Original Articles

Immunohistochemical and Ultrastructural Localization of Leptin and Leptin Receptor in Human White Adipose Tissue and Differentiating Human Adipose Cells in Primary Culture

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Leptin is mainly produced in white adipose tissue and acts both at distant sites and locally at the tissue from which it originates. The cellular and subcellular localization of leptin and its receptor (Ob-receptor [Ob-R]) and their relationship to various stages of fat cell maturation have not been characterized as yet. Therefore, we analyzed leptin and Ob-R by using reverse transcriptase–polymerase chain reaction, immunohistochemistry, and ultrastructural immunogold labeling in human white adipose tissue and in human adipocyte cell cultures at early and late stages of differentiation. Both leptin and its receptor were present in mature unilocular fat cells. The thin cytoplasmic rim of the adipocytes exhibited the strongest expression of both leptin and Ob-R. At early stages of differentiating human adipocytes, leptin was mainly expressed in multilocular preadipocytes, whereas the Ob-R was found predominantly on fibroblast-like cells. Other cellular components of human white adipose tissue were characterized by anti-CD31 for endothelial cells, anti-CD68 for macrophages, and antibodies specifically labeling B-cells and T-cells. In addition to fat cells, endothelial cells were immunopositive for the full-length leptin receptor. On the ultrastructural level, leptin was mainly found attached to cellular membranes and in small alveolate vesicle-like structures in the cytoplasm of adipocytes. Leptin was also present on the cell membranes of endothelial cells and macrophages. We conclude that the expression of the Ob-R in human white adipose tissue is not restricted to adipocytes but is present in resident endothelial and immune cells. Ultrastructural localization studies revealed an association of leptin with cell membranes and small vesicles. The cellular and subcellular distribution of leptin and its receptor suggests an important autocrine and paracrine role for leptin in human adipose tissue. *Diabetes* 49:532–538, 2000

Leptin is an adipostatic circulating hormone that is mainly produced in adipose tissue (1,2). This hormone decreases food intake and increases energy expenditure (2). As part of a feedback system that regulates fat cell stores in the human body, leptin closely correlates with body adiposity and weight changes. Although the molecular structure, regulation, and function of this hormone have been extensively studied, no reports exist regarding its subcellular localization and distribution in its main production site, the adipose tissue (3). White fat constitutes the bulk of all adipose tissue and is distributed throughout the body. It contains several types of cells, including adipocytes, endothelial cells, and immune cells (4,5). Adipocytes originate from fibroblast-like lipid-free precursor cells to mature gradually into lipid-laden "signet-ring" adipocytes (6).

Leptin mRNA and protein are expressed primarily by mature fat cells (7–9). However, in which compartment of the fat cell leptin is localized is not clear and neither is whether leptin is stored or associated with an organelle or secretory granule. Furthermore, the mRNA for the long active form of the leptin receptor has been demonstrated in human adipose tissue only by reverse transcriptase–polymerase chain reaction (RT-PCR) (10,11). From these studies, whether leptin receptors are expressed only by adipocytes or also by other cellular components of adipose tissue, including endothelial cells and resident immune cells, is not clear.

The aim of this study was to analyze the localization of leptin and its receptor in human adipose tissue and in human adipocytes sustained in primary culture at various stages of maturation and to investigate for the first time the subcellular distribution of leptin in human white fat tissue by ultrastructural immunolocalization.

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BSA, bovine serum albumin; DMEM/F12, Dulbecco's modified Eagle's medium/Nutrient Mix F12; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase–polymerase chain reaction.

RESEARCH DESIGN AND METHODS

Subcutaneous and visceral tissue specimens of white human fat were obtained from normal-weight human subjects (BMI <26 kg/m^2) undergoing abdominal surgery or surgical mammary reduction. The study was approved by the ethics committees of the Universities of Düsseldorf and Leipzig.

Cell culture. After removal, adipose tissue was immediately transported to the laboratory in phosphate-buffered saline (PBS) containing 20 mg/ml bovine serum albumin (BSA). The isolation of adipose tissue–derived stromal cells was performed as described previously with minor modifications (12,13). Adipose tissue was dissected from fibrous material and visible blood vessels, was minced into small pieces, and was digested for 90 min with PBS containing 20 mg/ml BSA and 250 U/ml collagenase type *Clostridium histolyticum* (Biochrom KG, Berlin, Germany). The dispersed tissue was filtered through nylon mesh (pore size 150 µm) and was centrifuged for 10 min at 200*g*. Sedimented cells were resuspended in erythrocyte lysis buffer containing 0.154 mol/l NH_4Cl, 10 mmol/l $KHCO_3$, and 0.1 mmol/l EDTA and were incubated for 10 min. The cells were then washed one time with PBS and finally were resuspended in Dulbecco's modified Eagle's medium/Nutrient Mix F12 (DMEM/F12) (1:1) containing 15 mmol/l HEPES and 2.5 mmol/l L-glutamine (Life Technologies, Karlsruhe, Germany) supplemented with 1.125 g/l $NaHCO_3$, 10% fetal calf serum, and 50 µg/ml gentamicin. Cells were seeded in four-well culture slides (Becton Dickinson, Heidelberg, Germany) at a density of 150,000 cells/well. The next day, most cells were attached to the slides. After washing twice with PBS, stromal cells were further cultured in a defined serum-free medium to induce differentiation into adipocytes. The adipogenic medium consisted of DMEM/F12 supplemented with 1.125 g/l $NaHCO_3$, 50 µg/ml gentamicin, 10 µg/ml transferrin, 100 nmol/l cortisol, 66 nmol/l insulin, and 200 pmol/l triiodothyronine. During the first 2 days, 20 µmol/l 3-isobutyl-1-methylxanthine and 1 µg/ml troglitazone were also added to the adipogenic medium. Medium was exchanged every 48 h. Within 16 days of culture, stromal preadipocytes differentiated into adipocytes. All buffers and media were adjusted to a pH of 7.4.

RNA isolation and PCR experiments. Total RNA was isolated from human white fat by using a silica gel–based membrane method with the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA content and quality were determined photometrically. The RNA was digested with ribonuclease-free deoxyribonuclease A (Boehringer Mannheim, Mannheim, Germany) and 1 U/µg RNA in 20 mmol/l Tris-HCl (pH 8.0) and 2.5 mmol/l MgCl for 10 min at 27°C. The reaction was stopped by incubation at 65°C for 10 min and the addition of 5% (vol/vol) EDTA (20 mmol/l). To control the complete digestion of DNA, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR was performed by using RNA as a template. A total of 5 µg of RNA was taken to synthesize cDNA by using the cDNA single-strand synthesis kit with oligo (deoxythymidine) primers (Pharmacia Biotech, Freiburg, Germany). The quality of the templates was confirmed by GAPDH PCR. PCR was performed in a GeneAmp 9600 thermal cylcer (Perkin Elmer, Überlingen, Germany) by using 36 cycles and 5′Cy5-labeled intron-spanning primer pairs for the full-length human leptin receptor (Ob-R), and for the short isoforms (Ob-R219.1–Ob-R219.3). The identity of the PCR products was proved by sequencing (Med Sequanus; Pharmacia Biotech). Experiments were reproduced with RNA isolation from three different adipose tissues as described previously (14).

Light microscopy and immunohistochemistry in white adipose tissue and fat cell cultures. Formalin-fixed normal white fat was sectioned, deparaffinized, and unmasked by using heat treatment in sodium citrate buffer (pH 6, 10 mmol/l) at 95°C for 15 min before blocking endogenous peroxidase with 0.3% H_2O_2 for 15 min. By following the manufacturer's protocol, the sections were preincubated with 2% normal swine serum and were exposed to the 1:50 dilution of the polyclonal goat anti-human leptin receptor antibody (Ob-R) (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min. The antibody was raised against a peptide corresponding to amino acid 1146-1165 of the COOH-terminus of the full-length Ob-R. As a negative control, the human Ob-R antibody was replaced by goat IgG (Dianova-Immunotech, Hamburg, Germany), and no nonspecific staining was observed. After incubation with biotinylated link antibody for 15 min and horseradish peroxidase (HRP)-labeled streptavidin for 15 min, visualization was achieved by immersing the sections in 3-amino-9-ethyl-carbazole (Dianova-Immunotech, Hamburg, Germany) for 10 min. Slides were counterstained with hematoxylin for 1 min, rinsed in water for 10 min, and mounted with glycerin gelatin. Hypothalamus was used as a positive tissue control (data not shown).

Immunostaining for leptin was performed in the same way by using a rabbit anti-human leptin antibody (Ob) (clone Y-20; Research Diagnostics, Flanders, NJ) suitable for Western blotting and immunohistochemistry. Negative control sample staining with nonimmune rabbit serum was performed and showed no nonspecific staining. For detection of endothelial cells, monoclonal mouse anti-human CD31 cells were used (Dako Diagnostika, Hamburg, Germany). Macrophages and monocytes were characterized by using immunohistological staining with anti-human CD68/KP1, α–1-antichymotrypsin, and anti-CD14 (Dako Diagnostika). Leukocytes, B-cells, and T-cells were identified by using LCA, L26, and CD3 antibodies (Dako Diagnostika). Cultured cells were washed with PBS, and chambers were removed from the slides. Cells were then dried in the air flow of the laminar bench for 1 h and were immediately stained or wrapped in tinfoil and stored at –80°C. Before immunohistochemical staining, cells were fixed in 70% ethanol for 10 min. Antigen detection was achieved with the LSAB+ Kit (HRP) and the AEC Substrate System according to the manufacturer's protocol (Dako Diagnostika) in combination with specific primary antibodies.

Electron microscopy and immunogold labeling. For immunoelectron microscopy, small pieces of human white fat tissue were fixed in 0.1% glutaraldehyde and 2% formalin for several hours and were embedded in acrylic resin. Ultrathin sections were mounted on 200-mesh uncoated nickel grids. Sections were floated on blocking solution, incubated for 1 h in the primary antibody, incubated for 1 h within secondary antibody–gold conjugate, and then rinsed in PBS and in water (Pelco, Redding, CA). Sections were stained with uranylacetate and lead citrate. The sections were examined with a Phillips CM10 electron microscope (Phillips Electronics, Mahway, NJ) and photographed.

RESULTS

Expression of leptin and leptin receptors in human white adipose tissue: RT-PCR. Both leptin and leptin receptor mRNA were demonstrated in human white adipose tissue. In addition to the long form of the Ob-R, the mRNA of the isoform Ob-219.1–Ob-219.3, which lacks a part of the intracellular domain, was identified (Fig. 1).

Immunolocalization of leptin and leptin receptor in human white adipose tissue in situ: light microscopy. In addition to mature fat cells, human white adipose tissue consists of stromal cells, blood cells, tissue macrophages, and endothelial cells. By using immunohistochemical analysis, leukocytes (anti-LCA) were found frequently in the adipose vessels. Subclassification of leukocytes in serial sections demonstrated mostly T-cells (anti-CD3 positive) with a few B-cells (anti-L26 positive). Macrophages immunostained with anti-CD68 or α–anti-chymotrypsin were frequently found in direct contact with mature adipocytes (Fig. 2*A* and *B*). Endothelial cells lining the vessels are clearly characterized by a CD31 antibody (Fig. 2*C*). Macrophages, lymphocytes, and endothelial cells were found more frequently in visceral fat than in subcutaneous fat (Fig. 2*A* and *B*).

Mature fat cells were strongly stained with anti-human leptin antibody. The most prominent staining was observed in the small cytoplasmic rim of the adipocyte (Fig. 2*D*). Immunostaining for the full-length Ob-R revealed a staining pattern similar to leptin itself. The cytoplasmic rim of the adipocyte provided the strongest signals (Fig. 2*E*). Other cellular components of the white adipose tissue also showed a positive staining for the leptin receptor; endothelial cells were clearly positive for the Ob-R protein (Fig. 2*F*). No difference was evident in the staining pattern of visceral versus subcutaneous fat.

Detection of leptin and leptin receptor in preadipocyte stromal cells in primary culture and in differentiated human adipocytes. Stromal vascular cells obtained from white human adipose tissue were inoculated in serum-containing medium. After 16–20 h, cells were in a preconfluent stage and demonstrated a fibroblast-like morphology. After 5 days of culture, cells were grown to confluency and started to accumulate small refringent lipid droplets. The early cell cultures contained few CD31-positive endothelial cells. At this stage, all stromal cells, preadipocytes with small lipid droplets, and undifferentiated fibroblast-like cells demonstrated a positive staining for leptin (Fig. 3*A*). However, the staining in the preadipocyte with small lipid droplets was more prominent (Fig. 3*A*). After 18 days of culture in serum-free medium inducing fat cell differentiation, 70% of the cells

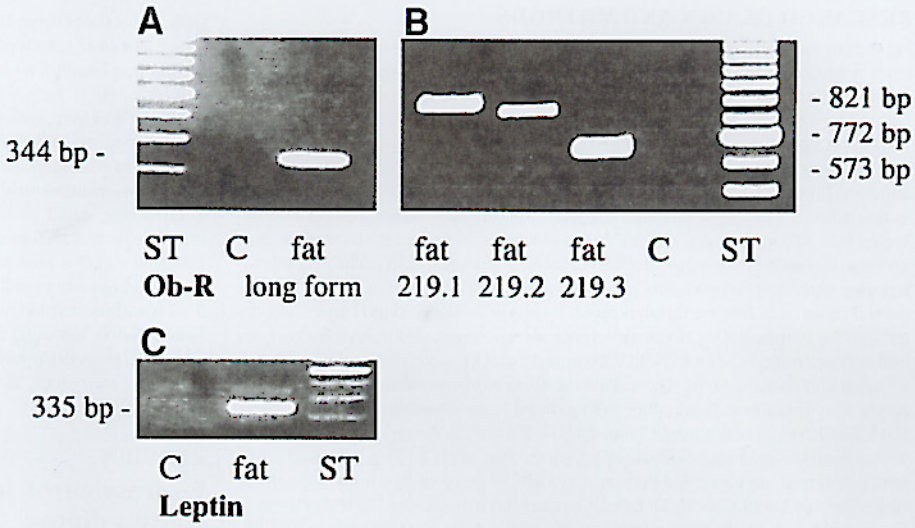

FIG. 1. RT-PCR detection of mRNA expression of the full-length Ob-R (*A*); the shorter Ob-R isoforms Ob-219.1–Ob-219.3 (*B*); and leptin (*C*) in human adipose tissue (fat). Negative control (C) without cDNA template in the sample; ST, molecular marker (100-bp ladder, Boehringer Mannheim).

demonstrated adipocyte-like morphology with triacylglycerol droplets. The differentiated adipocytes exhibited a marked staining for leptin. Similar to the in situ tissue preparations, no leptin staining occurred inside the lipid droplets (Fig. 3*B*). After 5 days of culture, only a few undifferentiated fibroblast-like cells were positive for the leptin receptor and

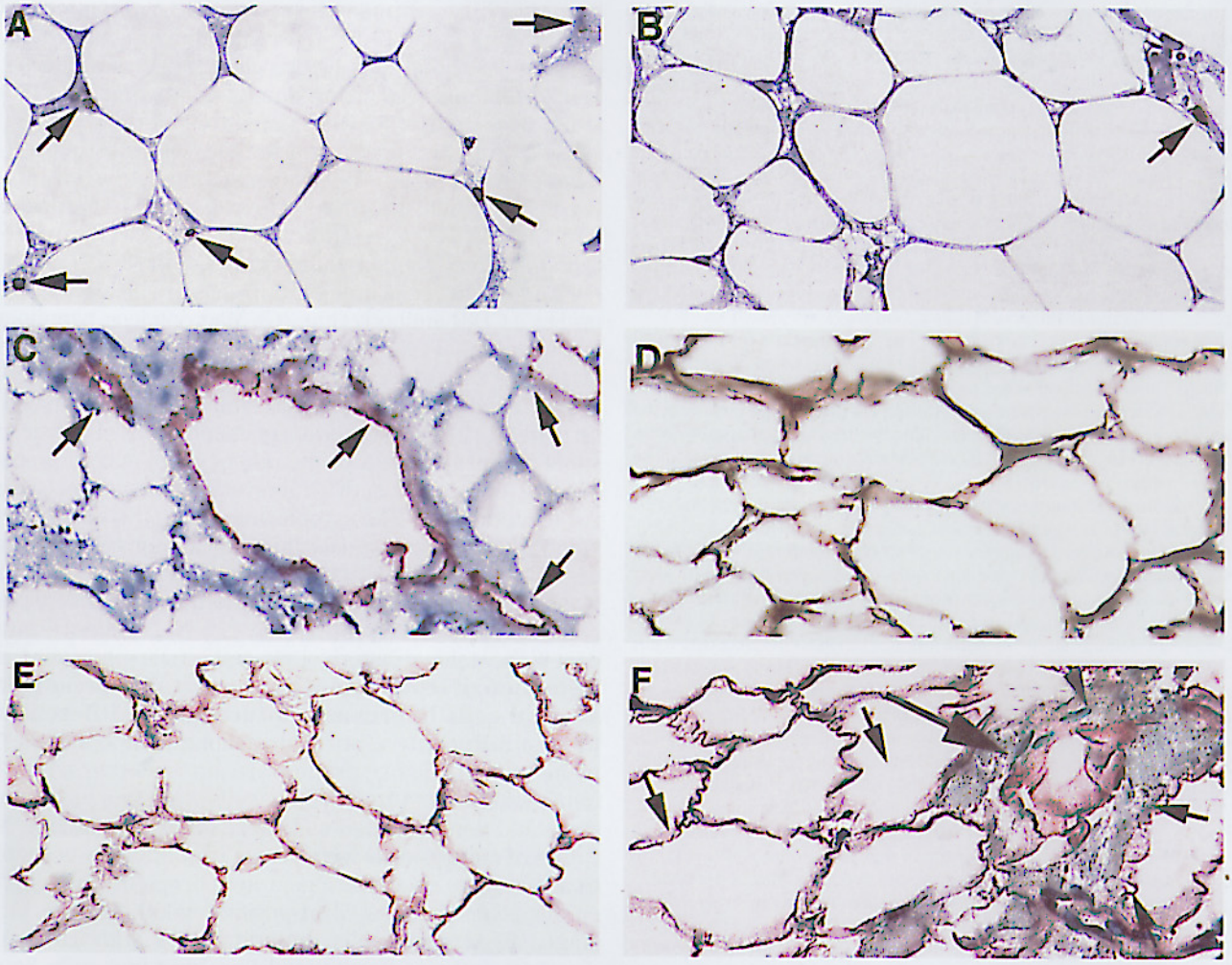

FIG. 2. Human white adipose tissue. CD68-positive macrophages (arrows) in direct contact with mature fat cells (×200) in visceral (*A*) and subcutaneous (*B*) fat. Macrophages are more frequent in visceral fat. *C*: Endothelial cells (arrows) immunostained with anti-CD31 lining small vessels in human white adipose tissue (×300). All adipocytes are stained with anti-human leptin antibody (Ob; Y-20). *D*: Note the strong staining in the cytoplasmic rim of the adipocyte (×200). *E*: The staining pattern for the full-length human leptin receptor (Ob-R; C-20) is similar to leptin itself (×150). *F*: Endothelial cells (arrows) exhibit an immunostaining pattern for the leptin receptor (×200).

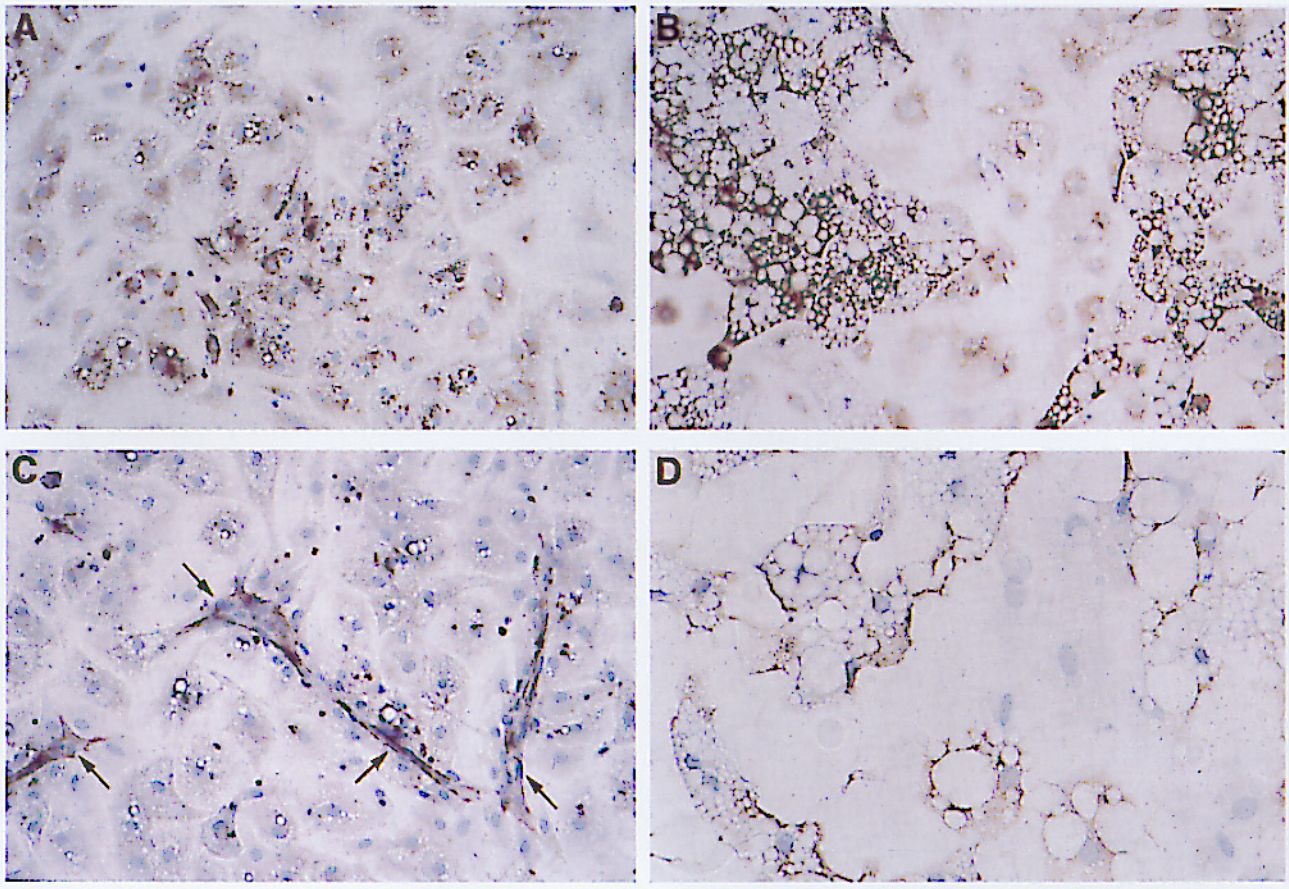

FIG. 3. Immunostaining of human fat cells in culture. *A*: After 5 days of culture, multilocular preadipocytes demonstrate a strong signal for leptin, whereas undifferentiated fibroblast-like cells exhibit only a weak staining (×100). *B*: After 18 days of culture in serum-free medium inducing fat cell differentiation, mature adipocytes show an intense staining in the thin cytoplasmic rim but not in the lipid droplets (×100). *C*: In 5-day cultures, some fibroblast-like cell preadipocytes exhibit prominent membrane staining for the leptin receptor (×100). *D*: In 18-day cultures, most differentiated fat cells express leptin receptor (×100).

exhibited a membrane staining pattern (Fig. 3*C*). At this stage of differentiation, the multilocular preadipocytes were negative for the leptin receptor. In contrast, after 18 days of culture, most differentiated fat cells showed a positive staining for the full-length Ob-R (Fig. 3*D*).

Ultrastructural localization of leptin in human white fat tissues. In human white adipose tissue, mature fat cells were characterized by a small oval-shaped nucleus with scarce perinuclear cytoplasm containing filamentous mitochondria, some endoplasmic reticulum, and a large number of vesicles. From this area, a thin rim of cytoplasm completely enveloped the central lipid inclusions (Fig. 4*A*). Consistent with the light microscopic findings, the small cytoplasmic rim of the adipocyte revealed gold particles that demonstrated labeling for leptin along the cell membranes (Fig. 4*B*). Leptin secretion was also found in the interstitial space between two neighboring fat cells. No leptin signal was inside the fat droplets (Fig. 4*A* and *B*). Although no large storage organelle for leptin could be detected in the adipocyte, small clustering of immunogold particles could be identified in alveolate or flask-shaped structures of uniform size (40–80 nm in diameter) attached to the cell membrane (Fig. 4*C* and *D*). Furthermore, in cultured preadipocytes, leptin was found in the cytoplasm of the cells (Fig. 5*A* and *B*). Although no leptin was inside the small lipid droplets, this hormone was found attached to the membranes of liposomes (Fig. 5*A*) and inside small vesicles along the cell membrane (Fig. 5*B*). Endothelial cells of capillary vessels were close to the surface of white fat cells. Most often, they were separated from these cells by their respective basement membranes and small bundles of collagen. Interestingly, leptin was frequently localized at the outer membrane of the endothelial cells, which formed frond-like folds (Fig. 5*C*). In addition, leptin was localized on collagen fibers (Fig. 5*D*) and on the outside of macrophages (Fig. 5*E* and *F*). Macrophages were characterized by typical primary and secondary lysosomes, a well-developed Golgi complex, small amounts of rough endoplasmic reticulum, elongated mitochondria, and microvilli.

DISCUSSION

Adipose tissue is not merely an inert form of connective tissue with the ability to store and release fat but rather is a dynamic cellular system that is continually active in the highly regulated uptake, storage, and release of lipids and in the secretion of hormones and cytokines, including leptin. Adipocytes express "functional" leptin receptors, and leptin was found on fat and other local cell membranes, which suggests an autocrine and/or paracrine role for this hormone. Leptin and its receptor were demonstrated by using both RT-PCR and immunohistochemistry. Quantitative RT-PCR and North-

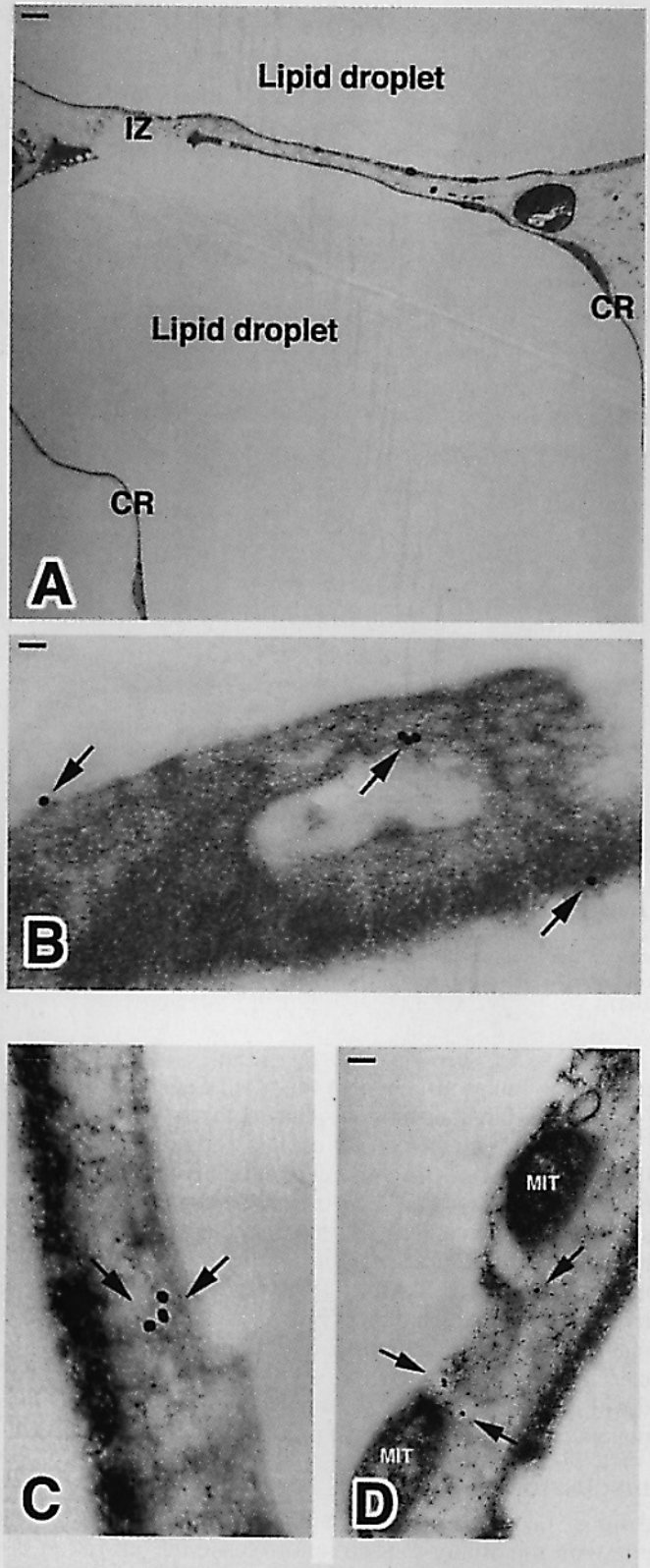

FIG. 4. Electron micrograph of human fat cells. *A*: Mature fat cells are characterized by a thin rim of cytoplasm (CR) surrounding a large central lipid inclusion (bar = 1.3 μm). *B*: No leptin signal is inside the fat droplets, but immunogold particles representing leptin molecules (arrows) are found along the cellular membrane (bar = 0.05 μm). Small clustering of immunogold particles occurs beneath the cell membrane frequently in alveolate flask-shaped vesicles (arrows) (*C* [bar = 0.05 μm] and *D* [bar = 0.2 μm]). MIT, mitochondria.

ern blot analyses previously showed that higher leptin mRNA expression is found in subcutaneous fat than in visceral fat. This finding was attributed to an increased adipocyte cell size and leptin gene expression in subcutaneous white fat cells (15–17). In our study, we found a higher content of macrophages, lymphocytes, and endothelial cells in visceral fat than in subcutaneous fat. This variation in cellular content of nonadipocyte cells may contribute to the differences in leptin expression between the two types of fat.

In our study, no large storage organelle for leptin could be detected in the adipocyte. This coincides with previous reports that leptin is newly synthesized and rapidly secreted from the cell (18). On the other hand, a study recently showed that insulin can induce some leptin secretion from presenting intracellular pools in the presence of a protein synthesis inhibitor in isolated rat adipocytes (19). This is consistent with our finding of a small clustering of immunogold particles representing leptin in the adipocytes in fat tissue in situ in humans. We will be interested to see whether the adipocyte can store more leptin under certain physiological or pathological conditions. The human adipocyte is known to have a complex system of vesicle formation. Thus, GLUT4 continuously recycles between the cell surface and an endosomal compartment in the adipocytes (20–22). Insulin decreases the rate of GLUT4 endocytosis while increasing its expression on the cell membrane (20–22). In addition, the adipocyte expresses several proteins that are involved in the regulation of exocytosis of neuroendocrine cells (e.g., Rab 3A) or a synaptic vesicle protein (e.g., synaptobrevine) (23,24). Therefore, our data suggest that, although leptin is not stored in large pools in the adipocyte, its cellular compartmentalization with vesicle-like alveolae may suggest a regulated exocytotic mechanism in its secretion. Colocalization of leptin with specific vesicle-associated proteins, fractionation, and studies using confocal laser microscopy will be useful in providing definitive proof of this concept. Analysis of the normal secretory process of leptin will be crucial in the search for abnormalities of leptin secretion in obesity, diabetes, and other diseases.

The binding of leptin to preadipocyte cell membranes and collagen fibrils is intriguing. Research recently showed that leptin acts on human bone marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes (25). Leptin resulted in a dose- and time-dependent increase in type 1 collagen, a decrease of adipsin and leptin mRNA levels, and a 50% decrease in lipid droplet formation (25). The findings suggest that leptin is "involved in a local ultra short negative feedback loop" (25).

Adenovirus-induced hyperleptinemia in normal rats resulted in fat loss, downregulation of lipogenic enzymes, suppression of the transcription factor peroxisome proliferator-associated receptor-γ, and upregulation of uncoupling proteins 1 and 2 and of the preadipocyte marker Pref-1 (26). Finally, the absence of fat in transgenic mice was associated with a 20-fold reduction in leptin levels and led to marked metabolic alterations including hyperlipidemia, insulin resistance, and diabetes (27). Together with these findings, our morphological data suggest that leptin may indeed act locally in fat tissue to influence the formation of mature adipocytes. Therefore, human adipose tissue may have an intrinsic autocrine feedback system controlling fat cell maturation. An impairment of this local feedback regulation could likely

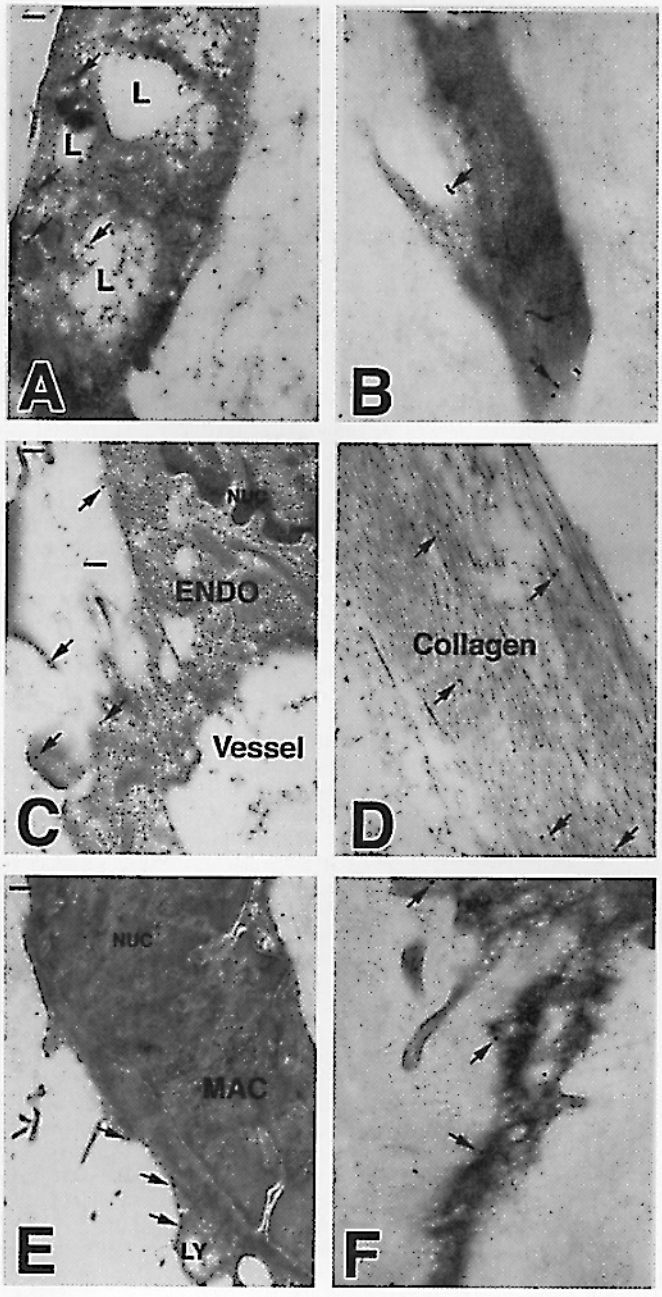

FIG. 5. Electron micrograph of human adipose tissue cells. In preadipocytes, leptin particles are localized on liposome membranes (L) and in small vesicles along the cell membrane (arrows) (*A* [bar = 0.3 μm] and *B* [bar = 0.2 μm]). Leptin binds to the outer cell membrane (arrows) of endothelial cells (ENDO) (*C* [bar = 0.3 μm]) and to collagen fibers (*D* [bar = 0.3 μm]). Leptin immunogold labeling is positive along macrophage (MAC) cell membranes (*E* [bar = 0.3 μm] and *F* [bar = 0.3 μm]). LY, lysosomes; NUC, nucleus.

cause or aggravate obesity and could also account for rapid resynthesis of fat and the frequent treatment failure observed in obese individuals.

Is leptin involved in a paracrine regulation of other cellular components in the fat tissue? Research recently showed that leptin promoted angiogenesis in cultured human endothelial cells and induced neovascularization in the corneas of rats (28,29). Therefore, our data, which demonstrate the expression of leptin receptor and binding of leptin to endothelial cells, suggest local regulation of adipose vasculature by leptin. Adipogenesis and angiogenesis are positively correlated during fat mass development, and capillaries are in close apposition to white fat cells (4). Triglycerides removed from the blood circulation are stored in fat cells. At the same time, however, neutral fat droplets break up, and lipids are released into the same blood vessels from which they entered the adipocytes. The exact mechanism that regulates storage and release of lipids (depending on the appropriate energy requirements of the organism and mediated by endocrine, paracrine, autocrine, and nervous inputs) is only partially understood. Again, based on our findings and on the possible effect of leptin on both adipocytes and endothelial cells, leptin may play a crucial role in balancing the process of fat accumulation and depletion.

Leptin receptors have been described on hematopoietic stem cell macrophages and lymphocytes (30). In our ultrastructural study, we found leptin molecules clearly binding to macrophages in the fat tissue. Leptin is a stress-related peptide that shares many features with cytokines (31–34). It regulates proinflammatory responses and the T-helper 1 (TH1) and T-helper 2 (TH2) immune response and is elevated in patients with acute sepsis (35–41). In fact, leptin appears to be involved in the immune defense of humans (35–41). It is expressed at high levels in bone marrow adipocytes, and phenotypic abnormalities in macrophages have been reported in leptin-deficient obese mice (42,43). Human fat produces copious amounts of interleukin-6 (44) and large amounts of tumor necrosis factor-α (45–47). However, the interaction of leptin with these cytokines is not clear at this point (48–50). We are only beginning to understand the role of adipose tissue in the regulation of the immune system and vice versa. Evidently, however, leptin participates in a local immune cell–adipocyte interaction in fat tissues.

In conclusion, our data demonstrate no large storage of leptin in adipocytes, localization of this hormone within small pinocytotic vesicles, and binding of this protein to cellular membranes expressing leptin receptors. Leptin receptors were present on adipocytes, endothelial cells, and macrophages, which suggests both an autocrine and paracrine action for leptin in human adipose tissue. Based on these findings, we suggest that leptin plays a major local role in the tissue in which it is produced. Elaborating on the mechanisms of the local actions of leptin should lead to a new understanding of the functions of human fat in both physiological states and in metabolic disorders.

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