Adipose cell differentiation in culture

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Summary

The isolation of preadipocyte cell strains from adipose tissue and from bone marrow, and the establishment of preadipocyte cell lines from embryonic and adult mouse, have been useful tools to study the process of adipose cell differentiation.

This process is regulated both by extracellular signals present in serum and by intracellular signals; the characterization of these signals is under investigation. During adipose cell differentiation morphological and enzymatic changes are dramatic and they are accompanied by qualitative and quantitative variations of the cell protein content. These changes include the induction of the enzymes of the fatty acid and triglyceride synthesizing pathways and a subsequent triglyceride accumulation. The development of hormonal responses to insulin and to β-adrenergics is also observed, and differentiated adipose cells behave essentially like mature adipocytes isolated from adipose tissue.

The present review will be devoted to the main events of adipose conversion in cell lines and cell strains, and to current work which concerns the identification of the triggering signals possibly involved in that process.

Cellular models

In vivo studies of adipose cell differentiation have been hampered by the lack of early and suitable markers for adipocyte precursors and for differentiating cells. As a result, up to now, one has been able to estimate the cellularity of adipose tissue only by counting the triglyceride-containing cells. From a developmental point of view, the role of the factors governing in vivo the cellularity of adipose tissue should be very important in the light of the susceptibility of mammalian adipose cells to hyperplasia and hypertrophy observed after birth and/or after nutritional manipulations (1).

Over the last ten years cellular models have allowed an in vitro approach of the differentiation of both human and animal adipose cells. Cell strains and cell lines of preadipocytes which have been used are summarized in Table 1.

Among the most obvious advantages of using adipose cells in culture are: i) the easiness to conduct in vitro long-term experiments since the viability of adipocytes isolated from adipose tissue does

Abbreviations

LPL , lipoprotein lipase
MGL , monoglyceride lipase
T3 , triiodothyronine
dbcAMP , dibutyryl-cAMP
MIX , 1-methyl-3-isobutylxanthine
PG , prostaglandins
FCS , fetal calf serum
EDGF , eye-derived growth factor
PDGF , platelet-derived growth factor
FGF , fibroblast growth factor
VLDL , very low density proteins.

Molecular and Cellular Biochemistry 49, 17–31 (1982). 0300-8177/82/0491-0031/$03.00.

Table 1. Preadipocyte cell lines and cell strains.

	Species	Origin	Caryotype	Reference
Cell lines				
1246	Embryocarcinoma cells injected into C3H mouse	T-984	Aneuploid	7
3T3-L1	Swiss mouse embryo	Variant embryo fibroblast	Aneuploid	4, 14
3T3-F442A	Swiss mouse embryo	Variant embryo fibroblast	Aneuploid	5
ob17	C_{57}BL/6J ob/ob adult mouse	Adipocytes	Aneuploid	6
PFC6	C_{57}BL/6J ob/ob adult mouse	Stromal cells of epididymal fat pad	Aneuploid	unpublished
HGFu	C_{57}BL/6J +/? adult mouse	Adipocytes	Aneuploid	unpublished
ST13	ddN mouse	Mammary carcinoma	Hypotetraploid	8
Cell strains				
	Rat	Stromal cells of epididymal fat pad	Not determined	11, 13, 21
	Bovine	Stromal cells of subcutaneous adipose tissue	Not determined	17
	Human	Stromal cells of omental adipose tissue	Not determined	10
	Mouse	Bone marrow	Not determined	9
	Rat	Bone marrow	Not determined	58
	Human	Bone marrow	Not determined	58

not exceed a few hours; ii) the possibility to control the cell environment; and iii) the possibility to study morphological and biochemical events occurring during adipose conversion.

Cell lines in contrast to cell strains, which may contain different cell types in some unknown and variable proportions through passages, present stable phenotypic properties through a large number of generations despite their aneuploid caryotypes.

In cell lines the program of differentiation is transmitted by cell division and the expression of the program – the adipose conversion – will take place essentially after proliferation (vide infra). It could be hypothesized that the rather extensive chromosomal rearrangements present in cell lines could affect their susceptibility, allowing adipose conversion to occur in vitro. The situation could be at variance with the situation in vivo where environmental conditions are likely to be different. However, the cell susceptibility does not seem to be different in vitro and in vivo, since injection of undifferentiated 3T3-F442A cells into athymic mice is followed by the development of differentiated typical fat pads (2).

The first preadipocyte cell line established in 1974 was 3T3-L1 (3). It was isolated as a clonal subline derived from the established fibroblast line 3T3, taking advantage that the latter shows a low but significant frequency of adipose conversion. The clone 3T3 was originally evolved from the disaggregated cells of late fetuses of Swiss mouse (4). Similarly, by serial selection beginning with 3T3-clone 18, which showed a very low frequency of adipose conversion, it was possible to obtain 3T3-F442A cell line; 3T3-F442A cells present a higher susceptibility to the adipose conversion than 3T3-L1 cells (5). Therefore, in both cases, a low proportion of the 3T3 cells are able to develop a cellular transition from an insusceptible to a susceptible state, and the frequency of this transition can be increased by selection through serial cultivation.

Clonal cell lines ob17 and HG-Fu were established from the epididymal fat pad of adult ob/ob mouse and of its lean counterpart. With the method used for isolation, they arise most likely from 'dedifferentiated' adipocytes (6). In contrast to ob17 line, PFC 6 line was obtained in parallel from the stromal-vascular fraction of epididymal fat pad which contains preadipose cells (vide infra).

Clone 1246 was isolated from the myogenic clonal cell line T984 grown under clonal conditions, which was itself isolated from a mouse teratoma (7).

The origin of ST13 line is quite different from that of other cell lines. ST13 line arises from the parental line ST which was itself established from a spontaneous mammary carcinoma of ddN mouse (8).

Preadipose cell strains were obtained from adipose tissue and bone marrow of rat and human (9–12). The stromal-vascular fraction of adipose tissue was used to isolate and culture a population of cells containing adipocyte precursors (13). However, the homogeneity of this initial population is difficult to control from experiment to experiment and seems to decrease with passages (vide infra).

Most detailed accounts have been obtained on 3T3-L1 cells over the past five years, but general features have emerged from studies of the different cell lines and cell strains.

Morphological changes during adipose conversion and the susceptible state

During the growth phase, preadipose cells present a fibroblast-like appearance. Their fibroblastic nature was ascertained by collagen synthesis for 3T3-L1, ob17 and bovine stromal cells (14–17); as expected collagen was found mainly of Type I and partly of Type III (15, 17). After the cells reach confluence, the cell shape changes. Cells become round, enlarge, start the differentiation program and will *later* accumulate lipid droplets in their cytoplasm. Small lipid globules may fuse ultimately 2–4 weeks post-confluence into a few or into a single fat globule. In agreement with these observations, undifferentiated 3T3-L1 cells contain actin-like stress fibers and synthetize actin (18) as well as bundles of intermediate filaments 19. Both structures disappear in differentiated cells in which actin content is decreased 4-fold, while the numerous lipid droplets are found at the border of the endoplasmic reticulum and closely associated to microperoxysomes and mitochondria (19). These observations would suggest that actin could play a role in morphological changes.

So far, the adipose conversion of cell lines and cell strains does involve a limited proportion of cells (40–90%). Typically, most cell lines will differentiate as colonies of fat cells separated from each other by cells insusceptible to adipose conversion (Fig. 1). For a given cell line, this proportion depends essentially upon the batch of serum being used, remains stable through a large number of passages under controled conditions and decreases thereafter. In cell strains obtained from rat adipose tissue the extent of adipose conversion varies with different parameters: age of the donor rat, anatomical site and purity of the stromal vascular fraction inoculated (20, 21). Subculture indicates that these cells multiply readily through passages but loose their ability to be converted to adipocytes (21). This observation suggests among possibilities an initial heterogeneity of the cell population with a selective advantage through passages for fibroblast-like cells distinct from adipocyte precursors. In any case, the adipose conversion is not an uniform process, not even within a given group of cells. In a group of susceptible cells the accumulation of lipids start in central cells while cells at the edges are still multiplying (Fig. 1).

In ob17 cells, recent experiments allow us to conclude that both susceptible and insusceptible cells are already present during the growth phase. Each cluster of fat cells arises from individual susceptible cells through divisions (22). Insusceptible cells can acquire the susceptible state if they are allowed to divide. This phenomenon can be observed either by making a 'wound' in areas of resting undifferentiated cells or by eliminating the differentiated cells after centrifugation and reinoculating the population of undifferentiated cells.

Enzymatic changes during adipose conversion

As could be anticipated, the spectacular morphological changes are accompanied by a large increase (50- to 100-fold) in the cellular triglyceride content and by the induction of a large set of enzymes related to fatty acid and triglyceride synthesis (Fig. 2). It is important to realize that the specific activity values are due to a combination of two factors which may vary *independently* (vide infra), viz. the number of differentiated cells relative to the total cell population *and* the net enzyme content per cell.

During the differentiation process of 3T3-L1 cells the synthesis of more than 50 cellular proteins is enhanced significantly (23). A coordinate rise (24) in most of the enzymes of de novo fatty acid synthesis (20- to 50-fold) and of some of the enzymes of the triglyceride pathway (30- to 100-fold) is observed, in contrast to enzymes of complex lipid biosynthesis which show no increase (25). Glutamine synthetase, not related to lipogenesis, is also increased more than 140-fold (26, 27). After their induction, the total content of the different enzymes increases

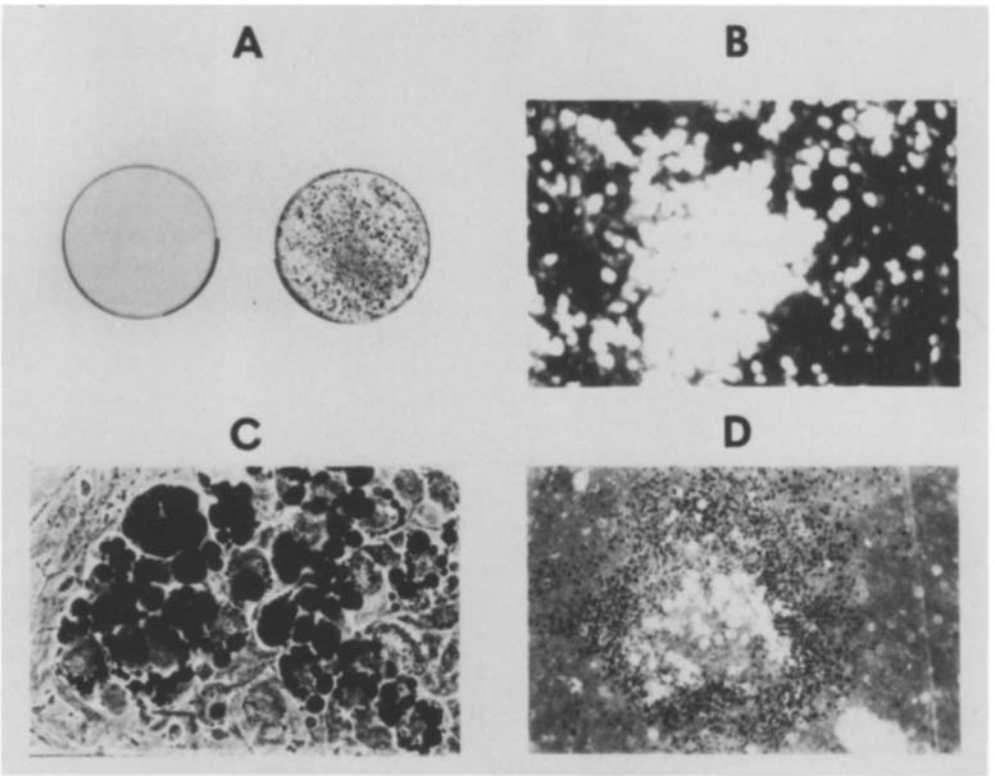

Fig. 1. Micrographs and autoradiographs of ob17 cells during adipose conversion.

(A) Oil Red O staining of cells were maintained 2 days (left) and 18 days (right) after confluence in the presence of 10% fetal calf serum, 170 nM insulin and 1.5 nM T3. Fat cell clusters appear in dark and usually contain several hundreds of lipid-filled cells

(B) 16-day post-confluent ob17 cells in the same medium as in (A). Magnification ×75. Differentiated cells are present in clusters and appear in white (no lipid staining)

(C) 13-day post-confluent ob17 cells in the same medium as in (A) and fixed with osmium tetroxyde. Magnification ×300. Lipid droplets of variable size appear in dark within cells

(D) Confluent ob17 cells were maintained as in (A). [^{3}H]thymidine incorporation was performed between day 8 and day 10 and cells were processed immediately for autoradiography. The autoradiograph shows a developing cell cluster, in which cells proliferate at the periphery while differentiated cells are already visible in the central area. The autoradiograph shows also that cell labelling is almost absent outside clusters, that in areas of insusceptible cells. Thus post-confluent mitoses occur specifically in susceptible cells which convert later into adipose cells.

(28–33) and accounts approximately for 40% of the total cytosolic proteins in 3T3-F442A cells (34). The expression of the differentiation program is completely abolished if exponentially growing cells are treated with 5-bromo-2′-deoxyuridine, a thymidine analog incorporated into DNA whose mechanism of action is not really known (14, 35). No triglyceride accumulation is observed under these conditions. This accumulation normally depends upon the availability of metabolites furnishing the glycerol and the fatty acid moities. On the one hand, glycerol-3-phosphate dehydrogenase, which is essential in furnishing the glycerol moiety as glycerol-3-phosphate, shows an increase of 2–4 orders of magnitude. This is due to the emergence of an enzyme that is not expressed in the growing state (31). On the other hand, depending upon the medium composition and upon the cell line, the fatty acyl moiety is derived partly from exogenous serum triglycerides through hydrolysis and partly from endogenous fatty acid synthesis. For instance the adipose conversion of 3T3-F442A is not affected by depriving the cells from serum lipids (36) while, in contrast, ob17 cells rely essentially upon an exogenous source (7). Similarly inhibition of de novo fatty acid synthesis does not affect lipid accumulation in 3T3-L1 cells (25).

So far, the earliest phenotypic expression of the

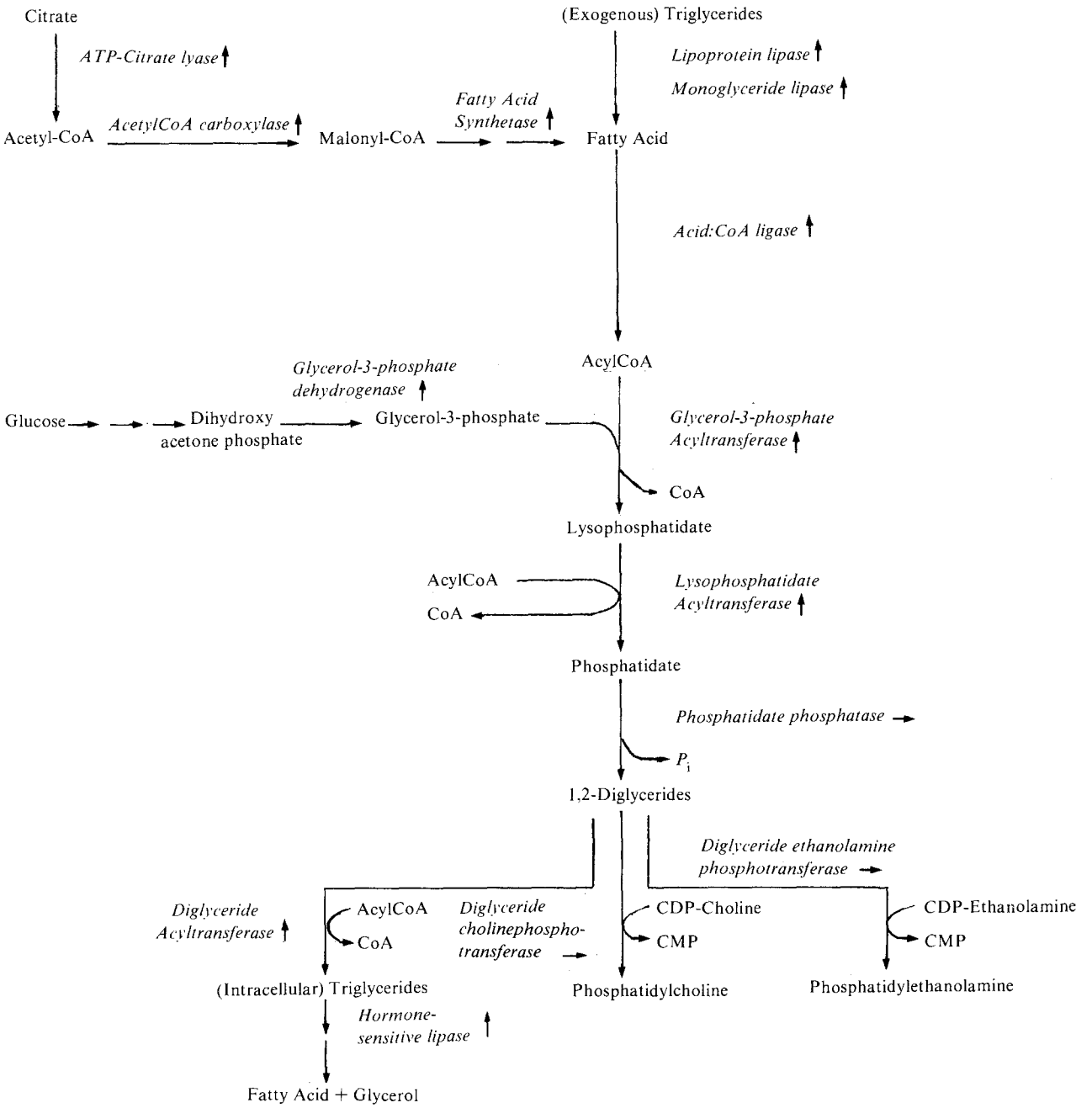

Fig. 2. Main metabolic pathways involved in adipose conversion. Vertical arrows indicate enzyme induction during adipose conversion. In contrast to the triglyceride-pathway enzymes, no induction is observed for phosphatidase phosphatase and for enzymes involved in complex lipid biosynthesis (horizontal arrows).

differentiation program is the development of lipoprotein lipase activity after the cells reach confluence* (37–39). The appearance of LPL corresponds in ob17 cells to a net increase in enzyme content per cell and is independent of the presence of serum triglycerides as substrates.* In vivo LPL, whose specificity leads to the formation of monoglycerides, is secreted by adipose cells and partly recovered at the luminal surface of endothelial cells, likely bound to heparan sulfate (42). In vitro most of LPL is heparin-releasable and is detected at the cell surface by indirect immunofluorescence experiments.* Of interest is the fact that in ob17 cells an increase in MGL activity is observed parallel to that of LPL. Both enzyme activities are abolished in 5-bromo-2'-deoxyuridine-treated cells. These results are in favour of the hypothesis that LPL and MGL both are active in the assimilation of exogenous triglycerides (43).

The large changes observed in the activities of the different key enzymes raise the question of what mechanisms are involved in the modulation of their synthesis and degradation. First, it is most likely that changes in activity are directly related to changes in enzyme content, as already observed for acetyl-CoA carboxylase (24), fatty acid synthetase and LPL (vide supra). Second, pulse-labelling experiments in 3T3-L1 cells have clearly shown that, with fatty acid synthetase, the rate of degradation is essentially unaffected during differentiation, and that the increase in activity is entirely due to an enhanced rate of synthesis (44, 45). Elegant studies on 3T3-F442A cells have demonstrated that the amount of translatable mRNA is the main process of modulating the synthesis of fatty acid synthetase and of glycerol-3-phosphate dehydrogenase (18, 34).

The next important question is whether there is any relationship between changes observed for a single enzyme (or a set of enzymes) and changes observed for another enzyme (or another set of enzymes). H. Green and his colleagues have presented evidences for the distinction between 'primary' and 'secondary' enzymes, favouring the hypothesis that a primary increase in a few enzymes leads to changes in the concentrations of key metabolites, leading in turn to changes in the rates of synthesis of 'secondary' enzymes (46). Different lines of evidence support this assumption: i) as already mentioned, expression of LPL and MGL precedes that of the triglyceride-pathway enzymes (43); ii) arrest of endogenous fatty acid synthesis by depriving 3T3-F442A cells from exogenous biotin does not prevent the appearance of LPL and of glycerol-3-phosphate dehydrogenase, in contrast to glycerol-3-phosphate acyltransferase and malic enzyme which are expressed at low levels (36); and iii) the use of agents affecting the cAMP content of differentiating 3T3-442A cells (isoproterenol, theophylline, dbcAMP allows the demonstration that the transcription of mRNA coding for fatty acid synthetase, malic enzyme and glycerol-3-phosphate dehydrogenase is affected differently (18).

In conclusion, the different approaches seem to exclude an operon-type response, in which a single gene would control a coordinate rise in the amounts of all the enzymes involved in the differentiation process.

Effects of hormones and drugs during adipose conversion and the development of hormonal responses

Mature mammalian fat cells have been studied in great detail and are known to respond to a variety of hormones, including insulin and different lipolytic hormones. For the sake of clarity, it is helpful to distinguish between: i) long-term hormonal and drug effects on adipose conversion; ii) changes in receptor level and in hormone sensitivity during adipose cell development; and iii) hormonal responses of differentiated cells. Easy conclusions can be drawn from this depending on the existence of a differentiation process occurring as extensively, synchroneously and rapidly as possible. Unfortunately, it is generally the case (but not always, see below), since: i) some cells are not converted to adipose cells, and ii) the rate of conversion varies within individual clusters and between cells within a given cluster.

* A transient reduction in poly(ADP-Ribose) synthetase activity has been recently reported as an earlier event in differentiation of 3T3-L1 cells. This transient reduction in activity is assumed to be related to changes in chromatin structure (41).

* Vannier, C., Négrel, R. and Ailhaud, G., unpublished observations.

Long-term hormonal and drug effects on adipose conversion

Exposure for 2 days of confluent 3T3-L1 and 1246 cells to a mixture of dexamethasone and MIX compressed the time course of adipose conversion into one week. A large proportion of cells (70–90%) is converted and differentiation occurs quite uniformly rather than in fat clusters (47–50). At higher concentrations than that used for dexamethasone (0.25 μM) representative of a glucocorticoid, mineralocorticoids (deoxycorticosterone) and sex steroïds (progesterone) are also effective (47). MIX, dexamethasone, indomethacin or $PGF_{2\alpha}$, present individually, are also capable of having a lower but positive effect on the 3T3-L1 adipose conversion (48), while PGE_1 and MIX are mildly effective on the conversion of adipocyte precursors of rat adipose tissue (12, 21). The mechanism of action of these drugs is presently unknown. The continuous presence of MIX is not required and this drug does not seem to act via cAMP since dbcAMP and 8-bromo-cAMP are ineffective in 3T3-L2 cells (51, 52). It has been suggested for dexamethasone that inhibition of prostaglandin E_1 synthesis or its precursors or products could play a role in triggering the activation of the differentiation program of 3T3-L1 cells (47). This hypothesis is difficult to assess since no data are available on the synthesis of prostaglandins by these cells and since the relationships between cessation of growth and prostaglandin synthesis on the one hand, and adipose conversion on the other, are not clear-cut. For instance, in ob17 cells, the adipose conversion begins by a limited proliferation followed by growth arrest (vide infra). The latter is parallel to a striking decrease in prostaglandin synthesis (PGE_2 and 6-keto-$PGF_{1\alpha}$; ref. 53). However, the potent inhibitory effect of exogenous $PGF_{2\alpha}$ on adipose conversion, accompanied by a potent rise in prostaglandin biosynthesis, is not abolished even in the presence of low concentrations of dexamethasone and indomethacin that are able to block prostaglandin synthesis (54). Therefore, if necessary, the cessation of prostaglandin production does not seem to be sufficient to allow differentiation to proceed under conditions in which $PGF_{2\alpha}$ is active as a mitogen and where a continuous cell proliferation is observed. An alternative explanation would be that prostaglandin synthesis is a mere reflection of cell proliferation and fortuitously time-related to differentiation in resting cells.

Indomethacin, at concentrations much higher than those required to inhibit prostaglandin synthesis, as well as MIX, are able to accelerate the adipose conversion of ob17 and 3T3-L2 cells, respectively, *even* in the presence of 5-bromo-2′-deoxyuridine which normally prevents their differentiation (35, 55). Both drugs do not act by altering the amount of 5-bromo-2′-deoxyuridine present in DNA. As yet their action as 'promoters' of differentiation, which is shared by hypolipidemic drugs like clofenapate and clofibrate (55), is difficult to understand.

In the absence of any drug treatment, i.e. 10% faetal calf serum, a low but significant adipose conversion occurs within 2–4 weeks post-confluence. Insulin supplied by FCS ($<10^{-11}$ M) is needed *on a long-term basis,* since resting ob17 cells maintained in insulin-deprived FCS loose viability within 10 days (40). The accelerating effect of insulin on adipose conversion of ob17 cells is concentration-dependent (Fig. 3). Insulin is clearly active within a physiological range of concentrations. EC_{50} values (1–2 nM) are close to Kd values for the high-affinity binding sites for insulin ($\cong$1 nM; ref. 56), suggesting that insulin effects on adipose conversion are exerted through insulin receptors per se. In agreement with this hypothesis are experiments on ST13 cells where suppression of functional insulin binding sites by tunicamycin is concomitent with a lack of adipose conversion (57).

In contrast to preadipocyte cell lines, cell strains of marrow preadipocytes fail to differentiate in the presence of insulin (58). If hyperresponsiveness to residual insulin present in serum were excluded in the future, it might be possible to use insulin-dependence as a criterion to distinguish between preadipose cells that are related to the haematopoietic system and those that are not.

Long-term and positive effects of triiodothyronine at physiological concentrations have recently been reported on the adipose conversion of ob17 cells (59). T3 is effective via nuclear hormone receptor. As for insulin, T_3 is active providing the cells have entered their differentiation program and cannot overcome the inhibition of adipose conversion by growth in the presence of 5-bromo-2′-deoxyuridine (59). Similar effects of T_3 on the induction of fatty acid synthetizing and esterifying enzymes

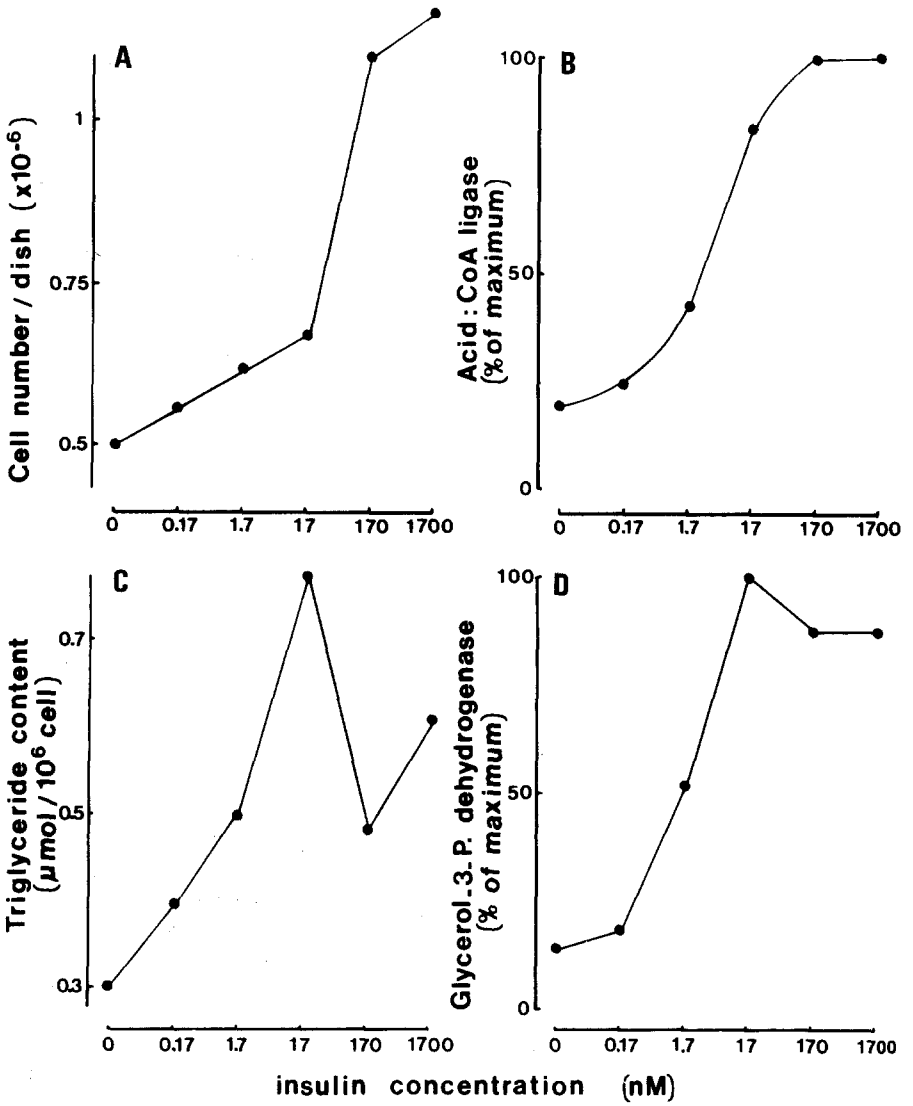

Fig. 3. Lipogenic and mitogenic effects of insulin on the adipose conversion of ob17 cells. Confluent cells were maintained in the presence of 10% FCS, 1.5 nM T3 and increasing concentrations of insulin and assays performed 16 days post-confluence. Lipogenic effects of insulin are effective within physiological concentrations and maximal at 17 nM (B, C and D) while the mitogenic effect of the hormone becomes only significant above the latter concentration (A).

have not yet been reported for other preadipocyte cell lines and cells strains, but these observations are in agreement with in vivo data obtained on fatty acid synthetase of adipose tissue from thyroidectomized rats treated with T_3 (60–62).

From agents shown to inhibit adipose conversion, when added at confluence and thereafter, should be excluded compounds which behave as potent mitogens and thus prevent the continuously proliferating cells to enter the differentiation program ($PGF_{2\alpha}$, PGE_1, EDGF, PDGF, acidified serum) (54, 63). This does not seem to apply for retinal and retinoic acid on ST13 cells (64) and for mouse interferon which is capable of inhibiting the 3T3-L1 conversion without mitogenic effect, while interferons from heterologous species are inactive (65). The mechanism of interferon action remains to be investigated.

Chronic exposure to agents known to increase cAMP levels (epinephrine, isoproterenol, dbcAMP)

affect the lipid accumulation of differentiating cells but do not prevent the cells to acquire the spherical shape characteristic of adipocytes. These agents cause a decrease in the rate of synthesis of lipogenic enzymes in 3T3-F442A (18); their effect is likely to be rapid, since a significant decrease in the rate of synthesis of fatty acid synthetase is observed after a short-term exposure of differentiating 3T3-L1 cells to isoproterenol or to dbcAMP (44).

Developmental changes in receptor level and in hormone sensitivity

Changes in receptor level and in hormone sensitivity during adipose cell development have been investigated in 3T3-L1 and ob17 cells. In 3T3-L1 cells, different groups have shown that a dramatic increase in the insulin-binding capacity (3- to 35-fold) is accompanying the differentiation, whether insulin (65) or a 'triggering cocktail' (47, 67, 68) (vide supra) is present in order to accelerate the adipose conversion. A potent increase has also been found in ST13 fibroblasts during differentiation into adipose cells (57). In 3T3-L1 this increase is primarily accounted for by an increase in receptor number with no significant change in receptor affinity, although a 5-fold decrease in affinity has been reported by some investigators (47). Elegant studies on 3T3-L1 cells using a heavy isotope density-shift method have clearly demonstrated that the rise in insulin receptor levels is due solely to a parallel rise in the rate of insulin receptor synthesis (69–71). The differentiation of ob17 cells is also accompanied by an increase in the number of their insulin receptors (1.5- to 2-fold) with no change in affinity. ob17 cells present a self-modulation of their insulin receptor levels after chronic exposure to insulin, as well as a loss of insulin-sensitivity (56).

Changes in receptor level are accompanied in differentiating 3T3-L1 cells by changes in glucose metabolism and insulin responsiveness (47, 68). Insulin is almost ineffective in exponentially growing and confluent cells on 2-deoxyglucose uptake, in contrast to post-confluent cells. However, in the latter cells the marked increase in insulin responsiveness is essentially due to a large decrease in the basal rate of transport with no significant change in the maximal rates after stimulation of the cells by supraphysiological concentrations of hormone. In contrast to insulin, the stimulatory effect of $PGF_{2\alpha}$ on 2-deoxy-glucose uptake is abolished during the course of differentiation of 3T3-L1 cells (72).

More significant is the 4-fold rise of glucose oxidation in the presence of insulin and the striking change in coupling between insulin binding and the biological response (68). In sparse cells a linear coupling exists between the two parameters. In confluent cells 15% receptor occupancy corresponds to 50% stimulation of response and full occupancy is needed for maximal response. In differentiated cells 50% of the response is obtained with less than 5% occupancy and 40% occupancy is sufficient for a maximal response. Changes in increased binding capacity do not seem to be coordinated with changes in hormone responsiveness, since the former lags significantly behind the latter during development (47). The mechanism of insulin action in 3T3-L1 cells via its specific receptor has been substantiated by using insulin-receptor antibodies in lieu of insulin. Both the expected acute and chronic metabolic responses were obtained, strongly suggesting that the receptor itself contains the information necessary to generate the subsequent effects (73, 74).

A development of responsiveness to lipolytic hormones is also occurring during adipose conversion of 3T3-L1, ob17 and 1246 cell lines as well as during that of adipocyte precursors of rat adipose tissue (49, 50, 75, 76). Since a cascade of events is happening between the hormone–receptor interaction and the activation of hormone-sensitive lipase (77), more than one critical step may be involved in the development of the lipolytic response determined by fatty acid or glycerol release from intracellular triglycerides.

The differentiating 3T3-L1 cells acquire the ability to synthetize cAMP in response to corticotropin and become more sensitive to the stimulation of adenylate cyclase provoked by β-adrenergics (75). This ability is correlated to the acquisition of a phosphodiesterase having a low Km for cAMP and sensitive to insulin (78). As in 3T3-L1 cells, responsiveness to isoproterenol is present in undifferentiated ob17 cells (76). However, the magnification in β-adrenergic responsiveness was observed as a function of development only during the period of time where the cells reach confluence; it decreases rapidly within a couple of days and slowly thereafter. No dramatic variations in the sensitivity to isoproterenol responsiveness occur during maturation of ob17 cells to adipose cells. In contrast to late

confluent cells, early confluent ob17 cells do not show the full lipolytic response provoked by isoproterenol, epinephrine and corticotropin and which involves first the cAMP-dependent activation of protein kinase and then the activation of hormone-sensitive lipase by phosphorylation. The development of a complete lipolytic response was also obtained in adipocyte precursors from rat and human converted to adipocytes, as well as in differentiated 1246 cells and 3T3-L1 cells (12, 49, 50, 79). Direct enzyme activity measurements in 3T3-L1 and 1246 cells have shown that, while the kinase activity remains stable, the hormone-sensitive lipase activity increases during differentiation (49, 80). Similar results have been recently obtained in ob17 cells (J. Khoo, personal communication). Therefore, the most critical and general event for adipose cells during differentiation is likely to be the induction of hormone-sensitive lipase.

Hormonal responses of differentiated cells

After differentiation, mature cells from different cell strains and cell lines have properties similar to those of adipocytes isolated from adipose tissue. Besides the full equipment in lipogenic and lipolytic enzymes, as well as the hormonal responses to insulin and to lipolytic hormones, differentiated 3T3-L1 cells chronically exposed to insulin manifest an elevated basal activity of 2-deoxyglucose transport and a blunted response to further stimulation of hexose uptake by insulin (81), which is in agreement with in vivo experiments on insulin-treated rats (82) and with in vitro experiments using intact human adipose tissue (83). A resistance to the stimulatory action of insulin on the transport of α-aminoisobutyrate also exists in differentiated ob17 cells exposed chronically to insulin; the stimulation is fully recovered within 3 days after insulin removal (40, 56).

The insulin resistance seen in vivo by glucocorticoid excess has been demonstrated in vitro by exposing chronically differentiated 3T3-L1 cells to dexamethasone and hydrocortisone. This insulin resistance occurs at hormone concentrations which are in the physiological range, showing that the effect of glucocorticoids is indeed direct on adipose cells via the glucocorticoid hormone receptor (84). Both in vitro and in vivo excess of glucocorticoids leads to a reduction in insulin binding with little change in receptor concentration, and also a marked decrease in the ability of insulin to stimulate 2-deoxyglucose uptake maximally.

The adipose conversion can be considered, therefore, as the result of the differentiated functions of specialized fibroblasts (Table 2) since the characteristic properties of converted cells are not shared by fibroblasts from rat and human skin (10, 11) and by fibroblastic cell lines.

The triglyceride accumulation is the most obvious feature of the adipose conversion and is indicative of a full differentiation process. However its reliability is questionable since: i) it can be affected in culture by many factors; ii) it is the last phenotype to be expressed; iii) it can be clearly dissociated from the induction of the characteristic enzyme markers of differentiation; and iv) cells different from adipose cells can accumulate lipid droplets in culture (85). First, addition of triglycerides either as VLDL (85) or as Intralipids (21) speeds up the process of triglyceride accumulation. This process is further accelerated by heparin through the release of lipoprotein lipase in the culture medium (20, 87). Second, the use of delipoproteinized or delipidized serum does not prevent the expression of at least the earliest stages of the differentiation program (LPL) but largely prevents the lipid accumulation (6, 36). Third, besides insulin and triiodothyronine normally present in foetal calf serum, unindentified factors are present at variable concentrations from batch to batch and should also play a role in adipose conversion.

Post-confluent mitoses and amplification of adipose conversion

The adipose conversion of preadipocyte cell lines may be postponed indefinitely if the cells remain in a continuous proliferating state. This situation is present in exponentially growing cells or in confluent cells exposed to different mitogenic factors ($PGF_{2\alpha}$, platelet extract, EDGF). Under usual conditions, i.e. foetal calf serum and insulin, the cells first reach a resting state where [^{3}H]thymidine incorporation into DNA and prostaglandin synthesis are minimal (22, 88). Subsequently, among the earliest events of differentiation taking place in 3T3-F442A and ob17 cells there is a mitogenic response of susceptible cells which appear later as

Table 2. Comparative main properties of isolated adipocytes and adipose converted cells.

	Cell lines					Cell strains				Mouse adipocytes
	1246[a]	3T3-L1[b]	3T3-F442A[c]	Ob17[d]	ST13[e]	Adipose tissue (rat)[f]	(human)[g]	Bone marrow (mouse)[h] (rat)	(human)[i]	
Morphological changes	+	+	+	+	+	+	+	+	+	+
Hormone sensitivity										
Short-term										
Catecholamines (3)	+	+	+	+		+	+			+
Corticotropin	+	+		+						+
Glucagon	+	-								-
Long-term										
Insulin		+	+	+	+			-	-	+
Triiodothyronine				+						+
Corticosteroïds (1)	+	+	-	-	+			+	+	+
		+ (2)								+
Enzyme induction										
Lipogenesis	+	+	+	+	+					+
Triglyceride synthesis	+	+	+	+	+					+
Lipolysis (LPL)	+	+	+	+		+	+			+
Triglyceride accumulation (3)	+	+	+	+	+	+		+		+

No indication corresponds to unavailable data.

(1) corticosteroïd effects on adipose cell differentiation.

(2) corticosteroïd effects on differentiated cells (insulin-resistance).

(3) Unesterified fatty acid outflow is significantly enhanced by perfusing bone marrow of dog tibia with isoproterenol (100). These experiments support indirectly the existence of true fat cells in bone marrow, i.e. cells susceptible to accumulate and to mobilize triglycerides.

[a] ref. 49, 50
[b] ref. 4, 5, 14, 18, 19, 25, 30, 38, 39, 44, 47, 84
[c] ref. 4, 18, 36, 38, 96
[d] ref. 6, 40, 43, 59, 76
[e] ref. 8, 57
[f] ref. 11, 12, 13, 20, 21
[g] ref. 10, 79
[h] ref. 9, 58
[i] ref. 58

clusters of fat cells. This mitogenic response is clearly shown by [^{3}H]thymidine incorporation into DNA and by autoradiographic experiments, as well as by a transient synthesis of prostaglandins. Elimination of cells in the phase of DNA synthesis with fluorodeoxyuridine or blockade of DNA synthesis with cytosine arabinoside abolishes the formation of fat cell clusters but does *not* abolish the adipose conversion of individual cells (22, 88). Therefore, this form of clonal selection increases the proportion of differentiated cells relative to the total cell population most significantly, although the initial percentage of susceptible cells should be low. It is likely that DNA synthesis is not required for the adipose conversion since the latter can occur in individual cells. However, the above experiments do not exclude the possibility of replication of limited portions of the genome. In any case post-confluent mitoses are beneficial to the extent of adipose conversion. This cell multiplication occurs only in the early stages of adipose conversion and fully differentiated adipose cells do not divide. It is difficult to assess the importance of this selective multiplication of susceptible cells in vivo. However, a parallel phenomenon may exist since differentiating adipocytes of rat adipose tissue present the highest rate of DNA synthesis as compared to stromal cells and to mature adipocytes (89–91).

Adipogenic factors and the role of serum

Adipose conversion may depend upon the existence of extracellular as well as intracellular signals. The nature of those regulatory factors is under investigation. Extracellular and regulatory signals should be present in sera of different species which are able to support growth and differentiation of

3T3-F442A cells, but foetal calf serum is by far the most effective for differentiation; similar observations can be made on ob17 cells. The existence of adipogenic factors in foetal calf serum has been inferred on the basis of experiments using cat serum on 3T3-F442A line (92) or a defined medium on 3T3-L1 line (93). Cat serum stimulates normal growth of 3T3-F442A cells but supports neither their differentiation nor the earliest stages of adipose conversion such as the characteristic change in cell shape and the transient post-confluent DNA synthesis. Foetal calf serum is required in addition to cat serum in order to 'trigger' the differentiation program (88, 92). The requirements of foetal calf serum for adipose conversion is also elegantly demonstrated by growing 3T3-L1 cells on polylysine-coated dishes in a defined medium containing insulin, transferrin, FGF and a new growth factor (Gimmel factor) purified to homogeneity from rat sub-maxillary gland (ref. 93 and D. B. McClure et al., unpublished results). This medium supports growth similarly to 10% foetal calf serum and confluent cells remains fully viable for many weeks. However, 1 to 2% foetal calf serum added at confluence, whether or not containing lipids, are still required for adipose conversion, indirectly demonstrating the necessity for adipogenic factors. Of interest is the fact that bovine pituitary extracts, assayed in the presence of cat serum, shows an adipogenic activity at low protein concentrations (50 μg–1 mg/l) (94). The adipogenic activity is at least 3 orders of magnitude higher in pituitary than in other organs and in foetal calf serum, and it has been suggested that the adipogenic factor of serum originates in the pituitary. Since the adipose conversion is estimated by specific activity measurement of enzyme markers such as glycerol-3-phosphate dehydrogenase, it is difficult to evaluate the relative contribution of the number of differentiated cells and of the net enzyme content per cell. It is likely that some factor(s) present in foetal calf serum are purely adipogenic, since the latter can support the differentiation of 3T3-F442A cells in which post-confluent mitosis were blocked by exposure to cytosine arabinoside (88). Proofs for the existence of external factor(s) controlling post-confluent mitoses and, consequently, the number of cells per cluster have been obtained on ob17 cells. Foetal calf serum treated in order to remove thyroïd hormones is able to support, in the presence of physiological concentrations of triiodothyronine, the adipose conversion of confluent cells which remain present as single cells or as clusters containing a few cells. Addition of an adipose conversion factor (ACF) for 2–3 days post-confluence restores the formation of large fat cell clusters similar to that observed in foetal calf serum (95). A similar factor(s) has recently been found in bovine pituitary extracts. Therefore, both foetal calf serum and pituitary extracts contain a factor(s) which modulates specifically post-confluent mitoses of susceptible cells and which can be considered as a specific mitogenic factor.

Recent work suggests that hemin and polyamines may behave as intracellular factor(s) modulating in some way the adipose conversion. Hemin enhances adipose conversion of 3T3-L1 and 3T3-F442A cells in the presence of both a high level (foetal calf serum) and a very low level (cat serum) of adipogenic factor (96). Hemin, which has no effect on the adipose conversion of ob17 cells, seems to increase the number of fat cell clusters. Its mechanism of action remains to be elucidated but its endogenous concentration may be critical at the earliest stages of adipose conversion since the latter is inhibited by aminotriazole (an inhibitor of heme biosynthesis) and since this inhibition is reversed by addition of hemin (95).

Along similar lines the requirement of endogenous polyamines in the differentiation process has been suggested quite recently, since α-difluoromethylornithine (an inhibitor of polyamine biosynthesis), added to early confluent and still differentiating 3T3-L1 cells, prevents their adipose conversion and provokes a significant decrease in the number of fat cell clusters. The formation of clusters was recovered by simultaneous addition of putrescine or spermidine (97). These experiments would suggest, as for hemin, that an increased accumulation of endogenous polyamines is needed for differentiation but only at a critical and early period after confluence; 3-deazaadenosine, an inhibitor of cellular methylation, has been shown to increase the triglyceride accumulation in 3T3-L1 cells twice (98). It would be interesting to carry out more detailed studies that might support the hypothesis that some critical methylation could trigger the process of adipose conversion.

The requirement of adipogenic factor(s) – still to be purified – for adipose conversion is not absolute,

since 1246 cells are able to differentiate in a serum-free medium containing insulin, transferrin and FGF. The differentiation process is only accelerated by a mixture of dexamethasone and MIX (49, 50). These interesting results indicate that the ability of 1246 cells to differentiate does not appear to depend upon (an) external signal(s) present in foetal calf serum. It is not yet known if these cells produce their own adipogenic factor(s) or are fully independent from a regulator of exogenous or endogenous origin. This point should be clarified by the use in serum-free medium either of coculture of 3T3-L1 and 1246 cells or of conditionned medium from 1246 cells on the adipose conversion of 3T3-L1 cells.

Future trends

Progress in the study of adipose cell differentiation has been rapid over the past six years. Among the major goals which can be foreseen are:

1) The better characterization and the purification of factors from serum and organs which are required for the adipose conversion of different preadipocyte cell lines. This study should help to establish the existence of a relationship, if any, between levels of adipogenic factors and physiopathological situations leading to changes in the development of adipose tissue.

2) The use of chemically defined media developed for preadipocyte cell lines in order to select and to grow primary and secondary cultures of stromal-vascular cells of adipose tissue. This approach should allow to obtain cell strains of preadipocytes devoid of other cell types, as already described for other tissues (99).

3) Further studies on cell lines and cell strains regarding the process of differentiation and the acquisition of the 'susceptible' state. It would be of interest to know whether or not the transition from insusceptible to susceptible state is a similar or a different mechanism from that observed in the commitment of cells during hematopoiesis. These studies, should be helpful for a better understanding on the nature of the initial signals which trigger the adipose conversion.

4) Investigations on the irreversibility of the adipose conversion are of interest since one cannot exclude that fully differentiated cells, which do not normally divide, may be able to do so after 'dedifferentiation' and 'reprogramming'. If it were the case it would help to understand, at least partly, the origin of the pool of adipocyte precursors involved in the hyperplasia of adipose tissue observed at the adult age in animals and in man.

5) More extensive studies on the synthesis and the degradation of key enzymes of adipose conversion regulated by insulin and lipolytic hormones, may reveal new perspectives in the modulation of these processes. The use of somatic cell genetics would be of interest in this respect by allowing the introduction of mutations into the lipogenic pathways of preadipocyte cell lines. The study of the resulting effects on adipose conversion would be of interest as well.

Acknowledgments

The author is greatly indebted to Drs P. Djian, C. Forest, D. Gaillard, P. Grimaldi, G. Serrero-Davé, C. Vannier and to Prof. R. Négrel for helpful discussions and criticism. The author wishes to thank Mrs. G. Oillaux for expert secretarial assistance.

This work was supported in part by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Délégation Générale à la Recherche Scientifique et Technique and by the Fondation pour la Recherche Médicale.

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Received March 18, 1982.