Annu. Rev. Nutr. 1992. 12:207–33

CELLULAR AND MOLECULAR ASPECTS OF ADIPOSE TISSUE DEVELOPMENT

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KEY WORDS: adipose cell differentiation, hormonal factors, signaling pathways, gene expression

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INTRODUCTION

During the past two decades, growth and development of white adipose tissue (WAT) and brown adipose tissue (BAT) has been extensively studied in

0199-9885/92/0715-0207$02.00

various mammals and humans. Despite a wealth of data, key information is lacking regarding the origin of fat cells and is minimal regarding adipose tissue development during embryogenesis and after birth. However, the relationships between WAT and BAT and the molecular mechanisms leading to adipose cell differentiation are now better understood. Quite recently, the importance of adipose tissue as an endocrine, autocrine, and intracrine organ has been established. In this review we examine the current understanding of the cellular and molecular mechanisms that ultimately determine the hyperplastic development of adipose tissue, and we discuss some of the controversial issues. When needed, references to older but important work are included.

Adipocytes represent between one third and two thirds of the total number of cells in adipose tissue. The remaining cells are various blood cells, endothelial cells, pericytes, adipose precursor cells of varying degree of differentiation, and, most likely, fibroblasts (54). Over the past few years, convincing evidence has accumulated concerning the existence of very small fat cells in addition to mature adipocytes (31, 43, 75). The relationships between a definition of cell types based upon ultrastructural studies and one based upon biological studies are summarized in Figure 1. Most of the cell population of adipose precursor cells corresponds presumably to those cells previously defined in developing and adult rodents as "interstitial cells" (14, 15, 52–54), "nonlipid-filled mesenchymal cells" (95), or "other mesenchymal cells," which includes both "undifferentiated" and "poorly differentiated" mesenchymal cells (100). From a biological perspective, it is generally accepted that adipose precursor cells arise from multipotential stem cells, the origin of which remains undecided. From an embryological perspective, the process of determination from multipotential stem cells leads to the formation of unipotential adipoblasts. Commitment of adipoblasts leads to the formation of preadipocytes, defined as cells that have expressed early but not yet late markers and that have not yet accumulated triacylglycerol stores. Thus, by definition, the population of adipoblasts and preadipocytes should correspond to interstitial cells or nonlipid-filled cells. Although adipoblasts are formed during embryonic development, it is unclear whether some remain postnatally or whether only preadipocytes are present. Clearly, biochemical or molecular markers are urgently needed to carry out decisive studies of the development of adipose tissue.

PROLIFERATION AND DIFFERENTIATION OF WHITE ADIPOSE TISSUE (WAT)

Embryonic Development

White adipose tissue cannot be detected macroscopically during embryonic life and at birth in rodents (rat, mouse), whereas it is present at birth in the

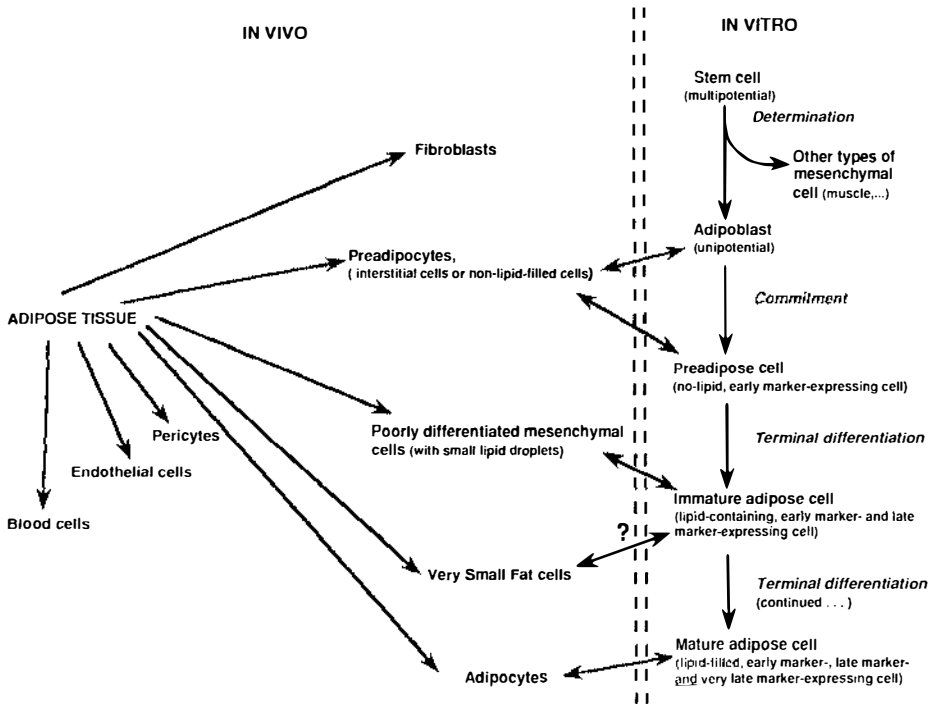

Figure 1 Relationships between morphological types in vivo and stages of cell differentiation in vitro. The adipocyte fraction corresponds to adipocytes and some very small fat cells. The stromal-vascular fraction corresponds to a mixture of the other cell types.

pig, rabbit, guinea pig, and human. The development of white adipose tissue has been studied in pig fetuses (66, 68, 69). Approximately at the beginning of the last third of the gestation period, large and small arterioles can be detected in the vicinity of small developing fat cell clusters that are surrounded by extensive stroma. Cell cluster size increases steadily with fetal age, but the number of clusters does not change significantly before birth. The importance of tissue vascularization is underlined by the fact that fat cell density appears to be positively associated with capillary density and that the largest fat cell clusters are located near the entry points of large blood vessels. The embryonic development of human adipose tissue takes place earlier than that of the pig, i.e. at the beginning of the second third of the gestation period in various sites (buccal, neck, shoulder, gluteal, perirenal) (16, 105–107). Adipose tissue appears and develops in those areas where it remains after birth. Initially, the aggregation of a dense mass of mesenchymal cells (mesenchymal lobules with no lipid accumulation) is associated with the organization of a vascular structure. Primitive fat cell clusters are then formed, composed of densely packed fat cells adjacent to capillaries. In the last stage, growth of

adipose tissue is mainly due to an increase in size of fat cell clusters surrounded by mesenchyme, which condenses rapidly and then thickens to form septa among the clusters. Both during pig and human embryogenesis, angiogenesis appears to be tightly coordinated with the formation of fat cell clusters in time and space. Therefore, the characterization of specific growth factors able to trigger and modulate the development of capillaries and fat cell clusters represents an important issue. A wide variety of ubiquitous angiogenesis factors has been described, some of which (TGF-β, PGE_2) are synthesized and secreted by adipocytes. Quite recently, however, monobutyrin (1-butyrylglycerol) has been described as a novel angiogenesis factor that stimulates motility (35). The biosynthetic pathway has been delineated recently, and monobutyrin appears to be a fat-specific angiogenesis factor (137). Conversely, microvascular endothelial cells from human omental adipose tissue [most likely mesothelial cells (130)] in culture secrete more insulin-like growth factor-I (IGF-I) and IGF-binding protein(s) than do epithelial cell types (79). Clearly, IGF-I mRNAs (and secreted IGF-I) in the human fetus are preferentially localized in fibroblasts and other cells of mesenchymal origin (61). It is now generally accepted that IGF-I acts as an endocrine hormone via the blood, and locally as a paracrine/autocrine factor, and that growth hormone (GH) controls its synthesis both in liver cells and in peripheral cells of mesodermal origin (70, 71). Taken together, these observations agree with in vitro data and suggest that the requirement for IGF-I in the adequate hyperplastic development of adipose tissue is met during embryogenesis.

Postnatal Development

Methods of determining fat cell number and size have been used to study the postnatal development of WAT, particularly in rodents. Unfortunately, they are not sufficiently accurate to detect modest changes in cell number and, in any event, they only count lipid-filled cells. In other words, preadipocytes (Figure 1), i.e. cells that have expressed early phenotypes but do not yet contain triacylglycerol droplets, are not counted. This technique excludes a priori a true estimate of the developmental potential of the various adipose depots. The expression of S-100 proteins may help solve this problem, because during the development of rat epididymal fat tissue, nonlipid-filled cells (interstitial cells) stain positively for these proteins while endothelial cells, pericytes, and fibroblasts do not stain (23). Numerous studies using ^{3}H-thymidine have been performed in order to distinguish between cell proliferation and the lipid-filling process. By combining pulse-labeling with ^{3}H-thymidine followed by autoradiography and histochemical visualization of α-naphtyl acetate hydrolase activity, i.e. most likely lipoprotein lipase (LPL) and/or monoglyceride lipase activity, Pilgrim (104) has been able to distinguish between lipid-free, mesenchymal cells (presumably adipoblasts), lipid-

free, esterase-positive cells (presumably preadipocytes), and lipid-filled, esterase-positive cells (adipocytes). The proliferative activity is highest in preadipocytes, which suggests that committed cells are able to proliferate, whereas fully differentiated cells lose the capacity to divide. Using a similar approach in mice, Cook & Kozak (26) have observed the highest labeling index in cells negative for glycerol-3-phosphate dehydrogenase (GPDH) activity, a late marker of adipose cell differentiation. A dramatic decrease of the labeling index precedes the rise of GPDH activity, which is detected subsequently in all triacylglycerol-containing cells. Thus it appears that early marker-expressing cells undergo mitoses before terminal differentiation takes place.

Sex- and site-related differences in body fat distribution are well known both in human and various animal species. Possibly, part of the explanation regarding the differential hyperplastic growth of adipose tissue lies at the cell level. In any event, the ability of adult rodents and human to increase the number of adipocytes, depending on the localization of the adipose depot, the nature of the diet, and the environmental conditions to which the animals were exposed, has been long known (39). Some controversy exists, however, as to whether the formation of new fat cells takes place during refeeding after a prolonged period of food deprivation. Initial reports indicated that such a regimen caused loss and recovery of endothelial and non-lipid filled mesenchymal cells only and that no loss or gain of fat cells occurred (95). Quite recently, a similar nutritional study in adult mice that used improved ^{3}H-thymidine autoradiography has shown that poorly differentiated mesenchymal cells can replicate. The labeling index reaches $\sim$ 10% at the sixth day of refeeding, giving rise to labeled adipocytes found near capillaries (100). Some loss of adipocytes appears possible under severe pathological conditions: a reduction in the number of fat cells from parametrial and retroperitoneal sites has been reported in diabetic rats (54), but the turnover of adipocytes clearly is a slow process. Proliferation of mature fat cells has remained a controversial issue. In vivo labeling experiments with ^{3}H-thymidine have shown that mature adipocytes do not incorporate the label to a significant degree. Recently, however, Sugihara and coworkers have reported (129) that approximately 2% of cultured adipocytes from very young rats can undergo mitoses in culture. Possibly, this phenomenon is related to the rearrangement of the extracellular matrix in vitro following collagenase treatment of the abdominal subcutaneous adipose tissue; this phenomenon might not take place in vivo to a significant extent.

Late Development

Since the pioneering work of Lemonnier (89), the hyperplastic development of adipose tissue in aging animals fed a high-carbohydrate or high-fat diet has

been thoroughly studied. Sprague-Dawley or Osborne-Mendel rats are able to increase the fat cell number in most of their adipose depots (retroperitoneal, perirenal) in response to a high-carbohydrate or a high-fat diet (39). The life-long potential to make new fat cells has been clearly illustrated in rodents. For instance, the perirenal fat depots of very old mice from both sexes contain large amounts of early markers of differentiation, i.e. A2COL6/pOb24 mRNAs, LPL mRNAs, and IGF-I mRNAs, indicating that preadipocytes at stages 2 and 3 are indeed present in these depots (4) (see section on sequential events and Figure 2). A similar conclusion can be indirectly drawn in humans, since a significant proportion of stromal-vascular cells from subcutaneous fat tissue of elderly men and women is able to differentiate in vitro into adipose cells (65). This finding indicates that adipose precursor cells, which are not likely to give rise to new fat cells, are indeed present in those individuals. This observation can also explain the acquisition of new fat cells, which is known to take place at the adult stage in normal subjects and obese patients.

Endocrine Regulation

Pituitary hormones may trigger and/or modulate directly or indirectly the formation of mature fat cells. In humans, GH deficiency and possibly hypothyroidism are accompanied by hypoplasia and hypertrophy of subcutaneous fat tissue (11). However, adipose tissue can be formed in hypophysectomized pigs (67, 109), which suggests that either GH is not obligatory or other hormone(s) act as substitute(s). Hypothyroidism in rats induces a transient hypoplasia, whereas hyperthyroidism induces a transient hyperplasia of retroperitoneal and epididymal fat tissues (90), suggesting a precocious formation of mature fat cells in response to triiodothryronine (T_3). The role of insulin in adipose cell differentiation has been investigated in streptozotocin-diabetic rats, taking advantage of the fact that after one month of diabetes the majority of adipocytes (*i*) have lost the bulk of their triacylglycerol, (*ii*) show a very small diameter, and (*iii*) contain several tiny triacylglycerol droplets (termed pauciadipose cells). After infusion of insulin by mini-osmotic pumps and pulse-labeling for 4 h with ^{3}H-thymidine, the labeling indices at days 1, 4, and 8 have been determined in the various cell types of parametrial adipose tissue (endothelial cells, interstitial cells, pauciadipose cells). A rapid and dramatic hypertrophic effect of insulin on pauciadipose cells transformed into adipocytes is observed, followed by a slower but potent effect of insulin on the proliferation of interstitial cells, which gives rise to neo-formed pauciadipose cells (53).

Glucocorticoids have been long known to increase adipose tissue mass via their hypertrophic effect. In Cushing's syndrome, hypercortisolism, which leads to centrally localized adipose tissue as in abdominal obesity, is likely accompanied by cell hyperplasia (110). A higher density of glucocorticoid

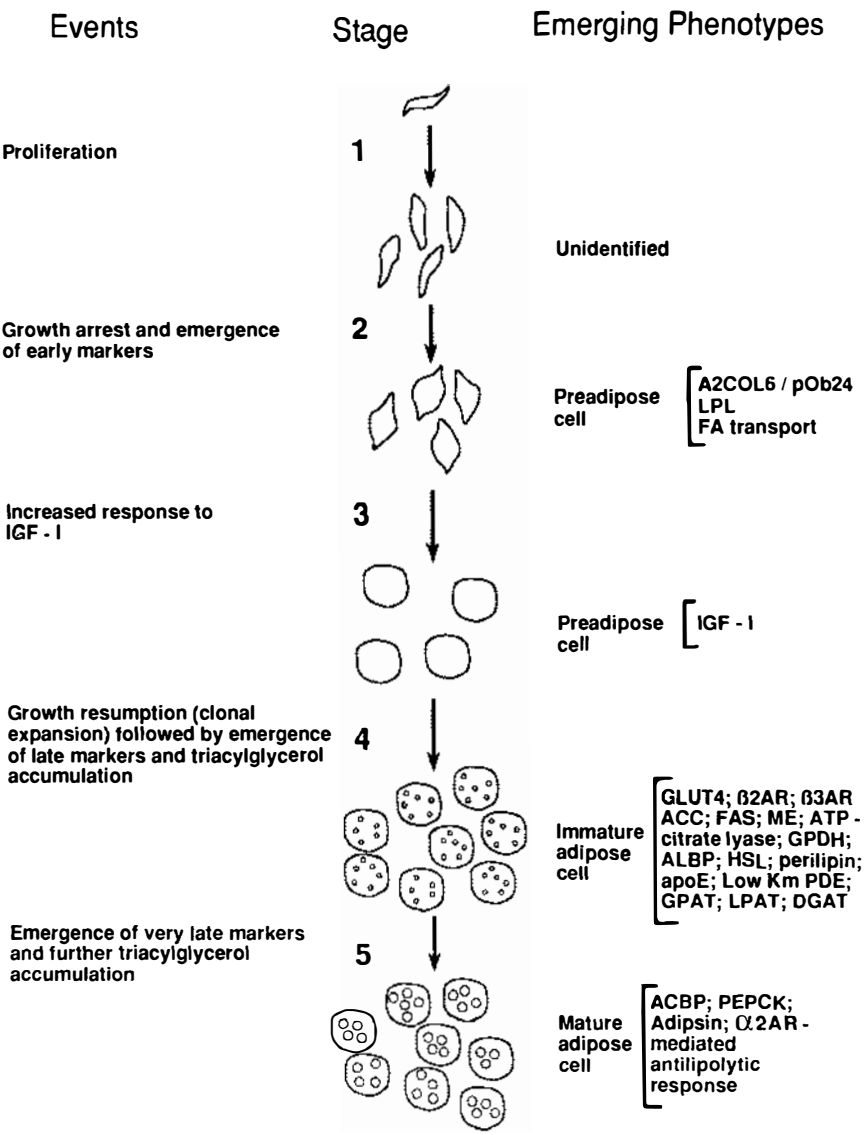

Figure 2 Multiple stages of adipose cell differentiation. The scheme is based upon data obtained with 3T3-L1, 3T3-F442A, and Ob17 cells as well as with rodent adipose precursor cells. The abbreviations are LPL, lipoprotein lipase; FA transport, fatty acid transport; A2COL6/pOb24, α2-chain of collagen VI; IGF-I, insulin-like growth factor I; GLUT-4, insulin-sensitive glucose transporter 4; ME, malic enzyme; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthetase; GPDH, glycerol-3-phosphate dehydrogenase; ALBP, adipocyte lipid-binding protein (aP2); HSL, hormone-sensitive lipase; apoE, apolipoprotein E; low Km PDE, low Km phosphodiesterase; GPAT, glycerophosphate acyltransferase; LPAT, lysophosphatidate acyltransferase; DGAT, diglyceride acyltransferase; ACBP, acyl CoA binding protein; PEPCK, phosphoenolpyruvate carboxykinase; GS, glutamine synthetase; β_2-AR, β_2-adrenoreceptor; β_3-AR, β_3-adrenoreceptor; α_2-AR, α_2-adrenoreceptor.

receptors is found in abdominal fat tissue than in other fat depots, and investigators have proposed that glucocorticoids may regulate this differential development (111). Glucocorticoids such as cortisol reportedly control terminal differentiation of human adipose precursor cells (see below). Thus one can envision that cortisol may play an important role in the development of cell hyperplasia and subsequently cell hypertrophy. The role of steroid sex hormones in the hyperplastic development of adipose tissue has not yet been documented, although low levels of testosterone in men or low levels of female sex hormones with increased levels of testosterone in women have been associated with increased visceral fat mass. This association may not be fortuitous; primates are unique in that a large proportion of androgens in men (40%) and a preponderance of estrogens in women (75% before menopause, ~ 100% after menopause) are synthesized in peripheral tissues (including adipose tissue) from adrenal precursor steroids (85, 86).

PROLIFERATION AND DIFFERENTIATION OF BROWN ADIPOSE TISSUE (BAT)

Considerable evidence now supports the view that BAT is a specific organ distinct from WAT. In most mammalian species, BAT develops during fetal life and is identifiable at birth. Studies on the prenatal development of BAT are scarce and have been hampered by the small size of BAT depots. Developmental changes of BAT were therefore examined in bovine fetuses: the emergence of uncoupling protein (UCP) unique to BAT mitochondria takes place during the last trimester of gestation (18). At birth both in bovine and ovine, the different adipose depots (perirenal, subscapular, retroperitoneal), except for the subcutaneous adipose tissue, can be considered as BAT (19). Taking advantage of the remarkable hyperplasia of adult rat BAT in response to cold acclimation and/or adaptation to hyperphagia, Bukowiecki and coworkers have studied the sequence of events leading to the differentiation of brown fat cells, under conditions where the labeling index increased 60–80 times over control values. After cold exposure for two days, the majority of labeled cells are interstitial and endothelial cells. The labeling index, which remains unchanged in endothelial cells with exposure time, decreases in interstitial cells whereas it increases first in poorly differentiated cells and later in brown adipocytes. Based upon these results and the determination of specific labeling frequency, i.e. the labeling index for a *given* cellular type independent of the other cellular types, the chronology for differentiation appears to be interstitial cells → poorly differentiated brown fat cells → brown adipocytes. Endothelial cells can be excluded as progenitors of brown adipocytes (15). The stimulation of BAT proliferation and differentiation is β-adrenergic-mediated, since continuous infusion of nor-

epinephrine or isoproterenol as β-agonists to warmth-exposed rats mimicks the effect of cold exposure, whereas the α-agonist phenylephrine is ineffective (52, 99, 114). Recent evidence obtained in adult dogs favors a β_3 receptor-mediated process (97). Using a novel β_3 adrenoreceptor agonist (ICI D7114) that has thermogenic and anti-obesity properties in this animal, a potent increase of the UCP content is observed in extracts of perirenal, peribladder, and pericardiac adipose tissue (116).

RELATIONSHIPS BETWEEN BAT AND WAT

Studies on the development of WAT and to a lesser extent of BAT have led to much controversy regarding the relationships between both tissues. The existence of distinct precursor cells (Figure 3) is assumed and is discussed later, but the existence of a single adipoblast giving rise to distinct brown and white preadipocytes cannot be ruled out. A possible transformation of brown adipocyte into white adipocyte, defined by the disappearance of UCP, is strongly suggested: UCP mRNA, which in bovine fetuses at birth reaches its highest level in the various adipose depots, is no longer detectable two days later. Similarly, ovine UCP mRNA disappeared within two days from peritoneal adipose depot and within six days from all adipose depots (18). Since the estimated number of adipocytes remains constant in bovine during the first week following birth, we assume that a true transformation of brown into white adipocytes has been indeed taking place (Figure 3). Despite a dramatic mitochondriogenesis, the reverse phenomenon does not appear to take place in the epididymal fat pad, when rats are exposed to severe cold stress (93). In mice, under similar conditions, inguinal WAT only appears as BAT, expresses UCP mRNA, and the developed mitochondria contain UCP; this expression ceases in rewarmed animals (92).

ADIPOSE CELL DIFFERENTIATION IN VITRO

General Considerations

The process of determination from multipotential stem cells to unipotential adipoblasts cannot be easily studied (see Figure 1). However, a determination-like process can be induced by treatment of mouse 10T1/2 and 3T3 cells (131) and hamster CHEF-18 cells with 5-azacytidine (118), whereas it is spontaneous in T984 cells isolated from a mouse teratocarcinoma (30). In most cases, these treated mesenchymal cells were able to differentiate into adipocytes, chondrocytes, and fibroblasts. Adipose precursor cells or adipoblasts (see Refs. 2, 3, and 4 for reviews) have been cloned from this mixed population of cells (1246 clonal line), from mouse embryo (3T3-L1, 3T3-F442A, A31T, TA1) or hamster embryo (CHEF-18), and from adult mice

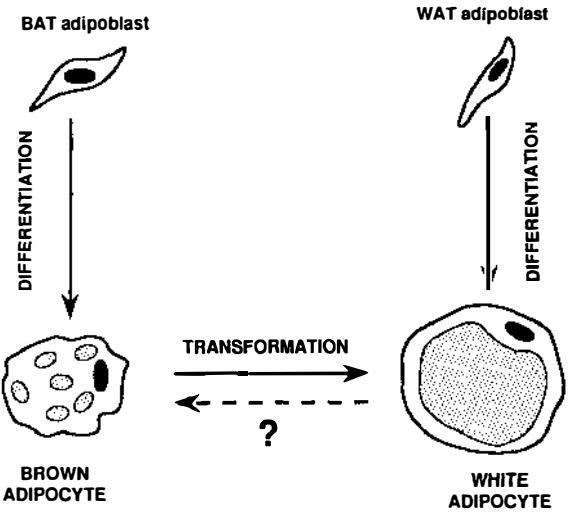

Figure 3 Distinct BAT and WAT precursors. Relationships between brown and white adipocytes.

(Ob17, HFGu, BFC-1, ST13, MS3-2A, MC 3T3-G2/PA6). When dealing with the various "preadipocyte" clonal lines, the process of adipose cell differentiation, which can be analyzed in vitro, corresponds to the phenotypic changes: adipoblast → preadipose cell (usually termed preadipocyte) → immature adipose cell → mature adipose cell (Figure 1). Some investigators have begun to study stromal-vascular cells from adipose tissues of various species, including humans (20, 32, 51, 57, 65, 115, 123); these cells are diploid but have a limited life span. Although the presence of adipoblasts cannot be excluded, the differentiation process of adipose precursor cells isolated from fat tissue corresponds primarily to the sequence: preadipose cell (preadipocyte) → immature adipose cell → mature adipose cell. This tentative conclusion is based upon the fact that rat, mouse, and human stromal-vascular cells contain the bulk of A2COL6/pOb24 mRNA and express LPL and IGF-I mRNAs, i.e. have already expressed early markers (stages 2 and 3 of Figure 2). Note that, when injected into animals at the undifferentiated state, cells from established lines (3T3-F442A, Ob17) or rat stromal-vascular cells develop into mature fat cells (4). When seeded at clonal densities, stromal-vascular cells from rat perirenal and epididymal fat depots show varying capacities for replication and differentiation, irrespective of donor age (34, 80, 135). At any given age, stromal-vascular cells from perirenal fat tissue showed a greater proportion of clones with a high frequency of differentiation than was found in epididymal fat tissue (34). In humans, stromal-vascular cells from abdominal fat tissue show a higher capacity for differentiation than those of the femoral depot (64), in agreement with clinical

observations regarding the differential adipose tissue development observed for both sites. Both in rat for the perirenal and epididymal adipose tissues (80) and in human for the subcutaneous adipose tissue (65), aging is associated with a decrease in the proportion of cells undergoing differentiation.

Sequential Events

Morphologically, after reaching confluence, fibroblast-like cells become round, enlarge, and accumulate triacylglycerol droplets in their cytoplasm. Once differentiated, mature adipose cells show most biochemical characteristics and hormonal responses, if not all, of adipocytes. The main events, based upon numerous studies by various investigators, are summarized in Figure 2. Growth arrest at the G_1/S stage of the cell cycle, rather than contact among arrested cells, is necessary to trigger the process of cell commitment (5). This commitment is associated with the emergence of potential regulatory genes such as A2COL6/pOb24 (29, 72) and clone 5 (96), LPL mRNAs and LPL activity (28), as well as the emergence of a selective uptake of long-chain fatty acids (1). The regulation of expression of these early genes takes place primarily at a transcriptional level and appears to be independent of various hormones which, in contrast, are required for terminal differentiation (28, 29). The expression of late and very late genes is associated with limited growth resumption of these committed, early marker-expressing cells. DNA synthesis of preadipose cells precedes terminal differentiation. At least one cell doubling has been consistently observed by various investigators using different cell lines and different culture media, and this process of clonal amplification of committed cells (defined as postconfluent mitoses) is limited both in magnitude and duration. Cell division of preadipose cells appears to be essential for terminal differentiation (defined by the emergence of GPDH activity), providing that cells are exposed to the appropriate hormonal milieu (5, 48, 84, 121). The observations made in vitro are in agreement with those made in vivo by Pilgrim (104) as well as by Cook & Kozak (26) concerning the relationships in rodent adipose tissue between cell proliferation and differentiation. However, a complete dissociation between growth resumption and terminal differentiation takes place in the case of human adipose precursor cells.

The process of terminal differentiation is characterized by the induction of late and very late markers. As shown in Figure 2, the enzymatic machinery required for lipogenesis and triacylglycerol synthesis is then turned on and is responsible for lipid accumulation. Among newly discovered late markers are GLUT-4 (17, 50, 60, 76, 83, 132), β_2- and β_3-adrenoreceptors (40, 41), perilipin (55), and apolipoprotein E (apoE) (142). Very late events include the emergence of acyl-CoA-binding protein (ACBP) (62) and α_2-adrenoreceptor-mediated response (119). In the case of GPDH, adipocyte-lipid-binding

protein (ALBP or aP2), and adipsin genes, this emergence is primarily due to increased gene transcription (25, 33). With respect to fatty acid metabolism, the increase in uptake of fatty acid is concomitant with the induction of LPL (Figure 3). Fatty acid entry then becomes sufficient to activate the transcription of ALBP and acyl-CoA synthetase (ACS) genes, whereas cholesterol can activate the expression of apoE gene (142). The time course of appearance of cholesterol ester transfer protein (CETP) mRNA and that of the corresponding protein are not known, but they are abundant in adipose tissue of mammals (74). Investigators report that the emergence of phospholipase A2 activity parallels that of GPDH activity in cells of the 1246 clonal line and in rat adipose precursor cells (49), which suggests an increase in the disposal of fatty acids (at position 2 of glycerolipids), including arachidonic acid required for terminal differentiation (46).

Changes in receptor level and hormone sensitivity during differentiation of 3T3 and Ob17 cells have been extensively described for insulin and lipolytic hormones (2). During the last few years, the characterization of β- and α_2-adrenoreceptors has expanded remarkably, and new strategies regarding the control of adipose cell hypertrophy can be considered (87). Quite recently, using specific ligands, Fève et al (40, 41) investigated the differential regulation of β_1-, β_2-, and β_3-adrenoreceptors in 3T3-F442A cells and that of their respective mRNAs. β_1-adrenoreceptors, detectable in growing, undifferentiated cells, increase their level up to 6-fold at growth arrest, whereas β_2-adrenoreceptors (under glucocorticoid stimulation) and β_3-adrenoreceptors emerge later, at a time when GPDH activity is also emerging. β_3-adrenoreceptors represent 90% of the total population of β-adrenoreceptors in differentiated 3T3-F442A cells. Since both these differentiated cells and mouse adipocytes share the same pharmacological properties with respect to β-adrenergics, β_3-adrenoreceptors likely play an important role in mediating catecholamine-induced lipolysis. Extrapolation of these observations to human adipose precursor cells remains questionable, because no β_3-adrenoreceptor mRNA has been reported in human fat cells (38).

Control of Terminal Differentiation of Preadipose Cells by Adipogenic and Antiadipogenic Factors

The critical role played by various hormones in regard to *differentiating* cells should not be confused with the role of the same hormones in regard to *differentiated* cells. So far, growth arrest at the G_1/S phase of the cell cycle appears sufficient to commit the cells and allow the expression of early markers (5, 28, 29). The combination of hormonal factors that trigger terminal differentiation of preadipose cells (stages 3 to 5 of Figure 3) remains difficult to define owing to the widely different experimental conditions used and the origin of the cells. When obtained in serum-supplemented medium,

the data are difficult to interpret, as illustrated recently by the expression of LPL gene in Ob17 cells, since the multiple effectors present in serum regulate its expression either negatively or positively (108). Therefore, serum-free, chemically defined media have been developed for the differentiation of adipose precursor cells from various clonal lines and those derived from various species including rat (32, 123), rabbit (115), porcine (51), ovine, (20) and human (65). For cells from clonal lines, the first hormonal requirement in serum-supplemented medium appears at stage 3 with GH activating the IGF-I gene at a transcriptional level (36). Induction can only occur after cell growth is arrested and early markers are expressed. Zezulak & Green reported that requirement of GH is followed by an increase in the responsiveness of 3T3-F442A cells to IGF-I; IGF-I in turn causes a mitogenic response, leading to clonal expansion, which is defined as post-confluent mitoses (143). This observation is at variance with another showing that exogenous IGF-I is obligatory and sufficient for the differentiation of 3T3-L1 cells (128). Under serum-free conditions, GH is required for differentiation of 3T3-F442A (59), and it has been suggested that GH drives committed cells to a special state of quiescence (the primed state), allowing them to respond to insulin, and then terminates differentiation. These conflicting results may be explained by the fact that cells may represent different developmental stages in the differentiation process and that, under serum-supplemented conditions, different hormones may play the same role while other hormones may merely overcome the inhibition of some serum factors. Data obtained under serum-supplemented and serum-free conditions indicate that GH is not required for the conversion of adipose precursor cells to adipocytes in rodents, domestic animals, and humans. If GH is indeed needed at some stage, a prior exposure of the cells to the hormone in vivo may be sufficient to "prime" the cells for terminal differentiation.

Triiodothyronine appears to be essential for the differentiation of Ob17 cells both in serum-supplemented and serum-free medium (5, 58). Removal of T_3 reduces by 75% the GPDH activity of 3T3-F442A cells under serum-free conditions (59), whereas addition of T_3 to serum-supplemented medium increases the number of differentiated cells, thus suggesting a selective multiplication of committed cells (42). The requirement for T_3 remains partial, if any, for the terminal differentiation of adipose precursor cells isolated from pig, rat, and rabbit adipose tissues. As in the case of GH, prior exposure to T_3 in vivo may explain these results.

Studies of insulin requirements have produced apparently conflicting results: when terminal differentiation of 3T3-L1 cells is induced in the presence of dexamethasone (or corticosterone) and 1-methyl-3-isobutylxanthine (MIX), either supraphysiological concentrations of insulin (active at least in part by binding to IGF-I receptor) or low concentrations of insulin and IGF-I

are required, both in serum-supplemented (3) and serum-free medium (63, 121). In the absence of both inducers, insulin appears more potent than IGF-I whereas, in their presence, the reverse is observed (10). Likewise, the optimal expression of GPDH activity and the maximal accumulation of triacylglycerol in rat and rabbit adipose precursor cells require insulin and IGF-I (32, 115). In any event, insulin, by regulating glucose transporters GLUT-1 and GLUT-4, increases the steady-state level of lipogenic enzymes and triacylglycerol stores. These results suggest a subtle interplay between insulin, IGF-I, and their receptors to modulate the maximal expression of terminal differentiation. MIX (added at confluence), which probably acts by raising intracellular cAMP concentrations via phosphodiesterase inhibition, has long been known to accelerate and amplify differentiation of 3T3-L1 cells; it has a similar effect on differentiation of rat and human adipose precursor cells. Because forskolin can substitute for MIX, it is likely that cAMP is a major signal leading to terminal differentiation (121). Arachidonic acid, which behaves as an adipogenic-mitogenic factor, triggers the production of cAMP and 1,2-diacylglycerol from polyphosphoinositide breakdown in Ob17 cells (46). Triggering of the cAMP and inositol phospholipid pathways is followed by at least one round of cell division, and within a few days the whole population of cells becomes differentiated. Indomethacin or aspirin prevents the arachidonic acid-induced differentiation, which would suggest that one or more of the three secreted prostaglandins (PGE_2, PGI_2, $PGF_{2\alpha}$) might be involved. Carbaprostacyclin ($cPGI_2$), a stable analog of prostacyclin, is indeed an efficient activator of cAMP production and terminal differentiation of Ob17 cells; its role in terminal differentiation has been extended to rat and human adipose precursor cells. $PGF_{2\alpha}$, which is able to activate polyphosphoinositide breakdown, dramatically increases the mitogenic-adipogenic effect of $cPGI_2$ that is present at submaximal concentrations (98). Antibodies against both prostaglandins (PGs) are able to counteract the adipogenic-mitogenic effect of arachidonic acid on terminal differentiation (27), thereby supporting the role of both prostaglandins in this process (4). Like $PGF_{2\alpha}$, GH promotes the formation of 1,2-diacylglycerol (37), but from phosphatidylcholine instead of polyphosphoinositide (21). Both GH and $PGF_{2\alpha}$ are thought to activate the PKC pathway, since 1,2-dioctanoylglycerol and phorbol esters can substitute for GH and $PGF_{2\alpha}$. In the process of terminal differentiation, the cAMP and IGF-I pathways may play a cardinal role, whereas the diacylglycerol and insulin pathways may play a modulating role (4).

The importance of controlling the stage of cell differentiation when examining any hormonal effect is illustrated clearly in the case of glucocorticoids. Some investigators have found that glucocorticoids stimulate the differentiation of 3T3-L1 cells (120, 121), TA-1 cells, and adipose precursor cells derived from rat, rabbit, and human adipose tissues (3). More recent studies

have examined the effects of dexamethasone and glucocorticoids on the proliferation and differentiation of rat adipose precursor cells (56, 138): glucocorticoids decrease markedly cell proliferation and enhance terminal differentiation. This stimulation appears to depend mainly on the presence of insulin and an optimal concentration of glucocorticoids, since inhibition of differentiation is observed at high concentrations and is a function of the duration of glucocorticoid treatment. Other studies have shown that corticosterone increases the metabolism of arachidonic acid and leads to an increase in the production of prostacyclin, triggering, in turn, cAMP production; this explains a posteriori the effectiveness of the dexamethasone/MIX cocktail used in a large number of studies and also the fact that glucocorticoids can substitute for arachidonic acid or PGs (47). Among steroid hormones, sex steroids such as β-estradiol, testosterone, and progesterone fail to enhance directly the differentiation of 3T3-L1 cells or of human adipose precursor cells, and only steroids with glucocorticoid activity are effective.

Chronic exposure to dehydroepiandrosterone and some structural analogues abolishes the emergence of GPDH activity and blocks differentiation of 3T3-L1 cells (126). The effect of vitamins and their analogues has been also investigated in 3T3-L1 cells: water-soluble vitamins (vitamin B6 group and vitamin C) stimulate GPDH activity and triacylglycerol accumulation, whereas many fat-soluble vitamins (vitamin A, D, E, and K groups) significantly inhibit both parameters (77). Ascorbic acid enhances synthesis and secretion of type IV collagen but not of laminin and entactin (101). Some drug treatments have been reported to enhance terminal differentiation: in 3T3-L1 cells and rat adipose precursor cells, a synergistic effect of various fibrates and different cAMP-elevating agents is observed (13). This requirement for cAMP has led Brandes et al to analyze cAMP-dependent protein phosphorylation: interestingly, both in the absence or presence of bezafibrate, a very early phosphorylation is observed in a 60-kDa acidic protein recovered in the nuclear fraction of dibutyryl cAMP-treated 3T3-L1 cells (12). A recent and intriguing observation (133) is the effect of butyrate in combination with insulin and dexamethasone in Swiss 3T3 cells, which do not usually differentiate into adipose cells. After growth arrest at G_1 induced with butyrate, in the absence or presence of MIX, these cells accumulate lipid droplets as well as LPL and ALBP mRNAs, but they do not accumulate adipsin mRNA. This observation is similar to the finding that, at high concentrations of indomethacin, activation of various differentiation-specific genes can occur by alternate and still unknown pathways, since synthesis of PGs is abolished under these conditions. The search for factors that act via paracrine mechanisms to trigger or enhance proliferation and/or differentiation of adipose precursor cells has shown that mature adipocytes isolated from human tissue release mitogenic factors (88), whereas those released from rat tissue promote

a potent increase in GPDH activity and triacylglycerol accumulation of cultured rat adipose precursor cells (127). Using these parameters as indicators of terminal differentiation, a factor of 63 kDa, distinct from known growth factors and hormones, has been characterized in rat and mouse serum (91).

Various factors that inhibit or abolish differentiation of adipose precursor cells are present in serum: platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) have been reported to be antiadipogenic factors for 3T3-L1 and 3T3-F442A cells, as have been epidermal growth factor (EGF) and TGF-β for rat adipose precursor cells (73, 124). The inhibitory effect of TGF-β appears to be independent of its mitogenic property. The situation remains unclear with respect to the effects of FGF and EGF (3). FGF added to serum-containing medium prevents the expression in TA-1 cells of some late differentiation-specific genes. FGF abolishes the expression of those genes in fully differentiated cells independently of its mitogenic properties, but, in contrast, basic or acidic FGF does not inhibit terminal differentiation of rat adipose precursor cells. EGF is required for terminal differentiation of 3T3-L1 cells (121), whereas it is inhibitory for terminal differentiation of rat adipose precursor cells (122 and Ref. 3 for review). In agreement with the latter observation, a recent report notes that subcutaneous administration of EGF to newborn rats results in a large decrease of the weight of inguinal fat pads, which suggests the delayed formation of adipocytes from preadipocytes (125).

The effects of tumor necrosis factor α (TNFα) have been studied extensively, as it can alter energy balance in vivo by a selective action on adipose tissue. In differentiated 3T3-L1, 3T3-F442A, and TA-1 cells, TNFα inhibits the expression of several differentiation-specific genes such as LPL, GPDH, adipsin, and aP2 mRNAS in a dose-dependent manner. Moreover, it stimulates lipolysis and induces a phenotypic "dedifferentiation" on a long-term basis (3). TNFα regulates the expression of procollagen genes in 3T3-L1 cells and of the TGFβ gene in TA-1 cells. The effects of TNFα on procollagen mRNA levels depend upon the stage of differentiation of the cells, as undifferentiated and differentiated 3T3-L1 cells undergo, respectively, a coordinate decrease or increase in the contents of types I, III, and IV procollagen mRNAs (136). On the other hand, TNFα within the same range of concentrations has been shown to kill 3T3-L1 cells or inhibit their differentiation (78); further studies are required to resolve these conflicting results.

Retinoids are able to inhibit terminal differentiation. Studies in 3T3-F442A cells indicate that this action is reversible and takes place after expression of LPL but before expression of GPDH and accumulation of triacylglycerol (102, 103). Moreover, retinoic acid can down-regulate adipsin expression in

differentiated 3T3-F442A cells, whereas the expression of other differentiation-specific genes (LPL, ALBP, and GPDH) remains unaffected (8). Thus, in vitro, the differential effects of retinoids depend upon the developmental stage of the cells.

At present, little is known about the intracellular components and mechanisms of the multiple signaling pathways involved in the control of terminal differentiation. The recent observations of Benito et al (9) indicate that Ras proteins are involved in the transduction signals initiated by insulin and IGF-I in differentiating 3T3-L1 cells. The c-Myc protein, whose gene expression is normally abolished at a time when early markers are expressed, prevents 3T3-L1 cells from entering terminal differentiation (44, 141). This blockage of differentiation is accompanied by a large decrease in the expression of genes encoding for the α1- and α2-chains of collagen I and the α3-chain of collagen VI. This observation should be kept in mind because the activation of the gene encoding for the α2-chain of collagen VI (A2COL6/pOb24) is the earliest event of differentiation so far reported (29, 72). These observations suggest that some extracellular matrix components may play a role in initiating events leading to terminal differentiation.

Brown Adipose Precursor Cells

An important observation made independently by Rehnmark et al (112, 113) and Kopecky et al (82) supports the existence of distinct BAT and WAT precursors (Figure 3): stromal-vascular cells isolated from the interscapular BAT of young mice are able after growth arrest to express UCP mRNA and UCP protein and to differentiate into lipid-filled cells. Pharmacological studies of the adrenergic response indicated that the stimulation of UCP gene expression is primarily regulated by β-adrenoreceptors (likely β_3) and that α_1-adrenoreceptors play an additional role. Insulin and T_3 are required for maximal expression. Under the same conditions, stromal-vascular cells isolated from WAT are unable to express UCP mRNA and UCP protein. As in the case of WAT, stromal-vascular cells from adult mouse BAT contain the bulk of A2COL6/pOb24 mRNA and thus should be considered as preadipocytes (Figure 3), although clearly, both for BAT and WAT, the coexistence of adipoblasts and preadipocytes cannot be excluded. In any event, these experiments show that mouse BAT and WAT arise from distinct adipose precursor cells (Figure 3). The possible transformation of BAT precursors into WAT precursors and the fine regulation of the expression of UCP gene have been recently investigated by Casteilla et al (20): in serum-free medium, the expression of UCP mRNA takes place in stromal-vascular cells from perirenal BAT of newborn lambs. Glucocorticoids promote a stimulating effect on this expression. However neither UCP mRNA is expressed nor a glucocorticoid effect is observed with precursor cells from 3-week-old lambs.

Therefore, the extinction of the expression of UCP gene is a rapid process in vivo, and a true conversion of BAT precursors into WAT precursors apparently occurs in ovine during this short period after birth.

ADIPOSE CELLS AS SECRETORY CELLS

Adipocytes are a major source of free fatty acids in mammals. Furthermore, both adipocytes and muscle cells have been long known as the main source of synthesized and secreted LPL. In the last few years, the concept that adipocytes behave as secretory cells for other proteins and metabolites has emerged. In a series of elegant experiments (24, 27, 81, 117), Spiegelman and coworkers were the first to show that adipsin, which is found in vivo in the circulation of both animals and humans, is produced and secreted in vitro by differentiated 3T3-L1 cells (constitutively and, more importantly, upon insulin stimulation) and mouse epididymal fat tissue (81). Both glycosylated forms of adipsin (37 and 44 kDa) are secreted, and considerably more adipsin is found in blood than in adipose tissue. Mouse adipsin is a secreted protease homologous to human complement factor D. Both adipsin and complement factor D are highly specific in clearing complement factor B when it is complexed with activated complement component C_3. In addition to adipsin, factor B and factor C_3 are also secreted by differentiated 3T3-F442A cells. These authors have proposed that adipsin and other factors of the alternative pathway of complement may play a role in the regulation of systemic energy balance in vivo (117).

Of utmost interest is the fact that CETP and apoE are synthesized by various tissues (muscle, heart, liver, adipose) in different species (hamster, rat, rabbit, human) but that, rather unexpectedly, mammalian adipose tissue and muscle appeared as major sources of CETP mRNA. Isolated hamster adipocytes synthesize and secrete active CETP, and analysis by in situ hybridization of adipose tissue reveals coexpression of CETP mRNA and LPL mRNA (74). In many ways, apoE resembles CETP, as it is produced in several peripheral tissues in which LPL is also expressed. During differentiation of 3T3-L1 cells, the expression of apoE gene takes place after that of LPL (142). In contrast to CETP, most of apoE is not secreted and remains cell-associated in differentiated cells. Thus adipocytes are another important extrahepatic source of CETP and possibly of apoE. Adipose tissue is known to contain the major cholesterol pool of the body both in rodents and humans; conceivably, locally synthesized apoE and CETP play a rôle in the local removal of adipocyte cholesterol. The capacity of adipose tissue as a secretory organ extends to sex steroid synthesis. Among the genes encoding enzymes responsible for the synthesis of androgens and estrogens from adrenal steroid precursors, investigators observed that the expression of P-450 aromatase

occurred earlier than that of GPDH during the differentiation of 3T3-L1 cells (140). The P-450 aromatase mRNA, the product of which is required for the synthesis of estrone and estradiol, is also expressed in rat and human adipose tissue. This gene as well as the genes encoding for the 3β-hydroxy-steroid dehydrogenase/Δ4-Δ5 isomerase and the 17β-hydroxysteroid dehydrogenase (required for androgen and estrogen synthesis from dehydroepiandrosterone) are also expressed in a variety of peripheral tissues, including adipose tissue (86, 144). Large amounts of adrenal steroid precursors secreted in human and nonhuman primates are further metabolized by peripheral tissues. This synthesis accounts for 40% of total active androgens in men and for a higher percentage of active estrogens in women. This activity, termed intracrine steroid formation (85), may thus be quantitatively important owing to the weight of fat tissue in normal subjects and obese patients. It is tempting to postulate that intracrine steroid formation may also affect adipose tissue metabolism, as reported recently in the regulation of lipolysis by androgens in rat adipose precursor cells (139). Among metabolites of interest, and as already mentioned, differentiated 3T3-F442A cells have been shown to secrete monobutyrin, which appears as a fat-specific angiogenesis factor (35, 137). Together, these various observations emphasize that not only are adipocytes able to respond to hormonal signals originating from endocrine glands and possibly from other cell types in their immediate vicinity but they are also able to synthesize and secrete peptides and nonpeptide factors that can be recognized and used by other cells.

RESEARCH TRENDS

At the present time, a better estimate of adipose tissue cellularity appears feasible by using in situ hybridization techniques with cDNA or RNA probes corresponding to early markers of adipose cell differentiation. More importantly, the cloning and sequencing of master genes involved in the determination process, leading to unipotential adipoblasts, can be foreseen. We also hope that the cloning and sequencing of the mouse *ob* gene and the rat *fa* gene will shed some light on the initial event(s) leading to adipose tissue hyperplasia (45, 134).

The "reactivation" of BAT, owing to its partial or nearly complete disappearance in many mammals and humans after birth, deserves further study. To date, the increase in rodents and dog of total UCP content of BAT upon adrenergic stimulation appears to be due more to hyperplasia than to an increase in the UCP content per cell. The recent observation that fatty acids can regulate gene expression in adipose cells (6, 7) may introduce a link between the composition of diets and the hyperplastic/hypertrophic response of white adipose tissue; and the characterization of fatty acid-responsive genes

may also produce some clues about the development of insulin-resistant states and cell hypertrophy.

From a more general point of view, adipose tissue now appears to perform endocrine, paracrine/autocrine, and intracrine functions. As such, it may play a direct role in some regulatory events related to cholesterol metabolism and energy balance. The potential to analyze in humans the composition of the interstitial fluid surrounding adipocytes in vivo (94) will enable scientists to study the factors that may be involved in adipose tissue hyperplasia and hypertrophy.

SUMMARY

Both in animals and humans, before or after birth, angiogenesis appears to be closely coordinated in time and space with the formation of fat cell clusters. Monobutyrin, a novel fat-specific angiogenesis factor, may play a role in this process. The potential to acquire new fat cells appears to be permanent throughout life in both animals and humans, as revealed by in vitro experiments. Considerable evidence now supports the view that BAT and WAT are distinct organs; in addition, the existence of distinct BAT precursor cells is demonstrated by their unique ability to express the UCP gene. In bovine and ovine, the transformation of BAT into WAT is strongly suggested by the rapid disappearance after birth of UCP from the various BAT depots. Despite the initial cell heterogeneity of the stromal-vascular fraction, cultured stromal-vascular cells of adipose tissue are adipose precursor cells that show varying capacities for replication and differentiation, according to age and fat depot. Studies of adipose cell differentiation in vitro correspond to the sequence: adipoblast (unipotential cells) $\xrightarrow{\text{commitment}}$ preadipose cell (preadipocyte) $\xrightarrow{\text{terminal differentiation}}$ immature adipose cell $\xrightarrow{\text{terminal differentiation}}$ mature adipose cell (adipocyte). Cell commitment is triggered by growth arrest and characterized by the expression of early markers (A2COL6/pOb24; clone 5; LPL), whereas only terminal differentiation of preadipocytes requires the presence of various hormones. Multiple signaling pathways have been characterized and shown to cooperate in the process of terminal differentiation. The concept that adipose cells behave as secretory cells is now emerging from in vitro data, since secretion of various proteins (LPL, adipsin, CETP) and important metabolites (fatty acids, monobutyrin, androgens, estrogens, prostaglandins) takes place both constitutively and upon hormonal stimulation. This suggests that adipose tissue participates more directly than previously thought in metabolic activities and energy balance.

ACKNOWLEDGMENTS

G. Ailhaud and R. Négrel are professors of biochemistry and P. Grimaldi is an INSERM investigator. This research has been supported by funds from

CNRS and grants from INSERM, INRA, and ARC. We thank C. Dani, D. Gaillard, and C. Vannier for their valuable contributions over the years. The important contributions of a large number of other investigators are also gratefully acknowledged. Special thanks are due to G. Oillaux for expert secretarial assistance.

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