6). Consistent with that finding, we most often observed cell clusters containing the small adipocytes surrounded by lectin$^+$ cells in *db/db* mice that were 8 weeks old, an age when *db/db* mice are rapidly gaining body weight (Fig. 6). By contrast, very few adipogenic cell clusters were found in 30-week-old *db/db* mice (whose body weights had already reached a plateau) (Fig. 6). There were a number of crown-like structures in 30-week-old *db/db* mice instead, as described later. These suggest that adipogenesis involving formation of cell clusters is a major mechanism for adipocyte hyperplasia within epididymal fat in young *db/db* mice. That similar colocalization of small adipocytes and their associated lectin-binding cells was observed in adipose tissue from other obese models, including insulin receptor substrate 2 knockout and wild-type mice fed a high-fat diet (online appendix Fig. 7). This suggests that this adipogenic mechanism commonly occurs during the development of adipose tissue obesity. Unlike 8-week-old *db/db* mice, older *db/db*, *ob/ob*, and insulin receptor substrate 2–null mice all exhibited crown-like structures, in addition to adipogenic cell clusters (Fig. 6 and data not shown). This further suggests that at later stages of obesity, crown-like structures also occur in adipose tissue. Taken together, these findings suggest that during the development of obesity in adipose tissue, adipocyte precursor proliferation and adipocyte differentiation take place within cell clusters containing multiple cell types, including adipocytes, endothelial cells, and adipocyte-associated lectin-binding cells. Additionally, given that adipogenesis and angiogenesis appear to be tightly coupled within these clusters, we will hereafter refer to them as adipogenic/angiogenic cell clusters.

Coupled angiogenesis is essential for adipogenesis in obesity. To examine the extent of the coupling between adipogenesis and angiogenesis within the adipogenic/angiogenic cell clusters, we assessed the extent to which perturbation of angiogenesis would affect adipogenesis by treating *db/db* mice for 2 weeks with an anti-VEGF antibody. Mice treated with the anti-VEGF antibody tended to gain less weight than control animals, but the difference did not reach statistical significance within the 2-week experimental period (37.8 ± 0.31 and 38.9 ± 0.84 g, respectively; $n = 10$ animals in each group, $P = 0.13$) (Fig. 2*B*). However, we found that the weights of the epididymal fat pads of anti-VEGF–treated animals were significantly lower than those of control animals (0.81 ± 0.014 and 0.89 ± 0.011 g, respectively; $n = 10$ animals in each group, $P < 0.05$). At the cellular level, although anti-VEGF treatment did not affect the average size of the larger adipocytes (Figs. 1*C*, 1*D*, and 2*A*), it markedly inhibited formation of the smaller differentiating adipocytes (adipocytes with diameters <50 μm) (2.0 and 21.8%, respectively; n = 1,000 cells in each group) (Fig. 2*A*) as well as formation of blood vessel sprouts and adipogenic/angiogenic cell clusters. The inhibition of cluster formation was confirmed by counting the numbers of adipogenic/angiogenic cell clusters defined by the colocalization of vessel sprouting and the accumulation of small BODIPY-stained cells and their associated lectin-binding cells (db/+ 0.2 ± 0.1, *db/db* 5.6 ± 0.5, and *db/db* + antiVEGF 0.9 ± 0.2 adipogenic/angiogenic cell clusters per low-power fields; $n = 80$ images for each group, $P < 0.05$ vs. *db/db*). We also

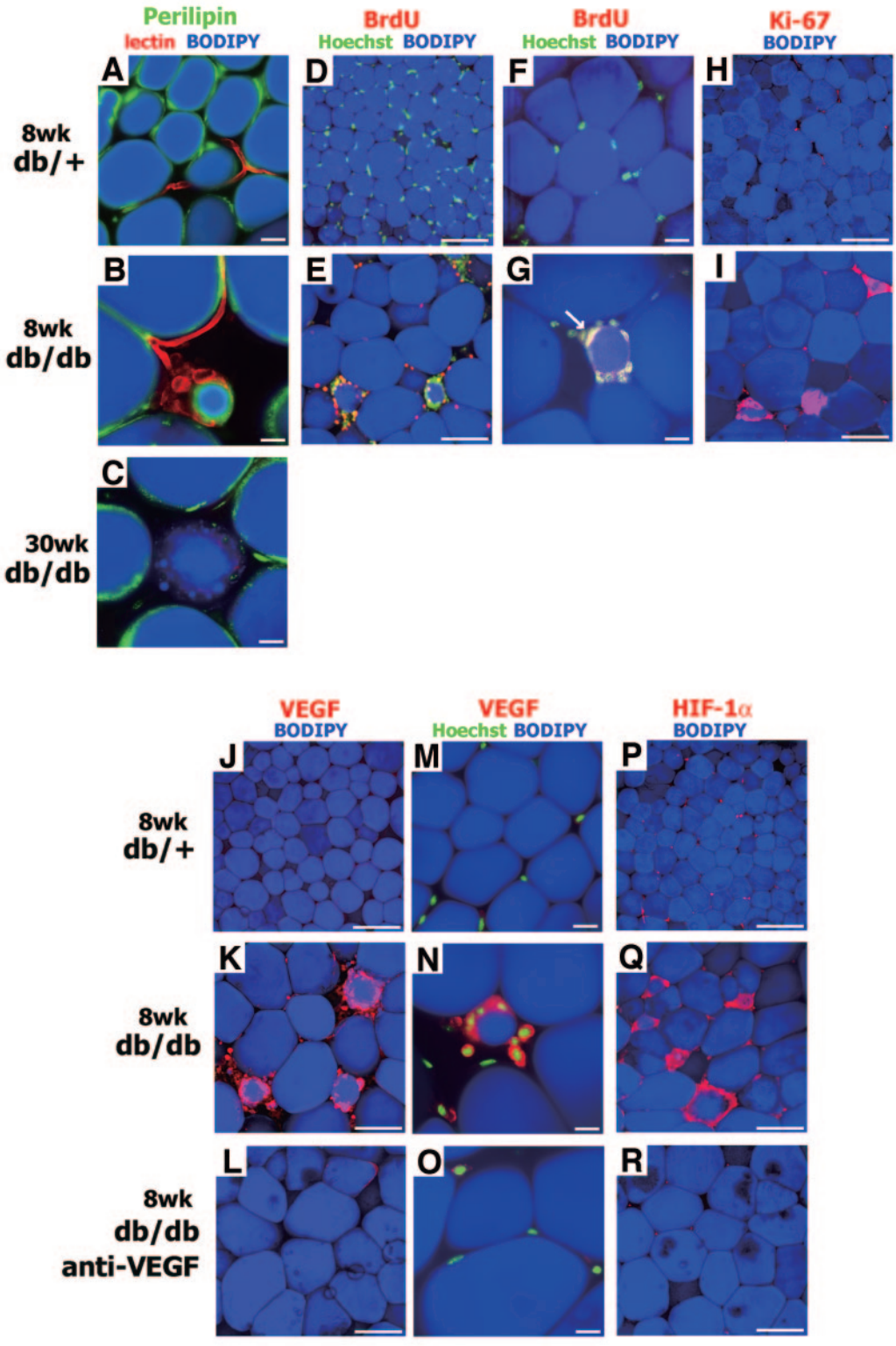

FIG. 4. Immunohistochemical analysis of fixed adipose tissue. Formaldehyde-fixed tissues from control 8-week-old db/+ (*A*, *D*, *F*, *H*, *J*, *M*, and *P*), 8-week-old *db/db* (*B*, *E*, *G*, *I*, *K*, *N*, and *Q*), anti-VEGF–treated 8-week-old *db/db* (*L*, *O*, and *R*), and 30-week-old *db/db* (*C*) mice were stained with antibodies against perilipin (*A–C*) (green), BrdU (*D–G*) (red), Ki-67 (*H* and *I*) (red), VEGF (*J–O*) (red), and HIF-1α (*P–R*) (red). The fatty acid was counterstained with BODIPY (blue) and the nucleus with Hoechst (*D–G* and *M–O*) (green). The small BODIPY$^+$ cells surrounded by lectin$^+$ cells in adipogenic/angiogenic cell clusters of 8-week-old *db/db* mice were positively stained for perilipin (*B*) and incorporated BrdU (*E* and *G*) (arrow; see online appendix Fig. 4), suggesting that the cells were small adipocytes that had recently undergone cell division. Adipogenic/angiogenic cell clusters can be distinguished from crown-like structures in 30-week-old *db/db* mice by the perilipin staining in small adipocytes and the lectin binding to surrounding cells (*C*; see also Figs. 1*E* and 5). The lectin$^+$ cells in adipogenic/angiogenic cell clusters were positively stained for incorporated BrdU (*E* and *G*) and Ki-67 (*I*), indicating that those cells were proliferating. VEGF staining showed that lectin$^+$ cells were strongly positive for VEGF (*K* and *N*). Scale bars represent 20 μm (*A–C*, *F*, *G*, and *M–O*) and 100 μm (*D*, *E*, *H–L*, and *P–R*); Z stacks are 4 μm (*A–C*, *F*, and *G*), 6 μm (*D*, *E*, *H–L*, and *P–R*), and 3 μm (*M–O*).

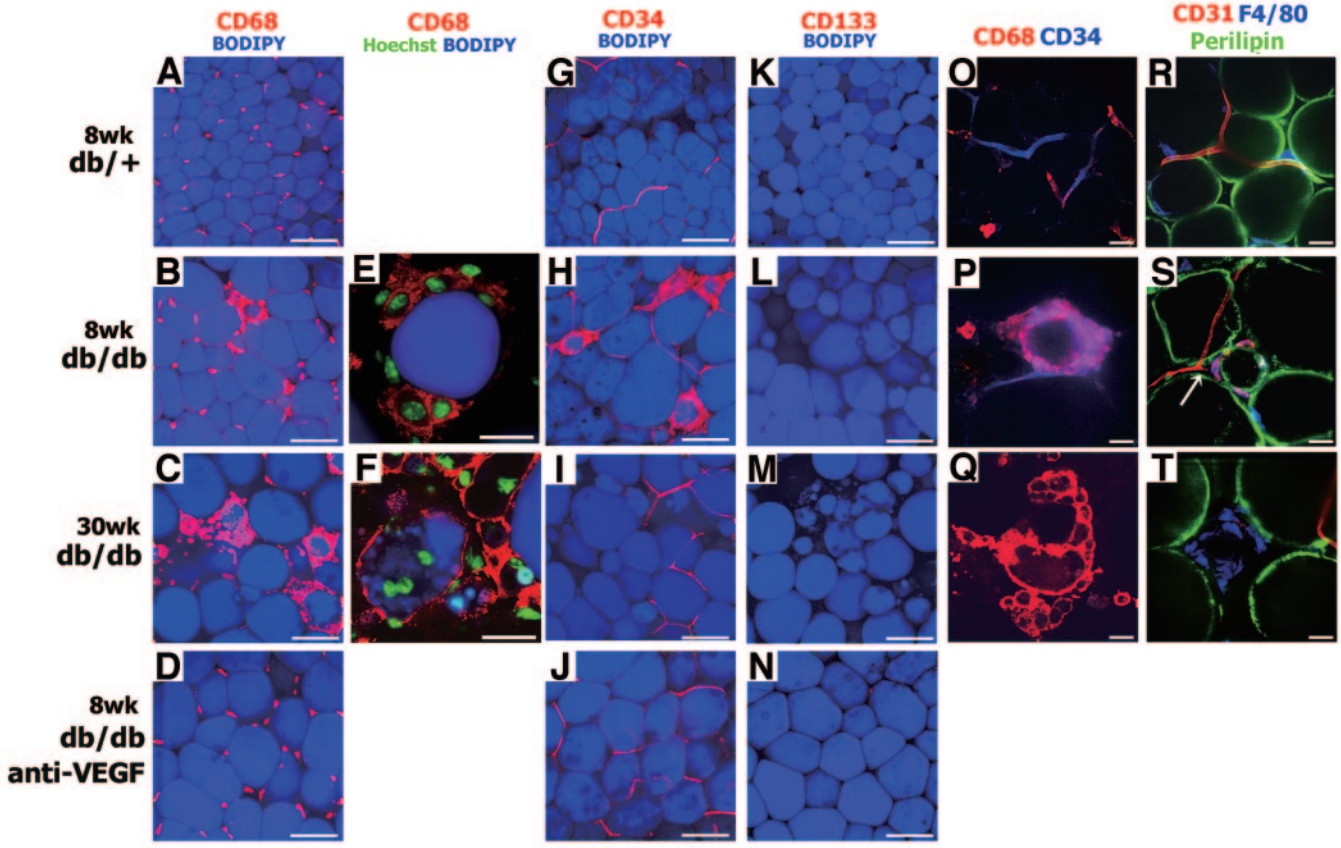

FIG. 5. Surface phenotypes of lectin-binding cells and small adipocytes. Formaldehyde-fixed tissues from control 8-week-old db/+ (*A*, *G*, *K*, *O*, and *R*), 8-week-old *db/db* (*B*, *E*, *H*, *L*, *P*, and *S*), 30-week-old *db/db* (*C*, *F*, *I*, *M*, *Q*, and *T*), and anti-VEGF–treated 8-week-old *db/db* (*D*, *J*, and *N*) mice were stained with antibodies against CD68 (*A–F*) (red), CD34 (*G–J*) (red), and CD133 (*K–N*) (red). The fatty acid was counterstained with BODIPY (*A–N*) (blue) and the nucleus with Hoechst (*E* and *F*) (green). The small lectin$^+$ adipocyte-associated cells in 8-week-old *db/db* were stained positively for CD68 (*B* and *E*) and CD34 (*H*) but were negative for CD133 (*L*), which can be distinguished from CD34$^-$ CD68$^+$ macrophages that were scattered in the stroma. Note that stromal CD68+ macrophages were not stained for CD34 (compare *B* vs. *H*). Double staining of CD34 (blue) and CD68 (red) confirmed that the lectin-binding small adipocyte-associated cells coexpressed CD34 and CD68 (*P*). Triple staining of CD31 (red), F4/80 (blue), and perilipin (green) showed that small perilipin$^+$ cells are surrounded by CD31$^+$ F4/80$^+$ cells (*S*). *S*: The image was constructed from thinner Z stacks (1.5 μm) to visualize the end of a sprouting blood vessel (arrow). Macrophages in crown-like structures in 30-week-old *db/db* mice were positive for CD68 (*C* and *F*) but negative for CD34 (*I*; compare *P* vs. *Q*). *F*: Multinucleated lipid-containing giant cells are shown. The lectin-binding CD34$^+$ CD68$^+$ cells disappeared following administration of anti-VEGF antibody (*D* and *J*). Scale bars represent 20 μm (*E*, *F*, and *O–T*) and 100 μm (*A–D* and *G–N*). Z stacks: 6 μm (*A–D* and *G–Q*), 3 μm (*E* and *F*), 4 μm (*R* and *T*), and 1.5 μm (*S*).

counted the number of adipocytes within the epididymal fat pads and found that they were significantly higher in *db/db* than db/+ mice (db/+ $0.97 \pm 0.17 \times 10^6$ and *db/db* $1.71 \pm 0.10 \times 10^6$ cells/fat pad; $n = 5$ animals in each group, $P < 0.05$) (Fig. 2*C*). Notably, adipocyte numbers were significantly reduced by anti-VEGF treatment (*db/db* + anti-VEGF $1.50 \pm 0.11 \times 10^6$ cells/fat pad; $n = 5$ animals, $P < 0.05$ vs. *db/db*). These results clearly show that in obese animals, anti-VEGF treatment inhibits not only angiogenesis, but also adipogenesis, and that the differentiation of adipocytes within the cell clusters is tightly coupled to angiogenesis, which requires VEGF. Anti-VEGF treatment also affected glucose and insulin tolerance (online appendix Fig. 8).

The role of VEGF in adipogenesis was further illustrated by time-lapse imaging of organ-cultured adipose tissue stained with lectin (20). The lectin-binding cells surrounding the small adipocytes migrated little in the absence of VEGF (online appendix Fig. 9 and Videos 1 and 2). By contrast, when VEGF was added to the culture medium (10 ng/ml), these cells exhibited both random migration and directional movement. In addition, the average speed of their migration was markedly increased by VEGF (control 98 ± 10 nm/min;, $n = 40$ cells from four animals and VEGF 245 ± 42 nm/min; $n = 34$ cells from four animals, $P < 0.05$), indicating that the migration of these adipocyte-associated cells was VEGF dependent.

Adipocyte-associated lectin-binding cells exhibit surface phenotypes that are different from stromal macrophages. The majority of lectin-binding cells within the adipogenic/angiogenic cell clusters did not appear to be components of capillaries and did not exhibit the typical morphological characteristics of endothelial cells, although lectin has been extensively used to identify endothelial cells in many tissues, including adipose tissue (19,23,24). For that reason, we next carried out immunohistochemical studies using fixed specimens to characterize the lectin-binding cells (Figs. 4 and 5). The adipocyte-associated cells stained positively for both BrdU and Ki-67, suggesting that they were proliferating (Fig. 4*E*, *G*, and *I*). These cells also were positive for CD68 (a common macrophage marker) (11) (Fig. 5*B*) but were negative for CD133 (a marker of primitive hematopoietic stem cells) (Fig. 5*L*), which suggests that they were of monocyte/macrophage lineage.

The adipocyte-associated cells also were strongly posi-

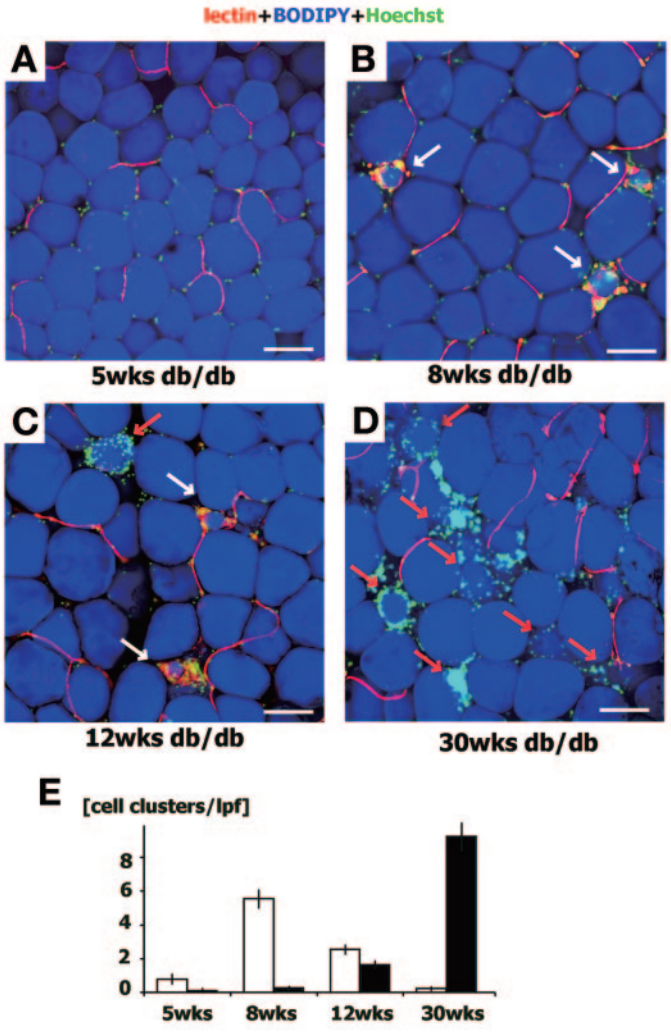

FIG. 6. Adipogenic/angiogenic cell clusters and crown-like structures in *db/db* mice of different ages. Epididymal adipose tissues from 5- (*A*), 8- (*B*), 12- (*C*), and 30-week-old (*D*) *db/db* mice were stained with lectin (red), Hoechst (green), and BODIPY (blue). *E*: The numbers of adipogenic/angiogenic cell clusters (□) and crown-like structures (■) per low-power field were determined from a total of 80 low-power fields from four animals for each group. Adipogenic cell clusters containing lectin$^+$ cells were rarely found in 5-week-old *db/db* mice (*A*). There were a number of adipogenic/angiogenic cell clusters (white arrow) containing lectin$^+$ cells in 8-week-old *db/db*, while very few crown-like structures were found (*B*). The number of adipogenic/angiogenic cell clusters declined in 12-week-old *db/db* mice, and crown-like structures (red arrow) coexisted with adipogenic/angiogenic cell clusters (*C*). In 30-week-old *db/db* mice, there were a number of crown-like structures, while very few adipogenic/angiogenic cell clusters were observed (*D*). Note that adipogenic/angiogenic cell clusters are characterized by the focal accumulation of lectin$^+$ cells (red), whereas crown-like structures are characterized by the focal accumulation of lectin$^-$ cells that often uptook BODIPY (blue). The positions of the lectin$^-$ cells are indicated by Hoechst (green). The scale bar represents 100 μm; Z stacks 6-μm thick.

tive for CD34, as were capillary endothelial cells within the adipose tissue (Fig. 5*H*). Approximately 80% of the lectin-binding cells also took up acetylated LDL (Fig. 3*D–F*), which is a characteristic feature of endothelial cells undergoing angiogenesis and of endothelial progenitor cells (25–27).

Coexpression of CD34 and CD68 on the surface of adipocyte-associated lectin-binding cells was confirmed by double immunostaining of adipose tissue from *db/db* mice (Fig. 5*P*). Neither CD68$^+$ CD34$^-$ nor CD68$^-$ CD34$^+$ cells were found within the adipogenic/angiogenic cell clusters. The former were found within stroma unrelated to adipogenic/angiogenic cell clusters, whereas the latter were present in the blood vessels adjacent to the cell clusters (Fig. 5*B* and *H*). Most likely, the stromal CD68$^+$ CD34$^-$ cells are macrophages, as has been previously reported (11,12,28). Also consistent with earlier reports, the CD68$^+$ CD34$^-$ macrophages were more abundant in *db/db* obese adipose tissue than in control adipose tissue (11).

To further characterize the surface phenotype of lectin-binding cells in the adipogenic/angiogenic cell clusters, we examined them by triple staining for the surface markers CD31 (platelet/endothelial cell adhesion molecule 1, an endothelial cell marker), F4/80 (a macrophage marker), and perilipin (an adipocyte marker). The small adipocyte-associated lectin-binding cells coexpressed CD68 and F4/80 (online appendix Fig. 10), and when triple stained for CD31, F4/80, and perilipin, F4/80$^+$ small adipocyte-associated cells also were positive for CD31 (Fig. 5*S*). In sharp contrast, CD31$^+$ endothelial cells in the capillaries were never positive for F4/80. Because F4/80$^+$ cells were negative for perilipin indicates they were not adipocytes. Thus, there were at least two distinct populations of lectin-binding cells in the obese adipose tissue: the CD31$^+$ CD68$^-$ F4/80$^-$ endothelial cells that composed the blood vessels and the CD31$^+$ CD68$^+$ F4/80$^+$ cells that surrounded the small differentiating adipocytes. The characteristics of lectin-binding cells, stromal macrophages, endothelial cells, and adipocytes in adipose tissue are summarized in Table 1.

The presence of CD34$^+$ CD68$^+$ cells in adipose tissue was also confirmed by flow cytometry analysis using stromal vascular fraction isolated from *db/db* and db/+ adipose tissues (online appendix Fig. 11). The flow cytometry revealed that the relative size of the CD68$^+$ cell population was larger in *db/db* mice (db/+ 29.2 ± 1.6 vs. *db/db* 40.0 ± 1.7%; $n = 5$ animals, $P < 0.05$), which is consistent with earlier reports (11,28). We also detected CD34$^+$ CD68$^+$ cells, and the relative size of their population also was increased in *db/db* mice (db/+ 2.2 ± 0.% vs. *db/db* 8.1 ± 1.2%; $n = 5$ animals, $P < 0.05$).

The CD34$^+$ CD68$^+$ lectin-binding cells were situated in the vicinity of ongoing angiogenesis, and high levels of VEGF by lectin$^+$ cells were detected within the adipogenic/angiogenic cell clusters (Fig. 4*K* and *N* and online appendix Fig. 12), suggesting that the CD34$^+$ CD68$^+$ cells may contribute to angiogenesis by producing VEGF (26,29). Most of the VEGF released by adipose tissue came from the nonfat cells (30), and CD34$^+$ cells in adipose tissue were known to secrete VEGF in vitro (31). Our results are in line with those findings and also demonstrate for the first time that adipogenic/angiogenic cell clusters are the sites of VEGF production. Moreover, reactive oxygen species (ROS) and hypoxia inducible factor (HIF)-1α also were observed in the adipocyte-associated CD34$^+$ CD68$^+$ cells in *db/db* mice (Fig. 4*P–R* and online appendix Fig. 13), suggesting the involvement of ROS and HIF-1α signaling in the production of VEGF (32).

Adipogenic/angiogenic cell clusters are morphologically and immunohistochemically different from crown-like structures. Recently, the focal infiltration of macrophages into the area surrounding dead adipocytes has been reported in older *ob/ob* and *db/db* mice and designated as "crown-like structures" (11,22,28). Crown-like structures have been shown to exhibit morphological features including *1*) the crown-like arrangement of macrophages, some of which form multinucleated giant cells

TABLE 1
Characteristics of cells in obese adipose tissue

Cells	Markers							
	CD68	F4/80	Lectin	CD34	CD31	Acetylated LDL uptake	Perilipin	CD133
Adipocyte-associated cells in adipogenic/angiogenic cell clusters	+	+	+	+*	+*	+*	−	−
Stromal macrophages	+	+	−	−	−	−	−	−
Macrophages in crown-like structures	+	+	−	−	−	−	−	−
Capillary endothelial cells	−	−	+	+	+	−	−	−
Adipocytes	−	−	−	−	−	−	+	−

*80% of cells.

surrounding individual adipocytes; *2*) adipocytes undergoing necrotic cell death, which is characterized by the disruption of membrane integrity, the degradation of the unilocular lipid droplet, and the appearance of multiple small lipid droplets; and *3*) the absence of perilipin immunoreactivity in dead adipocytes (22,33). As reported, we found a number of crown-like structures in adipose tissue in older *db/db* mice (Fig. 6). These crown-like structures contained relatively small adipocytes surrounded by small cells that were not bound by lectin (Fig. 6). The locations of these cell clusters appeared to be unrelated to the blood supply, and the small cells surrounding the adipocytes accumulated lipids (Figs. 1*E*, 5, and 6), suggesting that they were macrophages. Indeed, they were positive for the macrophage markers F4/80 and CD68 but negative for lectin and CD34 (Fig. 5*C*, *F*, and *I* and online appendix Fig. 14). Thus, they can be clearly distinguished from $CD34^+$ $CD68^+$ lectin-binding cells in adipogenic/angiogenic cell clusters. Multinucleated lipid-containing giant cells were also found in crown-like structures (Fig. 5*F*). The absence of perilipin staining indicated that adipocytes in crown-like structures were undergoing cell death, as previously reported (22) (Figs. 4*C* and 5*T*). Adipocytes within crown-like structures also exhibited discontinuities in the cell membrane and the degradation of unilocular lipid droplets that resulted in multiple small lipid droplets (Fig. 1*E* and 4*C*). Taken together, our imaging technique enables a clear distinction between adipogenic/angiogenic cell clusters and crown-like structures (Tables 1 and 2). It is worth mentioning that adipocytes within $CD68^+$ $lectin^-$ macrophage clusters were occasionally positive for perilipin, presumably indicating adipocytes were still in the process of cell death and crown-like structure formation.

In 8-week-old *db/db* mice, the vast majority of cell clusters were comprised of adipogenic/angiogenic cells (Fig. 6). The number of adipogenic/angiogenic cell clusters declined in 12-week-old *db/db* mice, and crown-like structures coexisted with them. In 30-week-old *db/db* mice, very few adipogenic/angiogenic cell clusters were found, whereas a number of crown-like structures were observed, indicating that at the late stages of obesity, crown-like structure formation frequently occurs in adipose tissue (22). Additionally, adipogenic/angiogenic cell clusters are the major mechanism of adipocyte hyperplasia in the early stages of obesity (Fig. 6*E*).

DISCUSSION

Despite detailed knowledge about the molecular mechanisms that control adipocyte differentiation in vitro, little is known about how adipogenesis progresses in vivo, particularly in obesity. Our live-cell imaging has revealed that adipogenesis takes place within adipogenic/angiogenic cell clusters that also contain various stromal cells and blood vessels and that angiogenesis is an essential part of adipogenesis in obesity.

Macrophages reportedly accumulate within obese adipose tissue (11,28) so that the presence of macrophage markers is significantly and positively correlated with both adipocyte size and body mass (34). However, little is known about the roles such macrophages play in adipogenesis and obesity. Although infiltration by macrophages of the area surrounding small adipocytes has been previously described in older obese animals (11,28), their functions have been related to adipocyte cell death (22). The present report is the first, to our knowledge, to describe adipocyte-associated cells that exhibit phenotypes of monocyte/macrophage lineages and play a role in adipogenesis. Results of the present study clearly demonstrate that in obese adipose tissue, there are two types of clusters of cells that exhibit at least some surface phenotypes of the monocyte/macrophage lineage such as CD68 and F4/80: adipogenic/angiogenic cell clusters and crown-like structures (Table 1). In the early stages of obesity of *db/db* mice, adipogenic/angiogenic cell clusters were the major cell cluster containing $CD68^+$ cells (Fig. 6*E*). Crown-like structures were scarcely found at the early stages of obesity. However, later in the development of adipose tissue, obesity crown-like structures were found more frequently as reported previously (11,28), while the number of adipogenic/angiogenic cell clusters declined (Fig. 6*E*).

Our results demonstrate two types of $CD68^+$ cells: $CD34^+$ $CD68^+$ cells associated with the small differentiat-

TABLE 2
Morphological and immunohistochemical characteristics of adipo-/angiogenic cell clusters and crown-like structures

	Adipocytes		Blood vessels	$CD68^+$ cells	
	Perilipin	Membrane discontinuity	Angiogenesis	Lectin binding	Multinucleated lipid-containing giant cells
Adipogenic/angiogenic cell clusters	++	−	+	+	−
Crown-like structures	−	+	−	−	+

ing adipocytes and $CD34^-$ $CD68^+$ macrophages scattered in the stroma. Although both cell populations are $CD68^+$ and $F4/80^+$, they may represent different cell lineages (Table 1). Indeed, the phenotype that includes CD34 and CD68 expression, lectin-binding, and acetylated LDL uptake fits the phenotypes of both endothelial cells undergoing angiogenesis and endothelial progenitor cells (29). However, $CD34^+$ $CD68^+$ lectin-binding cells were not found within blood vessels and did not exhibit the morphological characteristics of endothelial cells. Thus, the function and lineage of those $CD34^+$ $CD68^+$ cells are currently unknown. But, given the findings of recent studies showing that adipose tissue contains multipotent stem cells (9), it is tempting to speculate that $CD34^+$ $CD68^+$ cells might differentiate into endothelial cells and/or other cell types. Alternatively, they might support development of small adipocytes and angiogenesis. Production of VEGF in those cells may support this hypothesis. Future studies would definitely need to further characterize the lineage and function of $CD34^+$ $CD68^+$ cells.

Our live cell imaging of adipose tissue also revealed a close spatial and temporal relationship between angiogenesis and adipogenesis. This finding is consistent with those of earlier studies showing that inhibition of angiogenesis reduces adipose tissue mass and ameliorates obesity and insulin resistance (13–16), although these studies have not clarified how inhibition of angiogenesis leads to reduction in fat mass. Studies of the development of adipose tissue also have shown that angiogenesis precedes adipogenesis in embryos (35). However, it has been unclear how angiogenesis and adipogenesis interact and what role blood vessels play in adipogenesis in obesity. Our present findings clearly show that blood vessel sprouting is actively ongoing in the vicinity of clusters of differentiating adipocytes and adipocyte-associated $CD34^+$ $CD68^+$ cells (Fig. 5). Moreover, administration of anti-VEGF inhibited not only angiogenesis but also adipogenesis (Figs. 1 and 2), which provides direct evidence that angiogenesis is essential for adipogenesis in obesity.

Earlier studies also have shown that during postnatal development, VEGF expression within adipose tissue is correlated with fat weight (36). Our present findings shed light on the mechanism by which VEGF affects adipose tissue growth. In addition to inhibiting angiogenesis, administration of anti-VEGF also inhibited the accumulation of $CD34^+$ $CD68^+$ cells, thereby suppressing formation of adipogenic/angiogenic cell clusters (Fig. 5). It is thus likely that VEGF mediates the initial interactions between blood vessels, $CD34^+$ $CD68^+$ cells, and adipocyte precursors. The fact that VEGF receptor-1 is required for the recruitment of hematopoietic precursors and the migration of monocytes and macrophages (37,38) supports this model. Moreover, Fukumura et al. (16) recently reported that medium conditioned by endothelial cells contained VEGF and accelerated adipocyte differentiation, whereas treatment with a VEGF receptor 2–blocking antibody inhibits adipocyte differentiation. It is thus tempting to suggest that the adipocyte-associated $CD34^+$ $CD68^+$ cells not only accelerate angiogenesis but also support and promote the differentiation of preadipocytes via paracrine interactions (39–41).

ROS production and expression of HIF-1α within adipogenic/angiogenic cell clusters (Fig. 4 and online appendix Fig. 13) suggests their involvement in the signaling leading to VEGF production (42). Expansion of adipose tissue is achieved through enlargement of individual adipocytes and/or hyperplasia (7,11). Because a disproportionate increase in a cell's dimensions lengthens the distance oxygen must diffuse, one can reasonably expect that enlarged adipocytes are in a hypoxic state. Hypoxia is a potent initiator of angiogenesis that induces expression of a variety of cytokines, including VEGF, thereby inducing angiogenesis and the recruitment of progenitor cells (39,43,44). These recruited cells also produce VEGF, which further promotes angiogenesis and possibly stimulates the proliferation of preadipocytes through paracrine interactions with other cell types (e.g., endothelial cells) (29,40). Expression of HIF-1α is consistent with this scenario, though HIF-1α expression can be regulated by factors other than hypoxia (45). ROS production is also induced by a variety of environmental cues, including growth factors, making alternative scenarios possible. For instance, increased fatty acid levels may be sensed by preadipocytes or adipocytes and may trigger angiogenesis. Future studies will be needed to address the environmental cues involved in the initiation of the coupled adipogenesis/angiogenesis. That said, our present findings shed new light on the molecular interplay between angiogenesis and adipogenesis and provided a basis for the analysis and development of therapeutics targeting adipogenesis and adipose function.

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