review

Search for the adipocyte precursor cell and factors that promote its differentiation

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INTRODUCTION

Adaptation of the Coulter Counter to the study of adipose cellularity has provided an important tool for evaluating the relative contribution of size and number of adipose cells to lipid deposition in the body. With this technique, adipose cellularity has been investigated during normal growth and development and during onset of obesity in many different animal models and in different depots within each model (for recent reviews see 1–5). Although conditions have been identified in which adipose cells increase in number, it remains to be determined whether this increase represents lipid filling of differentiated cells or of cells recently differentiated from a pool of mitotically active cells. The ability to identify the adipose precursor cell, or preadipocyte, and the factors that influence its proliferation and differentiation would greatly aid the quest to understand, prevent, and cure obesity in humans. Even though the proliferating preadipocyte has yet to be identified as a separate, distinguishable cell type, much has been learned from examination of gross-structural and non-adipocyte aspects of adipose tissues and from recent advances of our understanding of hormonal, nutritional, and genetic effects on the development of adipose tissue. The body of information relevant to the search for the adipocyte precursor cell and factors that promote its differentiation are the subject of this review.

Studies that examined the gross-structural and non-adipocyte aspects of adipose tissue have largely been overlooked in recent years. In particular, the research of Wasserman (6–9), Liebelt (10–13) and Tedeschi (14–24) has pointed out the stromal-vascular and non-adipocyte aspects of adipose tissue in the fetus and in the growing and mature animal. This review will emphasize those studies that considered these aspects of white adipose tissue (WAT) development. Attempts to completely characterize adipocyte development (from presumptive to proliferating to mature differentiated cell) must be understood in terms of the development of all elements of adipose tissue at all levels of organization (from cell to tissue to depot). By studying lipid deposition at all levels of organization, new insight into the nature of the preadipocyte can be appreciated.

WHITE ADIPOSE TISSUE DIFFERENTIATION AND DEVELOPMENT

Research conducted before 1900 still provides the primary foundation upon which many histology texts rely for description of adipose tissue development. Several of these very early studies were conducted on brown adipose depots (25, 26), and other studies (27–31) involved typically white adipose depots. At the time of these early investigations, the physiological role of brown adipose tissue (BAT) and the conversion of BAT to WAT was not fully appreciated. Therefore, not surprisingly, two divergent theories on adipocyte development evolved from these early works. One theory, developed essentially from the study of WAT, stipulated that before lipid deposition there was no apparent cellular or tissue specialization and that immature or mature loose connective tissue simply filled with lipid. The other theory, proposed by students of BAT formation, contended that a high degree of

Abbreviations: WAT, white adipose tissue; BAT, brown adipose tissue; LPL, lipoprotein lipase.

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cellular and tissue specialization happened before or concurrent with lipid deposition. Subsequent work has proved the latter theory to be more feasible and relevant to the formation of WAT as well as BAT.

With respect to the latter theory, a close spatial and temporal relationship between adipocyte formation and blood vessel formation has been repeatedly observed (9, 26, 28, 30–49). Blood vessels and adipocytes either form simultaneously (28, 30, 31, 34–37) or capillaries appear before the adipocytes deposit lipid (8, 26, 32, 39–41). Regardless of the sequence, the blood vessels and first formed adipocytes are always found in close association. This anatomical relation is not without physiological significance. Lipoprotein lipase (LPL) is synthesized in the adipocyte and transferred to the luminal side of the capillary endothelial cells (50). In the lumen, this enzyme serves to hydrolyze circulating chylomicrons and very low density lipoproteins. A close spatial relationship between the adipocyte and capillary provides a minimal distance for the transport of LPL from adipocyte to capillary lumen and of free fatty acids from capillary lumen to adipocyte. Shorter distances, therefore, should lend a degree of efficiency to the situation. Recently (51), LPL activity was demonstrated in the lipid-free stromal-vascular fraction of immature adipose tissue. This discovery presents the possibility that the enzyme LPL may serve as a biochemical marker by which the prelipid accumulating adipocyte could be identified by fluorescent antibody techniques or other histochemical techniques.

Whether blood vessel formation precedes adipocyte formation or whether both features develop concurrently varies among species and among depots within species. Primitive organs of WAT are present in the human fetus from the third month of gestation (6). The primitive organs are ovoid bodies that vary little in size. Their appearance is conspicuous because of the network of capillaries and cells that they possess. In the vascular system of the primitive organ, single branches connect neighboring venules and arterioles. The cells of the primitive organ appear mesenchymal, except for the presence of small lipid droplets. Many of these cells serve as sites of lipid and glycogen storage in the human fetus. But it is the vascular system that first appears.

Arterioles and venules are found in areas within the human embryo that are destined to develop into the primitive fat organ (9). These arterioles and venules are conspicuous by virtue of a relatively thick layer of mesenchymal adventitial cells that surrounds them. As these vessels elaborate, mesenchymal adventitial cells also proliferate. This relation suggests that the primitive organ is formed by growth of endothelial and adventitial cells. By this vascular growth, the primitive organ and, therefore, the adipose lobules are built into connective tissue as separate entities and are not areas of connective tissue that convert directly to adipose tissue. This latter point pertains not only to development of primitive white fat organs but also to primitive brown fat organs in the human fetus (9).

In contrast to the developmental sequences within the primitive fat organ described in the human fetus, the primitive fat organ of the epididymal fat pads of the mouse is not estalished before lipid and glycogen appear in the cells. Instead, cellular lipid accumulates simultaneously with vascular growth and spreading of mesenchymal adventitial cells. The developmental process is otherwise identical to that present in other depots.

As indicated above, the developmental sequence of first vascular development then lipid accumulation in primitive organs of BAT and WAT are similar. Because of this relation Wasserman (9) suggested that both types of adipose tissue possess a common origin from the embryonic reticulo-endothelial primitive organ. The common origin concept is further supported by fine structural analysis (32, 42) and particularly by the fact that cultured interscapular BAT of the rat possesses typical white adipose cells after a minimal time in culture (52, 53).

The most consistent finding of many histological studies on developing WAT and BAT is that immature, developing adipocytes are present as cords of tightly clustered cells of various sizes. Contained within these cords and conceptually, at least, serving as the backbone are the endothelial cells of the vascular bed. Maximow and Bloom (54) described the simultaneous formation of primitive endothelium and primitive blood cells from the mesenchyme. In this process, mesenchymal cells proliferate actively and give rise to clusters of spherical basophilic cells (blood islands) connected with one another by strands of elongated cells. The peripheral cells of the blood islands and those of the strands become flattened and ultimately form lumens of primitive blood vessels. The central cells of the islands represent blast cells for various blood cells. This explains the observation of red and white blood cells in WAT and BAT. This concept is depicted diagrammatically in Figs. 78 and 79 of Maximow and Bloom's histology text of 1948 (54).

This concept explains the origin of the full range of cells common to mature red bone marrow that appear in the primitive WAT organ of the human (6, 9, 24) and the pig.[3] A variable number of lymph and haemolymph nodes are also present in BAT, hav-

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ing been identified in the interscapular BAT of the human (55–57), cat, and pig (57). The amount of lymphadenoid tissue in WAT is sensitive to adrenal insufficiency, stress, and splenectomy (24).

The cell cluster arrangement of the blood islands and the anatomical arrangement of the primitive fat cells in cords or cell clusters with a central capillary network make it enticing to hypothesize that the mesenchymal cells of blood islands could be early progenitors of cells destined to become adipocytes. But more direct evidence is necessary to verify this possibility. We can conclude, however, that the first identifiable adipocytes appear in the perivascular region and that other specialized cell types are contained within WAT.

Cells suggested as precursors to adipocytes include free endothelial cells (31, 37, 47, 59), perivascular reticulum cells (9, 21, 38, 41, 42, 60–62), macrophages (63), immature macrophages (31, 64–69), "fibroblast-like" perivascular cells (32, 39, 70), perivascular mesenchymal cells (54), and cells that can phagocytize particulate dyes (71, 72). Results from other studies have demonstrated the uptake of a particular dye by mature adipocytes (72–74) and lipid-depleted adipocytes (74, 75). This diversity of cell types and functions is also present in bone marrow tissue. Red and white blood cells, macrophages, plasma cells, and adipocytes are formed in bone marrow tissue (9, 21). The reticulum cells in bone marrow are phagocytic and have the potential to give rise to many of the various cell types present (76–78). Three types of bone marrow reticulum cells can be distinguished based on ultrastructure and morphology (77). The various ultrastructural and morphological appearances for these reticulum cells represent the full range of ultrastructure and morphological appearance described for preadipocytes. The physiological significance of these similarities is not known.

Napolitano (39) conducted an extensive electron microscopic study on the developing WAT of the inguinal and epididymal fat pads of newborn to 9-day-old rats. The stem cell of the presumptive epididymal fat pad was described as a spindle-shaped cell generally possessing four to five protoplasmic extensions directed in the long axis of the cell. These processes were long, tenuous, and generally devoid of inclusions. The cell organelles were primarily found in the perinuclear mass of cytoplasm. Rough endoplasmic reticulum was abundant and highly organized; mitochondria were small and spherical and had a simple internal structure. Nuclei were round to ovoid, with one to two small nucleoli and a homogenous distribution of intranuclear material of intermediate electron opacity. These cells were found throughout the presumptive epididymal fat pad and in rapidly enlarging adipose organs. Cells with similar ultrastructural characteristics were located adjacent to capillaries. As differentiation proceeded, cell shape changed from a spindle shape to the nearly spherical form of a mature adipose cell.

As lipid accumulation increased, the adipocytes passed through a sequence of stages in which certain cellular organelles and inclusions were modified. As the cells progressed through these stages, they were often closely associated with the endothelial cells lining the capillaries. More lipid appeared as small droplets on either side of the nucleus toward the poles of the cell. With the assumption of an ovoid form, much of the lipid coalesced into one droplet. Endoplasmic reticulum and Golgi membranes were less evident, but significant amounts of glycogen were observed, usually as puddles around the lipid droplets. Before the mature adipocyte stage, the number of endoplasmic reticulum profiles decreased progressively, and mitochondria appeared as spheres or long filaments. The mitochondria showed a simple morphology with few cristae that rarely extended across the organelle. Fine structural analysis of the development of the lipid-accumulating adipocyte by others has, in essence, confirmed this description (35, 38, 40, 42, 70, 79–83).

It was suggested (39) that the precursor cell of the adipocyte was a fibroblast or fibroblast-like cell. This association, however, may have been coincidental, rather than causal or sequential. The immature adipocyte contains an elaborate system of rough endoplasmic reticulum engaged in the synthesis and excretion of LPL. This organellular arrangement is analogous to that found in fibroblasts that are synthesizing procollagen and discharging it into the extracellular space. Therefore, morphological similarities between immature adipocytes and fibroblasts may be entirely coincidental and may preclude any distinction between preadipocytes and fibroblast cells (84).

ANALYSIS OF ADIPOSE CELLULARITY BY TRITIATED THYMIDINE

The lipid-laden adipocyte is generally considered to be incapable of mitotic activity. This conclusion is largely based on the absence of mitotic figures in adipocytes in histological preparations of adipose tissue. Simon (42) and Bell (33) reported that a fat cell loses its ability to divide once it begins to accumulate lipid.

In 1971, Pilgrim (85) used tritiated thymidine radioautography to study mitotic activity in fetal and early

postnatal subcutaneous adipose tissue of the rat. He combined radioautography with a histochemical technique for α-napthyl acetate esterase activity. Animals were killed 60 min after tritiated thymidine injection. High labeling indices were found in condensed groups of cells containing various proportions of mesenchymal cells, preadipocytes (esterase positive, no lipid), and adipocytes. Cell clusters that contained some lipid-laden cells had the highest labeling indices. Pilgrim (85) considered cells adipocytes (lipid-containing cells) when they contained empty vacuoles in the radioautographs. These vacuoles could have as easily contained glycogen because histochemical tests were not used to determine the vacuole constituents. This point is crucial because Pilgrim (85) reported that many lipid-containing adipocytes were labeled.

A problem in short-term tritiated thymidine radioautography is the inability to determine the ultimate fate of labeled cells. Fate can be determined only if animals injected with tritiated thymidine are killed after much longer time periods than the usual 15–60 min. Longer time periods would permit labeled cells to divide and terminally differentiate into specific cells and thereby allow one to state more precisely the nature of the mitotically active cells. Several long term studies have been reported. Hellman and Hellerstrom (86) injected tritiated thymidine into 1- and 3-day-old rats and killed the rats at different times between the 4th and 154th days of life. No attempt was made to classify tritium-labeled cells. For subcutaneous adipose tissue, the percentage of labeled cells at the different post-injection times tended to rise at first and then decreased progressively during the whole observation period. The frequency of labeled nuclei for rats at 4 and 11 days of age was approximately 60%; that at 5 months of age was less than 10%. The decrease in frequency of labeled nuclei with time was explained as a dilution effect resulting from cell division. The frequency of labeled nuclei decreased most between 11 and 32 days (from 65% to 20%) and thus indicated a time when large amounts of new unlabeled DNA were being added to the tissue. These results indicated that most DNA synthesis is complete by 32 days in the subcutaneous adipose tissue of the rat. However, as pointed out previously, no attempt was made to distinguish between adipocyte and stromal cell DNA synthesis.

Short and long term studies with tritiated thymidine were also used to study fetal and early postnatal adipose tissue in the pig (49). In the youngest fetuses, there was a period of intense mitotic activity before any lipid accumulation. During subsequent fetal development, clusters of tightly arranged cells were formed. Lipid accumulated only in these cell clusters. Mitotic activity was minimal during cell cluster formation and lipid accumulation. These results indicated the synthesis of a large bed of preadipocytes that formed into cell clusters that then accumulated lipid over a long period of time. In early postnatal pigs, labeling of cells was extensive within, and closely associated with, adipocyte clusters. If some of these laeled cells (postnatal pigs) were indeed preadipocytes, then this mitotic activity represented the synthesis of a second bed of preadipocytes.

Hollenberg and Vost (87) in a study on DNA synthesis in rat adipose tissue improved the method of nuclear labeling by fractionating the adipose tissue into adipocytes and stromal, vascular cells. The tritiated thymidine was still administered in vivo. With this modification, changes in DNA radioactivity with time could be followed in each of the two-cell pools. Hollenberg and Vost (87) reported that, in adipose tissue removed from 150–175 gram rats at 0–2 days after an injection, total tissue DNA radioactivity was constant, whereas the specific activity of the adipocyte fraction rose rapidly between 2 and 5 days and more slowly thereafter. They reasoned that newly labeled fat cells, initially collected in the stromal pool, required 2–5 days after completion of the DNA synthesis to accumulate sufficient lipid to be harvested in the fat cell fraction.

Recently, this type of approach was used by Greenwood and Hirsch (88) in an extensive study of the postnatal development of adipocyte cellularity in the rat. DNA specific activity was determined in adipocyte and stromal cell fractions at various times after rats were injected with tritiated thymidine at 9, 15, 22, 28, 35, 49, 90, and 150 days of age. In the 9- to 28-day old rats, an increase in adipocyte specific activity occurred at various times after injection. A bed of preadipocytes appeared to be synthesized during the second and third postnatal weeks; these preadipocytes took as long as 30 days to mature into adipocytes. The majority of preadipocyte synthesis was complete by the fifth postnatal week in the rat epididymal fat pad. After administration of tritiated thymidine to adult rats (89) there is a rapid appearance of label in the adipocyte fraction of collagenase digested adipose tissue. The existence of a rapidly proliferating cell type closely associated with mature adipocytes was suggested (89) to explain this finding. The possibility, however, that a small proportion of adipocyte nuclei had in fact incorporated the tritiated thymidine was not ruled out in these experiments (89).

Experiments involving adipose tissue from young rats given tritiated thymidine may be difficult to interpret. One study (89) compared the use of 500-micron nylon mesh to a 250-micron nylon mesh in

preparing collagenase liberated fat cells from 6-week-old and 3-month-old rats injected with tritiated thymidine. The smaller size mesh resulted in fat cell DNA specific activity which was a significantly lower proportion of the specific activity of stromal DNA when compared to the larger mesh. The 500-micron nylon mesh possibly allows aggregates of several cells to remain in the adipocyte fraction. These aggregates may be responsible for the accidental inclusion of a labeled stromal cell in the adipocyte fraction. Therefore, the use of the 500-micron nylon mesh may have confounded the results of some experiments (88).

In an experiment, involving young rats (3, 5, 10 days of age) injected with [^{14}C]thymidine, there was incorporation of label into the DNA of the adipocyte fraction in as little as 30 min after thymidine administration (90). Electron microscopic studies of the isolated adipocytes indicated a homogenous cell type characteristic of unilocular adipocytes. These authors (90) suggested that, early in postnatal life, some adipocytes may synthesize DNA for cell division after mobilization of cytoplasmic lipids. This contention is supported by studies in which mature adipocytes in culture lose considerable quantities of lipid and then acquire the ability to replicate (91).

Two studies already discussed (87, 88) report that the specific activity of the stromal-vascular fraction of adipose tissue declined over various experimental periods. The authors suggested that the migration or loss from the tissue of blood cells labeled in situ could result in lower specific activity. This suggestion seems reasonable on the basis of results obtained from pig adipose tissues (49). The in situ labeling of blood cells in developing and in mature adipose tissue may be extremely significant in terms of histogenic relationships discussed in the previous section.

Several studies have demonstrated increases in adipose tissue DNA content under a variety of influences (87, 92, 93). Characterization of the mitotically active cells with histochemical and histological and cell separation techniques would be useful. This information could greatly enhance our understanding of adipocyte formation.

ANALYSIS OF ADIPOSE CELLULARITY BY CELL CULTURE

Although the establishment of mitotic activity in adipose tissue in vivo has been established with relative ease, the actual precursor cell(s) of the fully differentiated adipocyte has been elusive in identification. The recent discovery of methods to culture cells that undergo proliferation and adipose conversion has made possible a new and promising approach to the analysis of adipose cellularity.

In the first report of the culture of human adipose tissue (94), the outgrowth of fibroblast-like cells was described. The fibroblast-like cells proliferated from the explants in a radiating fashion. The cells were spindle-shaped with several elongations. The nucleus was large and contained one to several nucleoli. Lipid inclusions were first seen around the nuclear zone and later in other parts of the cytoplasm. Inclusions were seen less frequently in the cellular extension. Others (95–97) have also shown that fibroblast-like cells having the characteristics of adipose cells can be obtained from human adipose tissue and grown in culture. Interpretation of these studies is limited because mature adipocytes displaying a unilocular adipocyte morphology were not observed. In addition, in a study by Dixon-Shanies, Radick, and Knittle (98), cultured adipose tissue cells presented a morphology distinctly different from that of the fibroblast because the adipose cells were polymorphic in form. Some cells had a polyhedral shape, while others were more fibroblast-like. Many small lipid droplets were seen in the cytoplasm of these cultured adipose tissue cells. Proliferation was slower in these cells than in the fibroblast cell cultures. Although rather long incubation times were necessary, the cultured adipose cells synthesized significantly more ^{14}C-labeled lipid from [^{14}C]glucose, but the fibroblast cultures had a higher metabolic rate. Lipid deposition and CO_2 production were not affected by addition of insulin to the medium. Although morphological and metabolic differences between the cultures of adipose tissue cells and fibroblasts were apparent, the authors acknowledge that the adipose tissue cells, even though capable of lipid accumulation, may not be adipocytes or even preadipocytes, because unilocular adipocytes were not demonstrated.

Complete differentiation of adipocyte precursor cells in culture was recently obtained, however, for cells isolated from the stromal-vascular fraction of adipose tissues of mature, as well as young animals (101). However, complete differentiation of adipocyte precursor cells isolated from mature animals could only be achieved by adjusting the incubation medium to 30% serum (99) or by addition of the potent phosphodiesterase inhibitor isobutylmethylxanthine to the medium (100). Because complete differentiation in the absence of isobutylmethylxanthine is achieved in stromal-vascular cells isolated from younger rats, Björntorp et al. (100) suggested that no new adipocytes are formed spontaneously in the adult (Sprague-Dawley) rat. This conclusion is in agreement with the Coulter Counter data of Hirsch and Han (102) and

the in vivo DNA labeling study of Greenwood and Hirsch (88).

The identification of the replicating preadipocyte or differentiated prelipid-filling adipocyte has yet to be revealed by use of cell culture techniques. Van and Roncari (99) described a fibroblast-like cell isolated from adult rats and humans that assumed adipocyte characteristics after the monolayer of cells reached confluence. Parallel cultures of skin fibroblasts showed no tendency to undergo adipose conversion (97, 99, 103). Björntorp et al. (100) determined that the responsible cell undergoing proliferation and adipose conversion was not an endothelial cell, but did not speculate beyond this point.

Recently (104–107), a cloned line of 3T3 cells was established from mouse embryo fibroblasts. Upon reaching a growth-inhibited, confluent state, cells of the 3T3-L1 subline spontaneously differentiate in vitro into cells possessing many of the biochemical and morphological features of adipocytes. Because coagulation factor VIII antigen is present in this cell line, 3T3-L1 cells may have originated from contaminating endothelial cells rather than from fibroblasts (108). Perhaps the more pressing issue concerning the 3T3-L1 cell is the fact that complete conversion of these cells to adipocytes that exhibit the unilocular, signet-ring morphology has not been shown. Green and Kehinde (109) recently reported that another murine preadipose cell line, 3T3-F442A, gave rise to typical fat pads when injected subcutaneously either in the flank or over the sternum of nude mice. The one limitation to this study was the inability to distinguish the injected cells until 1 to 2 weeks post-injection when flattened pads were visible. There is little doubt, however, that adipose conversion in 3T3 cell lines is qualitatively similar to that observed in vivo in adipose tissue. These similarities were recently reviewed by Green (110).

HORMONAL REGULATION OF ADIPOSE CELLULARITY

In the hypophysectomized fed and intact fed rat, formation and/or maturation of primordial fat cells were retarded when compared to intact fed rats (87). Inhibition of synthesis of stromal DNA was more marked in the fasted rats than in the two fed groups of rats. Growth hormone clearly stimulated thymidine incorporation into stromal DNA in all three groups. But only in the case of the intact fasted rats was DNA synthesis in the fat cell fraction enhanced by growth hormone. Murakawa and Raben (111) also observed this effect using an in vitro system to measure incorporation of thymidine into adipose tissue DNA. The abolishment by growth hormone of the inhibitory effect of fasting on DNA synthesis remains unexplained as to its physiological significance, but does allude to the possibility that growth hormone can influence proliferation of the preadipocyte.

The effect of insulin on DNA synthesis in the adipose tissue of adult rats has been investigated (112–115). Like growth hormone, insulin stimulated DNA synthesis in stromal elements other than primordial adipocytes when administered to normal animals. But it could not be determined whether the two hormones were augmenting cellular proliferation in the same or in a different population of cells. Insulin deficiency, on the other hand, inhibited the formation of new stromal elements and of adipocytes. Insulin administration during early life (birth, or at 1 or 3 weeks of age) influences only the size of the adipocytes but not the number of these cells. This finding is analogous to the findings in the adult rat (115).

In tissue culture, insulin stimulates DNA synthesis in human adipose tissue (116, 117). Esanu and Bray (117) studied DNA synthesis in adipose tissue of normal and obese humans. In vitro, DNA was synthesized to a greater extent in the adipose tissue of obese patients when compared to normal patients. In addition, insulin, human serum, and glucocorticoids stimulated incorporation of tritiated thymidine into DNA to a greater extent in the adipose tissue of the obese patients when compared to normal patients. The cellular fraction into which the tritiated thymidine was incorporated was not determined, but it may have been associated with cells in the stromal vascular fraction. This association is based on the findings of Ng et al. (95) who reported that stromal cells from the subcutaneous adipose tissue of obese, adult humans proliferated in cell culture. After 2 months in culture these cells accumulated significant amounts of lipid.

From the studies mentioned above concerning insulin action on fat cell precursor proliferation and maturation, particular cell type(s) responded to insulin with increased DNA synthesis. This increased activity could not be conclusively linked to specific adipocyte precursor cell proliferation or to adipose conversion. Insulin activity, however, was related to rate of lipid filling (maturation) of adipocytes.

In cell culture, insulin enhances the incorporation of precursors into cellular triglycerides in 3T3-L1 cells but does not seem to affect the probability with which the cells undergo adipose conversion (106). While the conversion of 3T3-L1 cells in the confluent stage is spontaneous, lipid filling of these cells is enhanced

by the addition of insulin to the media (106). The adipose conversion of these cells is associated with a 6- to 10-fold increase in specific insulin binding over that observed for undifferentiated control cells (118, 119). The increased lipid filling is partly enhanced by insulin through the stimulation of synthesis of LPL (120–122).

Other hormones have been tested on the established 3T3-L1 cell line for ability to stimulate precursor cell proliferation or to enhance adipose conversion. Epinephrine, isoproterenol, and dibutyryl cyclic adenosine monophosphate inhibit the incorporation of precursor metabolites into cellular triglyceride but do not affect the probability with which the cells undergo adipose conversion (106). The adipose conversion is also independent of cyclic AMP, but the process is enhanced by addition of 1-methyl-3-isobutylxanthine (122, 123). Treatment of confluent preadipocytes with dexamethasone shortens the time period required for adipose conversion (118). The process of adipose conversion is also stimulated by prostaglandin F^2_α (122, 123). The parent 3T3 cell line responds to fibroblast growth factor (108); the rate of proliferation is further enhanced by addition of dexamethasone. Hormonal regulation of proliferation and adipose conversion in 3T3 cells is reviewed in greater detail elsewhere (124, 125).

Björntorp et al. (101) characterized cells isolated from rat epididymal adipose tissue that proliferated in culture and developed into adipocytes. In this system, adipose conversion was promoted by the addition of heparin, prostaglandin E, isobutylmethylxanthine (phosphodiesterase inhibitor), and cholera toxin (potent adenylase cyclase stimulator) to the medium that already contained 20% horse serum. Theophylline, however, whose action is also mediated through inhibition of phosphodiesterase activity, did not promote adipose conversion. Therefore, the involvement of cyclic AMP in adipose conversion in this model system is uncertain. Because the other phosphodiesterase inhibitors promoted adipose conversion, it is tempting to suggest that the cells isolted by Björntorp et al. (101) are insensitive to theophylline.

Evidence of hormonal stimulation of the proliferation of preadipose cells in culture was recently reported (126). The influence of 17α- and 17β-estradiol was studied on adult human omental adipocyte precursor cells grown in culture. 17β-Estradiol stimulated the multiplication of human adipocyte precursors and had no effect on cell size. 17α-Estradiol, on the other hand, affected neither cell proliferation nor cell size. No sex difference was elucidated when cell culture results of studies on cells isolated from males was compared to results of tests on cells isolated from females.

NUTRITIONAL REGULATION OF ADIPOSE CELLULARITY

In addition to hormonal regulation of adipocyte proliferation, investigators anticipate that dietary control of lipid-storing capacity would involve alterations in adipocyte numbers. Most of the information available on the effects of nutrition on adipose cell proliferation has been obtained in laboratory rodents. The most widely cited of these studies is that by Knittle and Hirsch (127). In tests determining the effects of early neonatal nutrition on adipose cellularity, rat litter sizes were adjusted. From birth, male pups were raised in litters of either 4 or 22. The number of measurable fat cells in 20-week old rats from large litters (22 pups) was reduced to 43%. The authors concluded that early nutritional experiences can permanently modify adipose cell number and size in the rat. In addition they postulated that similar nutritonal experiences in man were of prime importance in producing hyperplasia and hypertrophy of adipose tissue found in obese subjects. Although this study has generated much interest and excitement in nutritional manipulation of adipose cell proliferation, some problems of interpretation of their data are serious. Because of the single impact of this important piece of work, the limitations of this study are cited here. The authors have acknowledged most of these limitations in subsequent publications.

Knittle and Hirsch (127) suggested that small litters (4 pups) produced a situation of caloric excess and that this caloric excess led to a stimulation of adipose cell proliferation. In fact, the small litters received a normal intake, and the large litters (22 pups) experienced caloric restrictions. Wurtman and Miller (128) demonstrated that rats grown in litters of 2, 4, 8, or 12 grew at similar rates and developed the same amount of body fat. Only pups grown in litters of 16 experienced a decrease in body weight gain and body fat. When their data are recalculated on the basis of the effect of restriction as a percentage loss of the control (or smaller litters, 2, 4, 8, and 12), the loss of body weight was 35.5% and the loss of total DNA was 35.0%. Tulp, Gambert, and Horton (129) demonstrated a 62% decrease in adipocyte number in protein-malnourished rats, and only a transient delay in adipocyte proliferation in energy-restricted rats. They concluded that the predominant effect of protein deprivation on adipocyte proliferation was proportional to the extent of overall growth retardation. Other investigators suggest similar general effects of restriction during preweaning on overall growth in rats (130–133) and mice (134–136). Although most of these studies support the conclusions

of Knittle and Hirsch (127) regarding a change in cellularity, there are no studies of simple caloric excess during the preweaning stage and its effect on adipose tissue development. Also, no data suggest that dietary restriction selectively influences adipose cell proliferation in the absence of the general depression in total cell proliferation (i.e., cell proliferation in other organs).

Another limitation of the experiments that relate nutritional manipulation to adipose cell proliferation is the interpretation of the Hirsch and Gallian (137) method for measuring adipocyte numbers. The originators of this method acknowledge that it is uncertain whether the increases in adipocyte number in their studies were the result of the formation of new adipocytes or merely the result of the enlargement (and thus detectability) of small adipocytes formed early in life (138). The Coulter-counting of osmium fixed cells is limited to those fat cells that reach 25 microns in diameter. For a recent comprehensive review of this methodology, refer to the recent report of Gurr and Kirtland (4).

Allen (2) showed two populations of adipocytes in extremely fat animals and suggested that once the larger adipocytes reached a certain size, new adipocytes were recruited. More recently investigators showed that an increase in adipose cell number in mature rats can result from overfeeding (138). In both of these studies (2, 138), at least two phenomena are possible. First, the recruitment of new cells may simply be a filling of existing differentiated adipocytes with lipid. Second, the stimulation of primary preadipocyte proliferation and their subsequent differentiation into adipocytes may account for the increased number of adipocytes. No data have been provided to distinguish between these two phenomena. Furthermore, we still have no evidence for the mechanism(s) or signal(s) that cause adipocyte proliferation during nutritional manipulation.

The approach to manipulation of adipocyte number by neonatal nutrition is based on the concept of a critical phase of adipose cell mitosis. Several observations were cited earlier which demonstrated a period of maximum DNA synthesis in adipose tissue. More recent information has challenged this concept. By dietary manipulation, adult rats can be stimulated to produce new adipocytes (138). Increased tritiated thymidine incorporation into DNA of adipocytes occurred after only 2 days in mature rats on a high fat diet (139). Some humans with adult onset obesity have excess numbers of adipocytes (137, 140). Lipectomy results in cellular regeneration in adult rats (141–144). In addition, the use of genetic models of obesity has demonstrated that fat cell multiplication continues in certain obesities for an abnormally long period of time (145). These observations and observations of fat cell multiplication in cell culture of cells obtained from adult rats and humans (99, 100) seriously challenge the concept of early maturation of adipose tissue. However, these exceptions to the critical phase hypothesis do not rule out the possibility that adipocyte numbers are more likely to be influenced during early development, especially in the normal individual.

Results from an important series of experiments by Greenwood and co-workers (146, 147) demonstrate that activity levels of adipose DNA polymerase and thymidine kinase are maximum during early development (in the preweaning rat). Adipose cell proliferation in the fetal pig is also extremely active (49). These observations support the concept that the mechanisms for stimulation of adipocyte proliferation are more apt to be sensitive to nutritional status during fetal and neonatal growth and development.

GENETIC REGULATION OF ADIPOSE CELLULARITY

Adipose cellularity is sensitive to genetic regulation. In the ob/ob mouse and Zucker (fa/fa) fatty rat, the animals have an increased number of adipocytes when homozygous for the recessive, obese gene. In other genetic models, obesity is characterized only by an increase in adipose cell size. Little data are available on obese animals in the preobese stage. The lack of data is caused by an inability to identify lean and obese animals at an early age. The obese Zucker rat can be identified at 5 to 7 days of age by sizing fat cells from inguinal fat pad biopsies (148). At 7 days of age obese Zucker rats have larger adipocytes and higher lipoprotein lipase activity than their lean controls (148, 149). These changes are observed in the absence of hyperphagia and hypertriglyceridemia (148) and, therefore, may represent a primary defect in the obese Zucker rat (148, 149).

The development of adipose tissue has been studied histologically in yellow obese (YO) and yellow normal (YN) mice (150–154). These studies were carried out on YN and YO mice between 10 days and 6 months old. Because differences in body weight are not significant until the mice are 6 months old, these animals were studied primarily in the preobese state. Epididymal fat cells were larger in the YO mouse by 63 days of age, whereas subcutaneous fat cells were much larger in the YO mouse only at 6 months of age. Subcutaneous and epididymal fat cell numbers were similar at 10, 21, and 63 days, whereas the YN mouse had many more fat cells than the YO mouse at 6 months. On the other hand, subcutaneous fat cell

number and size are greater in the overtly obese (ob/ob) mouse than the lean control (152). A larger fat cell size in the obese animal is the most consistent finding of histological studies on inherited obesity in mice.

Mast cell numbers have been determined in ob/ob, yellow obese, and New Zealand obese (NZO) mice (150, 151, 153, 154). The number of mast cells present in adipose tissue of the ob/ob mouse is 5-fold higher than the number present in the lean animals. Mast cell number is 3-fold higher in yellow obese mice, whereas the NZO mice have normal numbers of mast cells. The significance of the differences in mast cell numbers remains to be elucidated.

Hausberger (156) examined the adipose tissue of yellow, ob/ob, and mice made obese with gold-thioglucose injections for evidence of pathological changes. In markedly obese mice, the gonadal and perirenal fat pads had many macrophages, lymphocytes, and polymorphonuclear cells. Subcutaneous fat tissue showed few and only minor pathological changes in mice whose abdominal fat pads showed marked pathological changes. This abnormality may be present in adipose tissues of other genetically obese strains because it was not peculiar to one form of obesity as in the case of mast cell number (157). Hausberger's study of 1966 (156) is not clear as to the earliest time when "pathological" changes at the "microscopic" level can be detected in developing ob/ob or yellow obese mice. These cellular characteristics could have been present at a very early age in the obese animals. Hausberger (156) does indicate that those cells peculiar to pathological changes were present when pathological changes were not evident macroscopically. These cell types have been observed by many researchers in developing WAT tissue, lipid-depleted WAT and WAT from patients with abnormalities other than obesity. Possibly Hausberger's (156) microscopic observations are more significant in terms of adipocyte and adipose tissue development of the obese animal than they are in terms of purely age-related pathological changes.

The adipose cellularity of genetically obese animals was elucidated in recent years with Coulter Counter methodology. Kaplan, Trout, and Leveille (158) determined adipocyte size frequency distributions during the preobese and obese phases of development in lean and obese mice. The frequency of small cells continued to increase in the developing ob/ob mice. This increase resulted in bimodal adipocyte distribution of adipocytes after 10 weeks of age. At 3 weeks of age, the frequency distribution of adipocytes in ob/ob mice was significantly different from that in lean animals. At 26 weeks of age, adipocyte number and size were significantly larger in three depots of ob/ob mice than in lean mice (145). Ashwell, Priest, and Sowter (159) observed pockets of small multilocular adipocytes histologically in several depots of ob/ob mice ranging from 4 to 40 weeks old. These pockets were not found in lean mice. Therefore, bimodal adipocyte distribution patterns have been verified in the ob/ob mice by histological and Coulter Counter techniques. The Coulter Counter data (158) indicated the bimodal distribution at 10 weeks of age, whereas this morphological characteristic was described histologically at 4 weeks of age (159). The histological approach was much more sensitive to morphological differences due to obesity because of the cell size limit imposed in the Coulter Counter technique.

Johnson and Hirsch (145) determined adipose cellularity of three depots in six strains of genetically obese mice and in male gold-thioglucose-treated mice. Adipocyte hypertrophy was the consistent and usually dominant morphological explanation for adipose depot enlargement in genetic obesity and in obesity induced by gold-thioglucose. Only in the case of the ob/ob mouse did adipocyte hyperplasia accompany hypertrophy in depot enlargment. The cellular aspects of adipose tissue of lean and obese Zucker rats have been substantially reviewed (160). Adipocytes are increased in size and number in the obese Zucker rat when compared to lean littermate controls. The increase in adipose cell size is minimal in the gonadal pads and most evident in the subcutaneous depots. Apparent adipocyte hyperplasia continues well into adulthood for the Zucker obese rat. Cellularity of subcutaneous adipose tissue from USDA strains of genetically lean and obese pigs was studied by Steele, Frobish, and Keeney (161). High, low, and control backfat lines of Duroc pigs were studied at a constant age (100 days) and constant weight (95 kg). Adipocytes were greater in size and number in obese (high-backfat) pigs than in control and lean (low-backfat) pigs. Therefore, the ob/ob mouse, the Zucker obese rat, and the obese pig (high-backfat selection) are hyperplastic-hypertrophic obese animal models in terms of Coulter Counter data.

Characterization of the time of onset of increased adipocyte proliferation in the genetic models of obesity would greatly aid in the study of the milieu of factors that may regulate adipocyte numbers and size.

SUMMARY

Lipid deposition at the cellular level must also be considered at the tissue level. The perivascular origin of first-formed adipocytes has been confirmed over a number of species and adipose depots by many

investigators. Whether the proliferating preadipocyte is fibroblast, fibroblast-like, or reticulo-endothelial in origin is not yet resolved. However, several developmental aspects can be considered. LPL activity is present in the stromal-vascular fraction of lipid-free immature adipose tissue. The abundant and highly organized rough endoplasmic reticulum associated with the synthesis and removal of this protein from the cell (50) is analogous to the organellular arrangement found in fibroblasts synthesizing procollagen. This similarity in function and, therefore, in intracellular morphology, may explain why many investigators have described the preadipocyte as a fibroblast or fibroblast-like cell.

The arrangement of first-formed adipocytes as cords of tightly clustered cells and capillaries implies that preadipocytes likewise are arranged in this manner. The mesenchymal cells of blood islands prevalent in the immature fat organ are postulated to differentiate into epithelium and blast cells (54). A third component to which these cells differentiate may also include the adipocyte compartment.

Even though the precursor cell is not yet identified, evidence is accumulating to support the concept that preadipocytes can proliferate postnatally, and even in adulthood. Nuclear labeling, cell culture, lipectomy, and biochemical studies indicate the ability, or at least the potential, for preadipocytes to proliferate at any age. Therefore, some of the increase in cellularity measured by Coulter Counter techniques under a variety of conditions (age, hormonal, nutritional, and genetic influences) must be considered due to proliferative activity as well as to mere lipid filling of previously differentiated cells.

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