Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice

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Abstract Accumulation of visceral fat is a key phenomenon in the onset of obesity-associated metabolic disorders. Macrophage infiltration induces chronic mild inflammation widely considered as a causative factor for insulin resistance and eventually diabetes. We previously showed that >90% of macrophages infiltrating the adipose tissue of obese animals and humans are arranged around dead adipocytes, forming characteristic crown-like structures (CLS). In this study we quantified CLS in visceral and subcutaneous depots from two strains of genetically obese mice, db/db and ob/ob. In both strains, CLS were prevalent in visceral compared with subcutaneous fat. Adipocyte size and CLS density exhibited a positive correlation both in visceral and in subcutaneous depots; however, the finding that adipocyte size was smallest and CLS density highest in visceral fat suggests a different susceptibility of visceral and subcutaneous adipocytes to death. Visceral fat CLS density was 3.4-fold greater in db/db than in ob/ob animals, which at the age at which our experimental strain was used are more prone to glucose metabolic disorders.—Murano, I., G. Barbatelli, V. Parisani, C. Latini, G. Muzzonigro, M. Castellucci, and S. Cinti. **Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *J. Lipid Res.* 2008.** 49: **1562–1568.**

Supplementary key words obesity • macrophages • subcutaneous fat • adipocyte death

The incidence of obesity is rapidly increasing all over the world (1, 2). The phenomenon is a major public health concern, mainly because it carries an increased risk of death from obesity-associated disorders (3, 4). Obesity is frequently associated with metabolic syndrome; insulin resistance is widely suspected as the central starting event of the condition (1). Although the adipose organ of obese animals and humans is increased at both subcutaneous and visceral sites, visceral fat alone is responsible for the metabolic consequences of obesity (5–9). The reasons for this effect are unclear.

It has recently been shown that the adipose tissue of obese animals and humans is infiltrated by a pure macrophage population of bone marrow origin (10–14) expressing C-C chemokine receptor 2 (CCR2) (15). This phenomenon correlates with adipocyte size and body mass index (10). It is especially important because it is temporally associated with the appearance of insulin resistance (10, 16, 17). Furthermore, some cytokines held to have an important role in the pathogenesis of insulin resistance (TNF-α, IL-6, iNOS) seem to be more abundantly expressed in the macrophage-containing stromal-vascular part of adipose tissue than in the mature adipocyte fraction, suggesting that macrophages, not adipocytes, are the main producers of the cytokines responsible for the onset of insulin resistance (10, 16). Recent immunohistochemical experiments have confirmed that TNF-α and IL-6 are confined to macrophage-containing areas in the adipose tissue of obese mice (18). The reason for macrophage infiltration of obese adipose tissue was largely unknown until our group observed that the vast majority (>90%) of macrophages in obese mice and humans are found around dead adipocytes, where they may be resorbing the lipid remnants of these cells (11). This biological reaction results in characteristic morphological elements that we termed crown-like structures (CLS). CLS are easy to recognize at the light microscopic level, especially when macrophages are immunolabeled with specific antibodies. CLS thus identify dead adipocytes, and their number is a fair indication of the number of dead adipocytes and macrophages infiltrating the obese adipose tissue. If the cytokines responsible for insulin resistance are indeed produced by macrophages, CLS should be preponderant in visceral depots compared with subcutaneous fat in obese animals. Two recent papers seem to support this hypothesis, although one examined a visceral depot that is not found in

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animal number	db/db	db+	ob/ob	ob+
1	57	22	46	21
2	50	23	58	22
3	50	24	53	25
4	54	26	55	23
5	55	23,5	57	22
mean	**53.2**	**23.7**	**53.8**	**22.6**
SEM	**1.39**	**0.66**	**2.13**	**0.67**

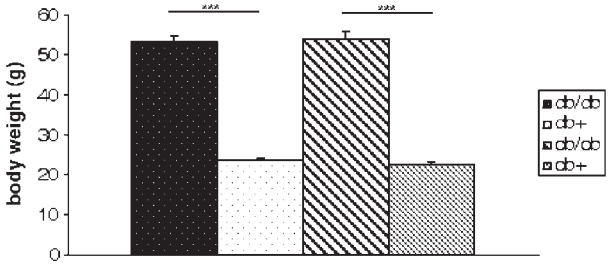


Fig. 1. Body weight (g) of animals at the time of euthanization. Mean ± SEM. *** $P < 0.001$.

humans (18) and the other analyzed only a small amount of adipose tissue in a heterogeneous population (19).

In this study, we thoroughly examined the visceral and subcutaneous fat depots of two different types of obese mice, db/db (strain Ks) and ob/ob (strain 6J), to establish whether dead adipocytes surrounded by macrophages (i.e., CLS) are more numerous in visceral than in subcutaneous fat. Such a finding would further support the adverse effect of visceral depots. We analyzed a large amount of adipose tissue from the depots that are most frequently responsible for human visceral obesity, whose portal drainage may have an important role in the pathogenesis of obesity-associated metabolic disorders.

MATERIALS AND METHODS

Animals

Twenty 14 week-old female mice were purchased from Harlan (Udine, Italy): five obese B6.V-Lep^{ob}/OlaHsd (hereafter ob/ob) and five lean control mice; five diabetic BKS.Cg^{-}+Lepr^{db}/+ Lepr^{db}/OlaHsd (db/db) and five lean control mice. Their weight is reported in **Fig. 1**. All animal procedures were in accordance with National Institute of Medical Research guidelines.

Mice were euthanized with an overdose of anesthetic (Avertin; Fluka Chemie, Buchs, Switzerland) and immediately perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 5 min.

Subcutaneous (inguinal) and visceral (mesenteric, omental, and perirenal) white adipose tissue depots were dissected using a Zeiss OPI1 surgical microscope (Carl Zeiss, Oberkochen, Germany) and assessed by light microscopy, immunohistochemistry, and morphometry.

The omental depot is seldom investigated in mouse studies (20), owing to its small size in these animals. Its anatomical site corresponds to that of other mammals, man included; its gross anatomy is shown in **Fig. 2**.

Light microscopy and morphometry

After dissection, depots were further fixed by immersion in 4% paraformaldehyde in 0.1 M sodium PB, pH 7.4, overnight at 4°C, then dehydrated, cleared, and paraffin embedded.

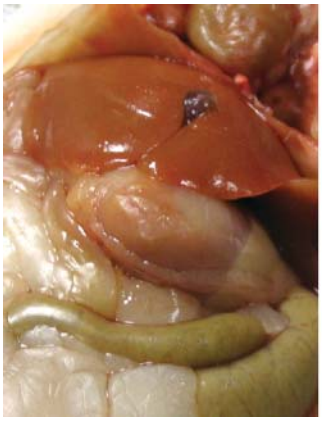
Two visceral depots with portal drainage (mesenteric and omental) (Fig. 2), a visceral depot with systemic drainage (perirenal) as well as the principal mouse subcutaneous depot (inguinal) were studied. Three sections from different levels (every 0.5 mm) of each depot were analyzed for adipocyte size and CLS density. For each level, 3 μm-thick serial sections were obtained, one for hematoxylin and eosin staining to assess morphology, and the others for immunohistochemical processing.

Adipocyte size was calculated as the mean adipocyte area of 300 random adipocytes (100 per section) in each mouse using a drawing tablet and the Nikon LUCIA IMAGE (version 4.61; Laboratory Imaging, Praha, Czech Republic) of the morphometric program. Tissue sections were observed with a Nikon Eclipse E800 light microscope using a ×20 objective, and digital images were captured with a Nikon DXM 1200 camera.

CLS density was obtained by counting the total number of CLS in each section compared with the total number of adipocytes and was expressed as CLS number/10,000 adipocytes.

Immunohistochemistry

For immunohistochemistry, 3 μm dewaxed serial sections were incubated with anti-MAC-2 (1:2,800; Cedarlane Laboratories, Paletta Court, Burlington,Ontario, Canada) and anti-perilipin (PREK 1:300, AS Greenberg, Boston,MA) primary antibodies according to the Avidin Biotin Complex method (16), as previously described (11). We used 3% hydrogen peroxide to inactivate endogenous peroxidase, followed by normal goat or



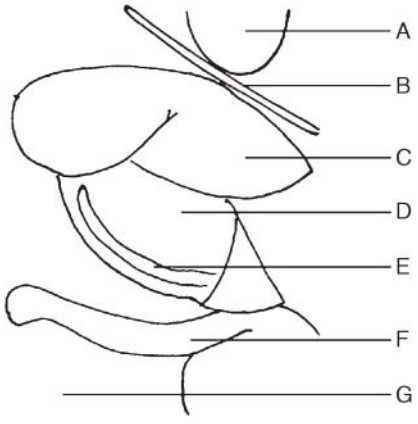


Fig. 2. Gross anatomy of the omental depot dissected under a Zeiss OPI1 surgical microscope. A = heart, B = diaphragm, C = liver, D = stomach, E = omental depot, F = colon, and G = mesenteric depot.

horse serum to reduce nonspecific staining. Consecutive serial sections were incubated overnight (4°C) with anti-MAC-2/galectin-3 (1:2,800) and anti-perilipin (1:300) primary antibodies. Biotinylated HRP-conjugated secondary antibodies were goat anti-rabbit IgG (PREK) and horse anti-mouse IgG (MAC-2/galectin-3; Vector Laboratories; Burlingame, CA). Histochemical reactions were performed using Vector's Vectastain ABC Kit (Burlingame, CA) and Sigma Fast 3,3′-diaminobenzidine as substrate (Sigma, St. Louis, MO). Sections were counterstained with hematoxylin.

Electron microscopy

Small tissue fragments were fixed in 2% glutaraldehyde-2% paraformaldehyde in 0.1 M PB, pH 7.4, for 4 h, postfixed in 1% osmium tetroxide, and embedded in an Epon-Araldite mixture. Semithin sections (2 μm) were stained with toluidine blue; thin sections were obtained with an MT-X ultratome (RMC; Tucson, AZ), stained with lead citrate, and examined with a CM10 transmission electron microscope (Philips; Eindhoven, The Netherlands).

Statistical analysis

Results are given as mean ± SEM. Differences between group means were analyzed by one-way ANOVA (InStat, GraphPad; San Diego, CA). Differences between groups were considered significant when $P \leq 0.05$. Linear correlations were calculated by nonparametric correlation (Spearman) performed using GraphPad Prism version 3.00 for Windows.

RESULTS

The body weight of the two strains of obese animals (ob/ob and db/db) was not significantly different, although both strains were significantly heavier (2.3-fold; $P = 0.001$) than the respective controls (Fig. 1). In lean mice, white adipocyte size was similar in all fat depots examined, but db/+ mouse adipocytes were 40% larger (**Fig. 3**) than those of their ob/+ counterparts ($P < 0.0001$). In both genetically obese mouse strains, white adipocytes were larger than in lean mice in all fat depots (Fig. 3). In db/db mice, adipocytes were 7.04-fold larger in omental fat, 6.2-fold larger in mesenteric fat, and 5.7-fold larger in perirenal fat; the increase was greatest in subcutaneous fat (8.1-fold). Adipocytes were also larger in ob obese than in lean mice (6.3-fold in omental fat, 7.5-fold in mesenteric fat, and 7.7-fold in perirenal fat). Again, the size increase was greatest in the subcutaneous depots (9.4-fold).

These data show that adipocyte size was significantly greater in the subcutaneous depots of both strains than in the pooled visceral depots: 1.3-fold both in db ($P = 0.002$) and in ob ($P < 0.0001$) obese mice (**Fig. 4**). MAC-2-immunoreactive macrophages were found in all depots of lean and obese mice. More than 90% were CLS (**Fig. 5**). They surrounded dead adipocytes containing various amounts of lipid remnants and had all the morphological, immunohistochemical, and electron microscopic characteristics described in our previous work (11). Dead adipocytes and macrophage infiltration in fat depots were quantified by measuring CLS density as CLS number/10,000 adipocytes (Fig. 3, **Fig. 6A**, **B**). CLS were more numerous in visceral than in subcutaneous fat in both mouse strains (db/db: $P < 0.0001$; ob/ob: $P = 0.006$) (Figs. 3, 4). The largest difference in CLS density was found between the subcutaneous depots and the mesenteric ($P < 0.0001$) and perirenal depots ($P = 0.004$)

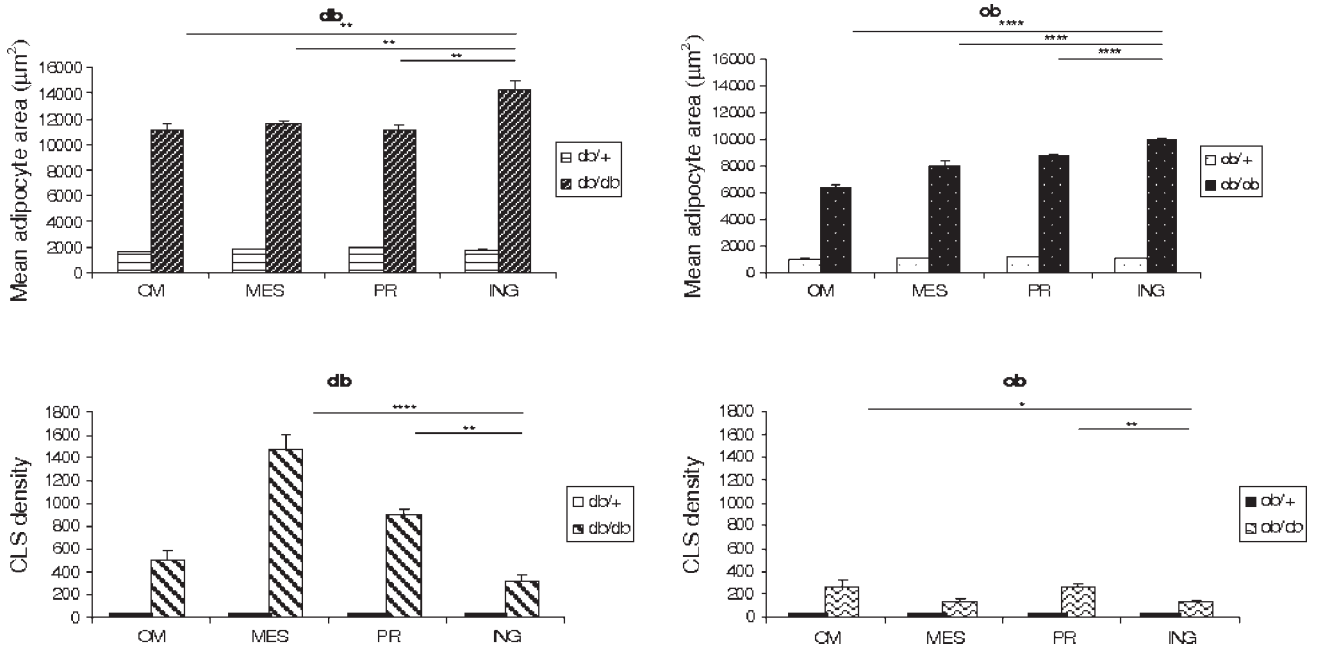


Fig. 3. Adipocyte size in three visceral fat depots (OM = omental, MES = mesenteric, and PR = perirenal) and in a subcutaneous fat depot (ING = inguinal) of lean and obese mice (upper panels). Mean ± SEM. Density of crown-like structures (CLS) in the visceral and subcutaneous depots of lean and obese mice (bottom panels). Mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

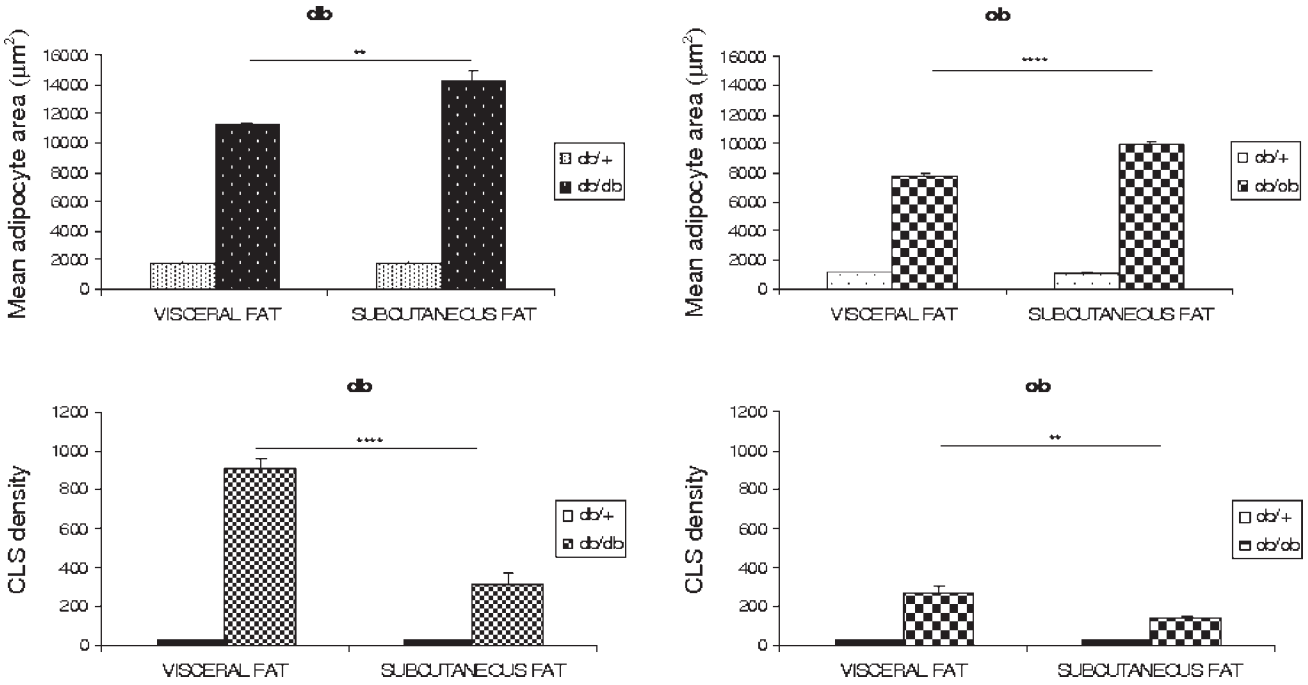


Fig. 4. Adipocyte size and CLS density in lean and obese mice. Data from the three visceral depots are pooled for direct comparison with the subcutaneous depot. ** $P < 0.01$; **** $P < 0.0001$.

in db/db mice, and between the subcutaneous depots and the perirenal ($P = 0.005$) and omental depots ($P = 0.04$) in ob/ob animals.

Such prevalence of dead adipocytes and macrophage infiltration was surprising because visceral depots exhibited the smallest adipocytes (Fig. 3) despite the positive correlation consistently found between adipocyte size and CLS density in both visceral and subcutaneous depots (**Fig. 7**). CLS density in the visceral depots of db/db mice was 3.4-fold greater than in the corresponding visceral depots of ob/ob mice [907.44 ± 48.05 vs. 267.08 ± 32.36; CLS number/10,000 adipocytes; $P < 0.0001$]. The largest difference was found in the mesenteric depot, where CLS density was 10.6-fold greater in the former animals. CLS density in the omental and perirenal depots of db/db mice was almost twice that of ob/ob mice (omental: 1.8-fold, $P = 0.04$; perirenal: 1.9-fold, $P = 0.01$).

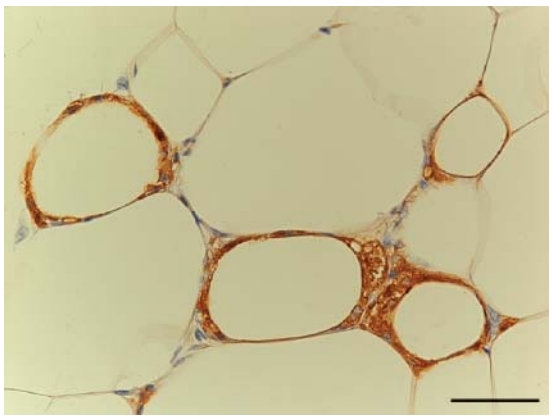


Fig. 5. Representative light microscopic image of CLS found in the mesenteric depot of a db obese mouse showing MAC-2-positive macrophages around remnants of lipid droplets of dead adipocytes. Immunohistochemistry: anti-MAC-2 antibodies (Cedarlane Laboratories; dil. 1:2800), Avidin Biotin Complex method in paraffin-embedded tissue. Bar: 50 μm.

DISCUSSION

It is well known that metabolic disorders in obese patients are most commonly associated with visceral fat accumulation (5–9). There is also growing evidence for the concept that the mild chronic inflammation of adipose tissue seen in obese patients and animals is associated with insulin resistance, which is probably the main cause of the other metabolic disorders (16, 21, 22). A time coincidence has been shown between adipose tissue infiltration by macrophages during weight gain and onset of insulin resistance (10, 16, 17).

In a previous work, we showed that >90% of macrophages infiltrating the adipose tissue of obese humans and animals are found around dead adipocytes, forming characteristic elements that we termed crown-like structures (11). Therefore, quantification of CLS yields a fairly accurate number of the macrophages infiltrating the fat depots. In this work, comparison of different visceral and subcutaneous fat depots of two strains of genetically obese mice indicated that visceral depots are the prevalent sites of adipocyte death and macrophage infiltration. The data suggest that the higher incidence of metabolic disorders associated with visceral fat accumulation could be due to a greater susceptibility to death of the adipocytes found

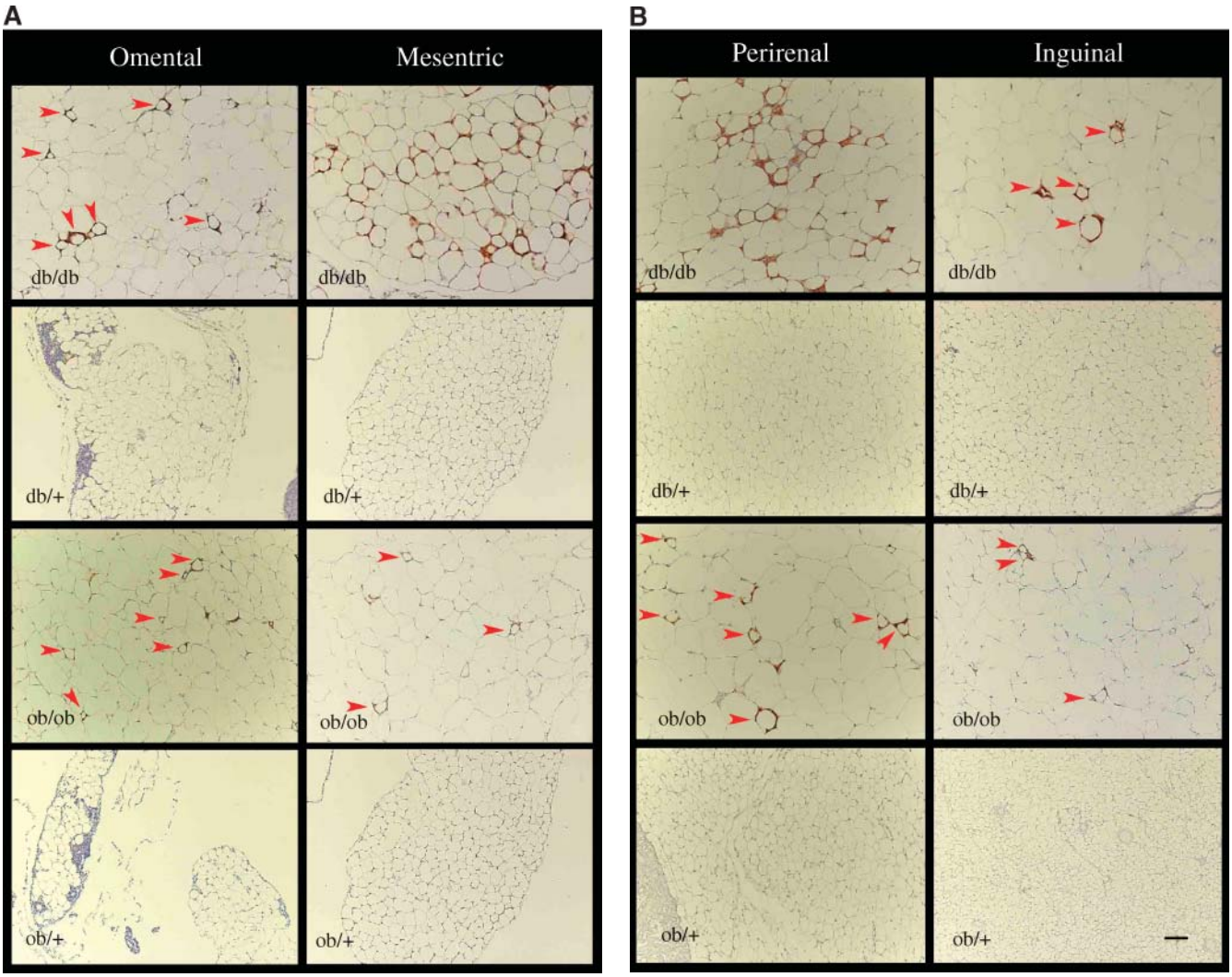


Fig. 6. Representative light-microscopic images from omental and mesenteric (A) and perirenal and inguinal (B) fat depots of lean and obese mice studied by MAC-2 immunohistochemistry. MAC-2-immunoreactive CLS are indicated by red arrowheads only when less than 10 were found in the field. Bar: 100 μm.

at this anatomical site compared with those found in the subcutaneous depot. This is in line with the view that adipocytes found in different depots have different properties (23, 24).

Visceral adipocytes have long been known to be smaller than their subcutaneous counterparts both in lean and in obese animals (25), and our data are consistent with this concept (indeed, visceral adipocytes were seen to be 20% smaller). The positive linear correlation found between adipocyte size and CLS density in all depots suggests that increased adipocyte size is the factor triggering macrophage infiltration, as also hypothesized in other studies (10). This suggests that visceral adipocytes reach the critical size triggering death before subcutaneous adipocytes. The established concept that larger adipocytes correlate with greater insulin resistance (26) could thus need to be reconsidered in its pathogenic mechanism.

Our data agree with recent findings on CLS density in a visceral (epididymal) and a subcutaneous depot in mice fed an obesity-inducing diet (18); however, the epididymal depot is not found in humans and is endowed not with portal but with systemic venous drainage. A recent paper supports a preferential visceral versus subcutaneous macrophage infiltration also in obese human patients (19). Some authors have hypothesized that the differences in size, function, and potential contribution to disease shown by the different fat depots may be due to regional intrinsic, differences, including differences in preadipocyte characteristics (23, 27). The greater propensity of visceral compared with subcutaneous adipocytes to die could thus be explained by intrinsic cellular differences among different fat depots. We, like most other investigators, studied adult (14 week-old), genetically obese mice, but Strissel et al. (18), recently reported that fat macrophage infiltration changes dynamically in mice fed a high-fat diet. Therefore different patterns at different ages cannot be ruled out. It is also interesting to note that CLS density in the visceral depots of db/db mice was about 3.5-fold higher than in

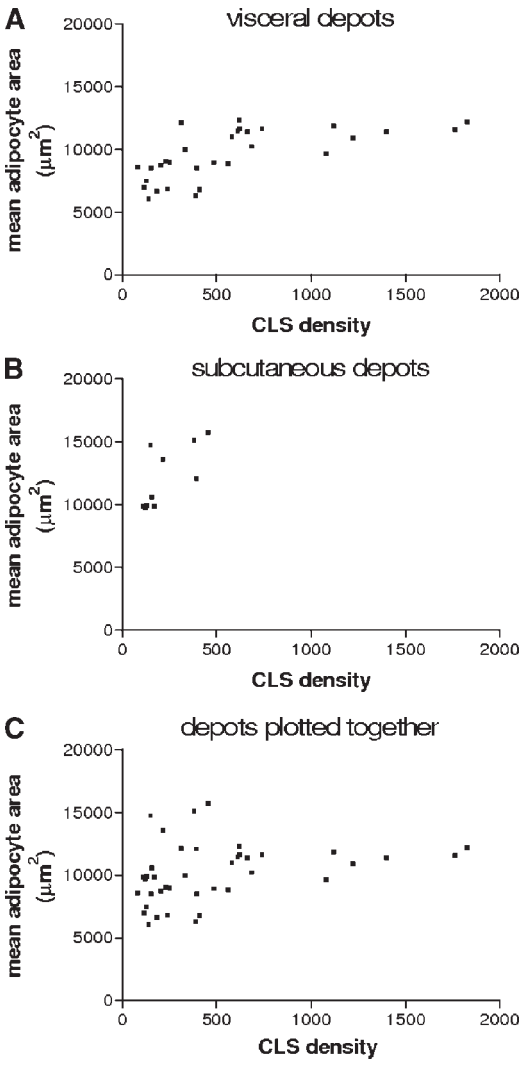


Fig. 7. Statistical correlations between mean adipocyte size and CLS density (number of CLS/10,000 adipocytes) in visceral depots (A), subcutaneous depots (B), and in all depots plotted together (C) from five ob/ob and five db/db mice. Mean adipocyte area was a strong predictor of CLS density in (A) visceral ($r = 0.72$, $P < 0.0001$) and (B) subcutaneous ($r = 0.76$, $P = 0.01$) depots. Linear correlations were calculated by nonparametric correlation (Spearman). (r = Spearman coefficient; P = probability).

ob/ob mice. Although we did not measure blood chemical parameters, db/db mice with the genetic background and at the age of those used in the present study seem to show a more impaired glucidic metabolism (28).

In conclusion, our data suggest that despite a positive correlation between size of adipocytes and macrophage infiltration both in visceral and in subcutaneous depots, the visceral depots display a more intense infiltration, even though adipocytes in visceral depots are smaller than those found in subcutaneous depots. The higher susceptibility of visceral adipocytes to cellular death could be due to different intrinsic proprieties of the different depots, and could be causally related to the appearance of metabolic disorders, as suggested by other authors (18, 19).

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