Differentiation and function of rat adipocyte precursor cells in primary culture

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Abstract Precursor cells to adipocytes were purified from the epididymal fat pads of small rats and studied in primary culture. A culture system in which substrate and cofactors were not rate-limiting for complete adipocyte conversion was used by utilizing an agarose feeding-layer. Detachment of cells from the culture dish was prevented by addition of a viscous layer of culture medium, containing methyl cellulose. This sytem allowed quantitation and definite characterization of formed adipocytes, defined as cells accumulating a lipid droplet >20 μm in diameter. The cells could be subcultured but then gradually lost their adipocyte conversion ability. Age of the donor depressed the adipocyte conversion which, however, never seemed to stop completely. Prostaglandin E_1 and $F_{2\alpha}$ had no definite effect in the physiological concentration range while indomethachin possibly had a weak inhibitory effect. Insulin, heparin, and isobutylmethylxanthine increased adipocyte formation. Development of characteristic adipocyte functions with time was examined. Lipoprotein lipase activity was very low in the isolated precursor cells before culture, but developed in culture at confluence and was a thousand-fold higher within a few days. At this peak lipoprotein lipase activity was 50-fold higher than in mature adipocytes from the same donor animal. Triglyceride synthesis from glucose peaked in parallel but never reached the value of mature adipocytes and very little fatty acid was synthesized. Hormone-sensitive glycerol release developed at confluence and reached the level of activity of mature adipocytes. This study and previous work have indicated a role for the cyclase system in the development of adipocytes from precursor cells. Dibutyrylcyclic AMP caused an enhancement of lipoprotein lipase activity and adipocyte conversion. In suspension media, the nucleotide caused inhibition. These results are compatible with an effect of the nucleotide, not directly on lipoprotein lipase and cell determination, but via events taking place at confluence associated with cell to cell interactions. In comparison with previously described cells from an established cell line which undergo adipose conversion (3T3 cells), the cells described in the present work, like adipocytes, showed more metabolic activity in pathways for fatty acid incorporation from exogenous lipid sources (lipoprotein lipase activity) than from de novo synthesis. Furthermore, host-factors could be followed such as in the age- and site-dependence of adipocyte formation. Physiological stimuli such as insulin, lipid substrate, and heparin had effects on adipocyte formation. It was therefore concluded that this cell preparation has a potential of yielding information of physiological significance in studies of the regulation of adipocyte multiplication.—**Björntorp, P., M. Karlsson, P. Pettersson, and G. Sypniewska.** Differentiation and function of rat adipocyte precursor cells in primary culture. *J. Lipid Res.* 1980. **21:** 714–723.

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Recent work on the development of adipose tissue has demonstrated important differences in experimental obesity syndromes in the experimental animal (1). Both genetic and exogenous factors are involved. In obese man the development of an enlarged fat depot by the formation of an abnormal number of adipocytes is associated with obesity which seems difficult to treat successfully by ordinary therapy (2, 3).

In order to understand regulatory processes involved in the development of an enlarged fat depot, cells have to be isolated for study before they have developed to mature adipocytes that apparently do not multiply. Such cells have now been described (4–7) although all these cells can not be regarded with certainty as adipocyte precursor cells. It is necessary to demonstrate that these cells actually develop into adipocytes of typical morphology and function (8) and this has apparently not been done in all the above-mentioned reports.

From an established cell line from the Swiss mouse embryo, the 3T3 line, Green and Meuth (4) have isolated sublines of cells which accumulate triglycerides and form adipocytes. The functional characteristics of these cells have been studied in detail, i.e., activities involved in the accumulation of exogenous lipids as well as in the synthesis of fatty acids (9).

From the epididymal fat pad of small rats a cell

Abbreviations: IBMX, isobutylmethylxanthine; DBCAMP, dibutyrylcyclic AMP.

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fraction can be isolated that develops nearly quantitatively to adipocytes (10). These cells seem homogenous in primary cultures. They may not be comparable to the sublines obtained from the 3T3 cells, which are obtained from an established cell line, where in the process of subculturing, functional characteristics and development of regulation processes, etc. might well have been different from those occurring in vivo. Therefore, it seems important to characterize further the cells obtained in primary culture described here.

In the present study two aspects of the characterization of the precursor cells have been addressed. First, factors promoting the conversion of the precursor cells to adipocytes have been examined; second, the process of triglyceride accumulation has been studied. For the first study, methodological developments were necessary. When adipocyte development is studied it is important to ascertain that adipocytes are actually the end product. Characteristics such as density (10), total triglyceride content per culture (4), or lipid granulation (5) might be misleading particularly in quantitative measurements. It was therefore considered necessary to establish the number of adipocytes formed by actually counting the cells during the developmental phase. This was made possible by using a viscous culture medium and an agarose feeding layer which permitted full development of adipocytes under conditions where quantitative measurements could be performed.

EXPERIMENTAL PROCEDURES

Cell isolation

Male Sprague-Dawley rats of different ages were kept at a room temperature of about 22°C. The smaller rats were kept up to ten in each cage, while the larger rats were caged singly. The animals were given tap water and a commercial rat chow ad libitum. The diet (EWOS, Södertälje, Sweden) contained by weight 5% fat, 55% carbohydrate, and 22.5% protein, plus minerals and vitamins. The smallest rats were also given boiled potatoes.

The rats were kept in the laboratory at least 3 days before they were killed by exsanguination under ether anesthesia. The fat depots were removed aseptically. Epididymal fat pads were removed by cutting close to the epididymis. Retroperitoneal and abdominal fat depots were removed as previously described (11) and scapular fat was removed by dissecting fat from the back at the level of the scapulae.

Cells were isolated as previously described in detail (10). In short, cells were liberated with collagenase,

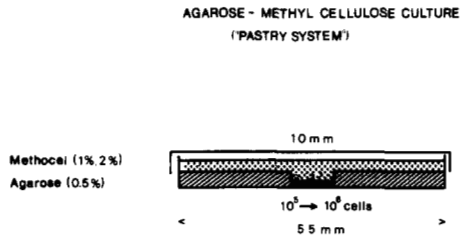

Fig. 1. The "pastry" system for culture of cells for quantitation of adipocyte conversion. Agarose (0.5%) (striped area) with a 10 mm diameter center well, containing about 10^6 cells (about 10^5 cells added) on which medium containing 2% Methocel (0.2 ml) (densely pointed area) was added. Another 5 ml of 1% medium containing 1% Methocel added on top (thinly pointed area). For other details, see "Experimental procedures."

and adipocytes were removed by flotation. Cell aggregates, consisting mainly of endothelial cells,[3] were separated off by filtration, and the filtered cells were then pelleted for further culture.

Cell culture

Cells were cultured in plastic Petri dishes (55 mm in diameter) containing medium 199 supplemented with glucose, insulin, a triglyceride emulsion, serum, and an antibiotic (cephalotin) as previously described (10). In these ordinary culture systems medium was exchanged every second day. Cultures were kept at 37°C in an atmosphere of 5% CO_2 in air saturated with water.

When quantitative conversion of cells to adipocytes was followed, cells were cultured with an agarose feeding layer (**Fig. 1**). Agarose (Type II, Sigma, St. Louis, MO) in distilled water (10 g/liter) was heated and stirred in a boiling water bath until it was dissolved. The solution was then autoclaved, and cooled to about 37°C. This solution was then diluted with concentrated medium 199, serum, triglyceride emulsion, insulin, and cephalotin to obtain the desired concentrations of constituents (10) and a final agarose concentration of 0.5% (w/v). Eight ml of the agarose medium was poured into a 55 mm diameter plastic Petri dish which was then cooled at 4°C until the contents congealed. In the center, a round well (1 cm^2) was punched out with a stainless steel ring; 10^5 cells were suspended in 0.1 ml supplemented medium 199, placed in the central well and allowed to form a monolayer, which usually occurred within 2 days. Thereafter the medium in the well was exchanged with 0.2 ml of a similar medium, made viscous with methyl cellulose (Methocel, Dow Chemical Co., Midland, MI) to a concentration of 2% (w/v). This layer was viscous

[3] Björntorp, P., M. Karlsson, P. Pettersson, and G. Sypniewska. Unpublished results.

enough to prevent the cells from detaching from the bottom during development. Another layer of less viscous medium (1% Methocel, 5 ml) that could be exchanged, was added on top of the previous layer and the agarose feeding layer. This culture system then formed a "pastry" with a total of about 10^6 cells in monolayer in the well, about 75 μmoles of glucose and about 30 μmoles of triglycerides in serum lipoproteins and in added triglyceride emulsion. The volume of serum was 2.5 ml. About one-third of these amounts was in the exchangeable top layer of the Methocel-containing medium and was thus possible to renew.

Cell counting

All cells within an area defined by a net in the eyepiece of the microscope were counted in ten randomized sections of the monolayer. Cells were considered to have developed into adipocytes when they contained a triglyceride vacuole which was >20 μm in diameter. The number of adipocytes is given in percent of total cells.

Cells were also counted in a Fuchs-Rosenthal blood cell counting chamber with 0.2 mm depth and $^1/_{16}$ mm^2 area after they were removed from culture by incubation for 10 min at 37°C in 0.125% (w/v) trypsin (Sigma, Type I, St. Louis, MO) in 0.15 M phosphate buffer, pH 7.0. When indicated, cells were removed with collagenase-containing medium in the same way as cells were liberated from the fat tissue, but with an incubation time of 10 min.

Cell function

Incorporation of [U-^{14}C]glucose into triglycerides and liberation of glycerol in the basal state and in the presence of norepinephrine (5×10^{-5} M) was measured as previously described (10). Lipoprotein lipase was assayed as described by Nilsson-Ehle, Tornquist, and Belfrage (12). Before the latter measurements, the culture medium in the Petri dish was exchanged with 2 ml of another medium containing 5% (v/v) fresh human serum and 1 IU heparin (Heparin, Vitrum, Stockholm, Sweden) in medium 199 for elution of lipase from the cells. Assay of lipase was performed after 10 min of elution. This time did not cause any significant inactivation of lipase in the elution medium as tested in separate experiments. The cells contained <5% of eluted lipase immediately after this elution. Thereafter lipase was assayed after 10, 20, and 30 min in new heparin-containing elution media.

Addition of 0.5 M NaCl or protamine sulfate (1 mg/ml) resulted in an inhibition of activity by 90 and 60%, respectively. Omission of serum in the assay system reduced activity by at least 80%. These characteristics suggest that the lipase activity assayed was that of lipoprotein lipase (13).

When metabolic activities were controlled in the "pastry" system, the agarose surrounding the central well plus the Methocel-containing medium in the central well could be removed en bloc leaving the cell monolayer on the bottom. These cells could then be tested for metabolic activities, as described above, or triglyceride contents.

Very low density lipoproteins were isolated from fresh human serum by ultracentrifugation at d 1.006 g/ml in a Beckman preparative ultracentrifuge. The top portion of the tube after centrifugation was dialyzed against 0.145 N sodium chloride and then concentrated to appropriate triglyceride concentration in a macrosolute concentrator (Minicon, Amicon Co., Lexington, MA). Triglycerides were determined according to Carlson (14).

Insulin antibodies from guinea-pig (kindly supplied by Pharmacia, Uppsala, Sweden) were tested for their inhibition of insulin action in the system described previously (10) where effects of added insulin on triglyceride synthesis from labeled glucose is determined. Antibodies equivalent to the inhibition of the action of at least 1000 μU of insulin were used in the experiments reported in the Results section. Isobutylmethylxanthine (IBMX) was from Aldrich-Europe (Beerse, Belgium); prostaglandin E_1 was obtained from Professor Sune Bergström, Karolinska Institutet, Stockholm, Sweden; prostaglandin $F_{2\alpha}$ was from Upjohn, England; indomethacin was from Merck, Sharpe & Dohme (Rahway, NJ); dibutyryl-cyclic 3′,5′ adenosine monophosphate (DB-CAMP) (monosodium salt) was from Sigma, St. Louis, MO; and norepinephrine (noradrenalin) was from Apoteksbolaget, Stockholm, Sweden.

RESULTS

Testing of culture conditions

In the "pastry" system, the total amount of glucose and triglyceride substrate was in about 10-fold excess at the beginning of culture if all cells in the monolayer were to fill up to a diameter of 20 μm. Nevertheless, at least each 4th day the top layer was exchanged to make certain that substrate or cofactor deficiency was not a reason for the failure of cells to develop into adipocytes. Adipocytes appeared with time in a sigmoid curve. In the experiment in **Fig. 2,** no more adipocytes appeared after about 11 days of culture. Exchanging medium at day 10 did not prevent leveling off of the curve.

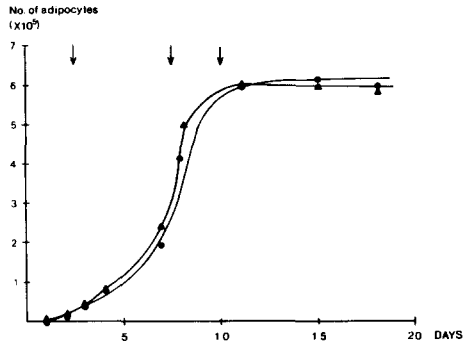

Fig. 2. Duplicate samples cultured in "pastry" system. Medium containing 1% Methocel exchanged at arrows. For further experimental details, see Experimental procedures section.

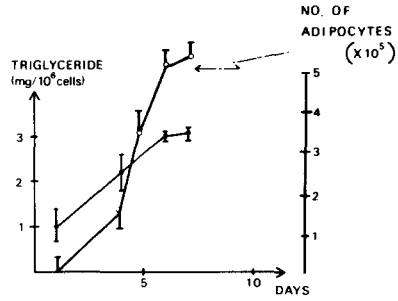

Fig. 4. Accumulation of triglyceride and appearance of adipocytes in the central well of the "pastry" system containing cells from the same original pool. Each observation point is the mean ± SEM of four to seven observations. Substrate as in "pastry" system; medium not exchanged.

In cultures without substrate saturation, addition of increasing amounts of triglyceride in the form of either a triolein emulsion or isolated very low density lipoproteins resulted in not only an increased rate of formation of adipocytes but also in an increased number of adipocytes formed (**Fig. 3**).

The technique selected for registration, viz. counting adipocytes with a triglyceride droplet > 20 μm in diameter, will by necessity cause a sigmoid curve of adipocyte development. Measurements of triglyceride contents of the cells in monolayer showed an apparently linear curve (**Fig. 4**) with time. This suggested zero-order kinetics and that substrate and cofactors were not rate-limiting for adipocyte formation in the "pastry" system.

Selected functional characteristics of cells cultured for 7 days in the "pastry" system are shown in **Table 1.** In comparison with adipocytes, the triglyceride synthesis was lower but lipoprotein lipase considerably higher. The activities correspond to those seen in cells of the same degree of development in the monolayer system without agarose and Methocel (see section on Development of cellular function).

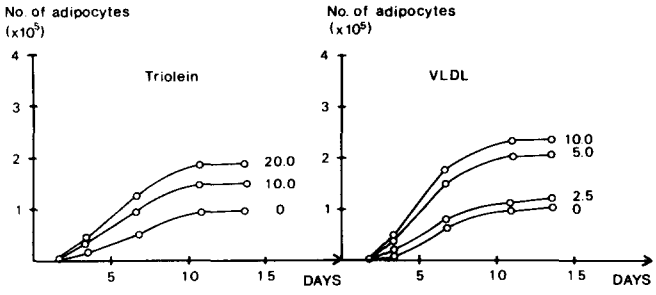

Fig. 3. Dependence of the formation of adipocytes in culture on triglyceride contents of culture medium. Left panel: culture in ordinary "pastry" system without serum but with an equivalent of VLDL-free serum added. Triolein triglyceride (Intralipid) (mg) present as indicated. Right panel: culture in ordinary "pastry" system without serum and triolein (Intralipid) but with an equivalent VLDL-free serum added. VLDL triglyceride (mg) present as indicated.

Subculture of cells

In these experiments cells were cultured in the ordinary system in primary and subsequent subcultures. In parallel, 10^5 cells were also inoculated in the "pastry" system to evaluate the remaining potential of the cells to adipocyte conversion. It was possible to subculture the cells through at least ten passages, but as seen in **Table 2,** after the first subculture the number of adipocytes became gradually smaller. This occurred after removal of cells by both trypsin or collagenase. The number of cells inoculated in the "pastry" system seemed to be of importance as well because when the inoculated cell number was split 1:2 still fewer adipocytes were found. Addition of IBMX (0.5 mM) to the system did not change the results (not shown).

Influence of age on adipocyte conversion

Table 3 shows the influence of age of the donor rat on the formation of adipocytes in the "pastry" system. In the smallest rats about half of the cells were converted spontaneously to adipocytes. With age

TABLE 1. Functional characteristics of cells cultured for 7 days in system with agarose feeding layer under methyl cellulose-containing medium

	Adipocytes[a]	Cultured Cells	Cells Before Culture
Triglyceride synthesis from [U-^{14}C]glucose (μmol/10^8 cells per hr)	2.8 ± 0.3[b]	1.1 ± 0.3	0.5 ± 0.2
Lipoprotein lipase activity (mU/10^8 cells)	42.0 ± 5.0	315.0 ± 32.0	2.0 ± 1.0

[a] Diameter: 31 ± 3 μm.
[b] Mean ± SEM; n = 4.

TABLE 2. Results of subculture on adipocyte conversion of cells

	No. of Experiments	Primary Culture	Subcultures				
			1	2	3	4	5
		(%)			(%)		
Subculture ratio 1:1	3–7	44 ± 3[a]	57 ± 2	26 ± 4	25 ± 2	8 ± 1	0
Subculture ratio 1:1 (collagenase)	2	45,47	45,61	27,42	27,11		
Subculture ratio 1:2	6	59 ± 2	29 ± 7	15 ± 2			
Subculture ratio 1:2 (collagenase)	4	47 ± 3	27 ± 3	12 ± 3	6 ± 1	<2	

[a] Means ± SEM.

About 10^5 cells inoculated in primary cultures and subsequent subcultures (Subculture ratio 1:1). Transfer of cells before monolayer formation with trypsin or (where indicated) with collagenase. In experiments with subculture ratio 1:2 the number of inoculated cells was halved between each transfer. Parallel cultures in system with agarose feeding layer under Methocel-containing medium to evaluate potential of cells for adipocyte conversion. Cultures performed until the number of adipocytes had leveled off (>12 days). Results expressed as number of adipocytes/total cells (%) in the agarose system.

this spontaneous conversion decreased, and in the oldest rats was about half that in the youngest animals.

There seemed to be a regional difference (**Table 4**). A decrease of spontaneously developing adipocytes in the epididymal fat region was observed in older rats, but this phenomenon was less pronounced in the perirenal region where spontaneous formation of adipocytes with age seemed to remain essentially unchanged. The results, in cells from these regions, seemed to be in contrast to the findings in the subcutaneous adipose tissue regions (abdominal and scapular). At the age of 28 days fewer adipocytes were found here and in the 84-day old rats essentially no adipocytes appeared.

Influence of various agents in vitro on adipocyte conversion

It is seen in **Table 5** that insulin was necessary for maximal adipocyte conversion, and that only low concentrations of insulin are needed. IBMX showed a significant but, on the average, a limited effect. The stimulatory effect seemed to be more pronounced when the spontaneous adipocyte conversion was low, as shown in one experiment in the table. Heparin showed a clear effect, apparently maximal at 5 IU/ml.

PGE_1 also showed an effect while this was not the case with $PGF_{2\alpha}$. Indomethacin probably produced a small but significant inhibition at higher concentrations (**Fig. 5**).

Development of cellular function

Fig. 6 summarizes the development of functions for triglyceride accumulation and hydrolysis in the cells that are converted to adipocytes. These cultures were performed in ordinary media without agarose or Methocel. From very low activities of lipoprotein lipase (Cf. Table 1) there was an increasing level, apparently starting at confluence of the cells as a monolayer on the bottom of the culture dish. At this point the activity of the cultured cells had exceeded that found in adipocytes developed in vivo in the donor rat. A further increase occurred up to a peak level at day 6 where the activity was much higher than in adipocytes, and about 1000-fold higher than in cells before culture. After peaking, the activity decreased again and this occurred while the cells detached from the bottom of the culture dish. Lipase activities present in the cells when heparin elution was performed, or having developed during a 30-min incubation, probably representing newly synthetized enzyme (15), showed essentially the same development.

Synthesis of triglycerides followed the same pattern

TABLE 3. Influence of the age of the donor rat on the adipocyte conversion of isolated cells from the epididymal fat pad

Rat age (days)	18	28	47	110	180
Number of rats	5	8	9	7	3
Adipocytes/total cells (%)	56 ± 3[a]	48 ± 1	42 ± 2	28 ± 1	27 ± 5

[a] Means ± SEM.

Cultures performed until the number of adipocytes had leveled off (>12 days).

TABLE 4. Influence of the age of the donor rat on the adipocyte conversion of isolated cells from different adipose tissue depots

Rat age (days)	28	42	84
Abdominal	23,22,24	18,19,17	2,4,3
Scapular	22,19,23	15,16,16	2,3,3
Perirenal	48,36,37	40,33,29	42,28,31
Epididymal	47,34,39	38,24,23	26,22,22

Cultures performed until the number of adipocytes had leveled off (>12 days). Results expressed as adipocytes/total cells (%); n = 3.

TABLE 5. Influence of various agents on the conversion of cells to adipocytes

	Percent	Number
Control (without added insulin)	42 ± 3	3
+ antiinsulin	15 ± 2	3
+ insulin (100 uU/ml)	41 ± 3	3
+ insulin (1000 uU/ml)	42 ± 3	3
Control	40 ± 2 (33)	4 (1)
+ IBMX (0.5 mM)	45 ± 1 (54)	4 (1)
Control	44 ± 3	3
+ heparin (1 IU/ml)	61 ± 2	3
+ heparin (5 IU/ml)	68 ± 2	3
+ heparin (10 IU/ml)	55 ± 3	3

Control system containing supplemented medium 199 (5 mM glucose, 40 mU insulin, 20% (v/v) serum, 0.5 (w/v) of triglyceride emulsion) except in first experiment where control system contained only the endogenous insulin of the serum added (giving a final concentration of 5 uU/ml). Anti-insulin added in excess, neutralizing the effect of 1000 μU/ml insulin on triglyceride synthesis from glucose in adipocytes. Means ± SEM. IBMX experiment with low spontaneous fat cell development given separately (within parentheses). Cultures performed until the number of adipocytes had leveled off (>12 days).

as that of lipoprotein lipase, but here the peak activity did not reach that in adipocytes. The majority of the glucose label incorporated into the triglycerides was found in the glycerol moiety (>80%), and consequently only a small part was in the fatty acids.

The development of activity responsible for glycerol release followed the same general time-curve as lipoprotein lipase activity. Catecholamine sensitivity

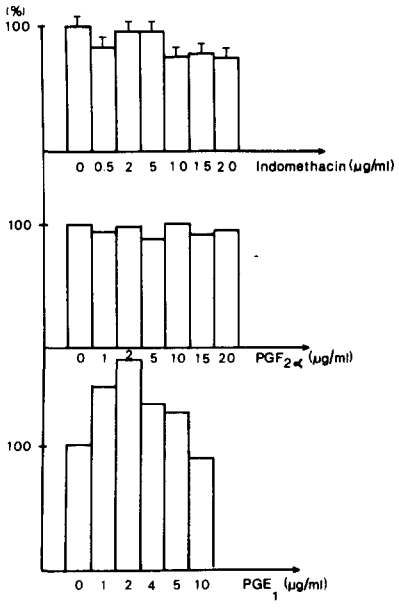

Fig. 5. Conversion of cells to adipocytes in the presence of indomethacin (n:4), $PGF_{2\alpha}$ (n:2) and PGE_1 (n:2). Results expressed as averages in relation to control experiments (4). Means or means ± SEM.

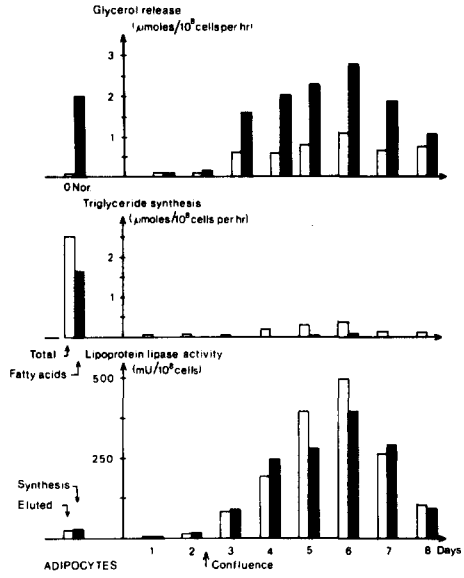

Fig. 6. Development of functions in cells converting to adipocytes. Comparison with adipocytes from the same donor animal (mean diameter: 28 ± 2 μm, left). Top panel: glycerol release without (open columns) and with 5×10^{-6} M norepinephrine (filled columns). Middle panel: incorporation of [U-^{14}C]glucose into triglycerides (open columns) and in triglyceride fatty acids (filled columns). Lower panel: lipoprotein lipase activity in initial heparin eluate (open columns) and in eluate after another 30 min incubation (synthesis, filled columns). For other experimental details, see Experimental procedures section. Approximate time for formation of confluent monolayer indicated. One representative experiment out of three to seven performed.

seemed to develop at confluence. The peak activity was of the same order as that in adipocytes.

Fig. 7 shows a similar experiment with the ordinary amount of insulin added to the endogenous insulin in the serum in the culture system compared with a system with no insulin added and the serum insulin neutralized with an excess of insulin antibodies. It is evident that insulin is necessary for the full development of lipoprotein lipase although activity appears also in the absence of insulin. In analogy to the experiments with the "pastry" system (Table 5) the formation of adipocytes and lipid accumulation was hampered in these experiments, and the cells detached early from the bottom of the culture flask in the presence of anti-insulin.

Influence of DBCAMP on lipoprotein lipase and adipocyte development

Previously described experiments (10), as well as results presented above, indicated that the cyclase system is involved in the formation of adipocytes. Addition of dibutyrylcyclic AMP to the culture system was therefore examined. **Fig. 8** shows that this resulted in lipoprotein lipase activity which seemed to develop earlier than in the system without addition. More

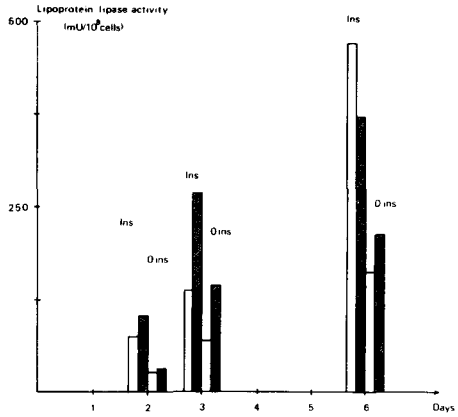

Fig. 7. Development of lipoprotein lipase activity in cells converting to adipocytes: influence of insulin. Parallel cultures with ordinary amounts of insulin (40 mU/ml) plus endogenous insulin in serum (3 uU/ml) (left pair of columns), and without added insulin (only endogenous insulin in serum, 3 uU/ml) plus excess of insulin antibodies (right pair of columns). Lipase in initial heparin eluate (open columns) and in eluate after another 30 min incubation (synthesis, filled columns). For other experimental details, see Experimental procedures section. One representative experiment out of three performed.

adipocytes appeared here also (not shown). It could not be decided from these results whether this enhancement of lipoprotein lipase and adipocyte development was due to a direct influence on cell determination or via an effect on the time for confluence. Contact inhibition of multiplication at confluence in this system does not occur simultaneously in the whole culture. At the beginning of culture before confluence, a cell clone thus contains all stages of cell development with a resting state after contact inhibition and the beginning of triglyceride accumulation in the center of the clone, while in the periphery of the clone, cells are still dividing.

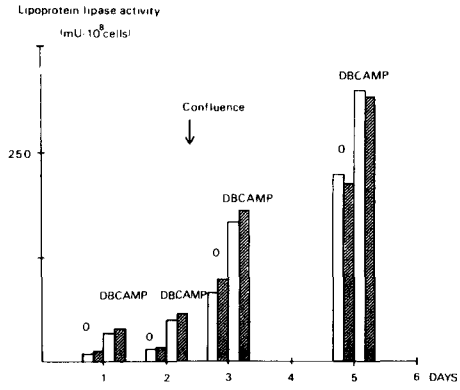

Fig. 8. Development of lipoprotein lipase activity in cells converting to adipocytes: influence of dibutyryl cyclic AMP (DBCAMP). Parallel cultures without (left pair of columns) and with 1 mM DBCAMP (right pair of columns). Lipase in initial heparin eluate (open columns) and in eluate after another 30 min incubation (synthesis, filled columns). For other experimental details, see Experimental procedures section. One representative experiment out of three performed.

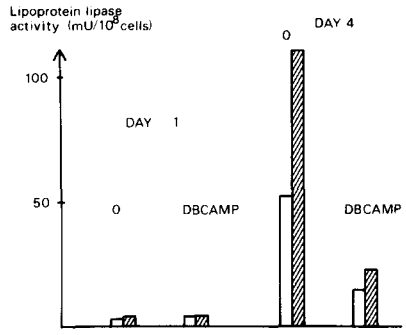

Fig. 9. Influence of dibutyrylcyclic AMP (DBCAMP) on lipoprotein lipase activity in cells in suspension media. Cells first cultured without DBCAMP during 1 day or to a stage after confluence (4 days) and then removed by trypsin and cultured for another 2 days in suspension media containing methyl cellulose with or without 1 mM DBCAMP. Lipase in initial heparin eluate (open columns) and in eluate after another 30 min incubation (synthesis, filled columns). For other experimental details, see Experimental procedures section. One representative experiment out of three performed.

To study further the effect of dibutyrylcyclic AMP, experiments were performed in suspension cultures where cell to cell interactions could essentially be avoided. Cells obtained from cultures during one day without DBCAMP were tested in suspension cultures with and without DBCAMP for 2 days. Here, essentially neither lipoprotein lipase activity developed in either of the systems nor any adipocytes (**Fig. 9**). Similarly, cells obtained after total confluence (day 4) in cultures without DBCAMP were cultured for 2 days with and without DBCAMP in media containing Methocel in order to keep cells suspended. Here the presence of DBCAMP caused an inhibition of lipase activity (Fig. 9).

DISCUSSION

The cell culture system

In the previous reports (8, 10), adipocyte conversion was evaluated tentatively by analyzing the distribution of cells according to their density. The lowest density class was suggested to consist of adipocytes or cells converting to adipocytes. A full development of cells to adipocytes was difficult to follow in this system because the cells detached from the bottom of the culture flask preventing quantitative morphological analysis. Furthermore, it was not possible to examine these cells functionally. The present "pastry" system was worked out to solve these problems. Detachment was prevented, and a large part of the culture dish supplied a limited number of cells with

substrate. In this system substrate and cofactors were probably not rate-limiting for the formation of adipocytes. Furthermore, as shown in Table 1, functional characteristics of these cells demonstrated that their triglyceride synthesizing capacity and lipoprotein lipase activity corresponded approximately to that at a similar degree of development without Methocel in the medium (Fig. 6). The lipoprotein lipase activity exceeded that of mature fat cells from the same donor rat while triglyceride synthesis was about half. In terms of average fat cell size (16), this rate of triglyceride synthesis was, however, probably not lower than that in the mature fat cells because the size of the cultured cells was less than half of that of the isolated adipocytes. These results show that the cells are not only converted efficiently to adipocytes in the "pastry" system, but they also fill up with triglyceride rapidly and have intact functional characteristics during this development.

The assay method in the "pastry" system allows only mature adipocytes to be registered because the criterion is that the cells should contain a triglyceride droplet > 20 μm to be counted. This qualification probably gives a high specificity for the assay system, and the risk for errors caused by counting cells other than fat cells should be small. On the other hand it is quite possible that this high specificity caused a too conservative, quantitative estimation of adipocyte formation. Cells with a cloudberry-like appearance, with the cytoplasm filled with several triglyceride vacuoles, were thus not counted as fat cells. The main purpose of the examinations of various agents in the present work was to test their qualitative effect conclusively, and therefore this approach, with a high selectivity of the assay method, was chosen. It is likely that from a quantitative point of view the formation of adipocytes was underestimated. This probably explains the lower percentage figures for adipocyte conversion caused by PGE_1 and IBMX in the present work as compared with the previous report (10). Furthermore, clearly a large number of preadipocytes were never expressed fully to adipocytes, within the definition used.

Subculture of cells

The previous report (10) indicated that the cells forming adipocytes can be subcultured repeatedly. This has also been reported with other cell preparations accumulating lipid (4, 5). In the present work this was confirmed. It turned out, however, that even if the cells multiply readily through passages they continuously lose their ability to be converted to adipocytes in the process of subculture. This has also been reported for the adipose sublines of the 3T3 cells (17). These results demonstrate the importance of an analysis of remaining cells of this type through subculturing.

Influence of age on adipocyte formation

Previous work has shown that with age there is a decrease in the tendency to spontaneous formation of adipocytes in preparations from the epididymal fat pad of rats; here IBMX could reveal a residual ability to cell multiplication with age (10). The "pastry" system presumably allows a more complete expression of spontaneous adipocyte development than the culture conditions previously utilized. This probably explains why in the present work there still seems to be a low but significant formation of adipocytes also in older rats. Apparently this residual ability to form new adipocytes varies in different regions. It seems to essentially disappear with age in the two subcutaneous regions tested, but remains in visceral depots, particularly in the retroperitoneal depot where the decrease with age is only discrete. It is of particular interest in this connection that Lemonnier (18) previously demonstrated the residual capacity of older rats, after fat feeding, to form new adipocytes in the retroperitoneal region. Faust et al. (19) have recently noted a similar finding in the male Sprague-Dawley rat where the number of fat cells increased by almost a factor of two. New fat cells were also found in other regions. Our previous report (10) also demonstrated the remaining ability of adipocyte formation in older rats after IBMX stimulation. It is noteworthy that the substrate for lipid filling in the "pastry" system is mainly fatty acids, in analogy with the results of fat feeding in the report of Lemonnier (18). This study seems to permit the tentative conclusion that new fat cells probably can be formed without an age-limit in the rat, particularly in certain adipose tissue regions. Whether there is a substrate specificity for such a development remains to be further elucidated.

Effect of various agents on adipocyte formation

In the present and previous work (10), the presence of IBMX, PGE_1, cholera toxin, and DBCAMP promoted the formation of adipocytes. These findings indicate that the cyclase system is involved in the adipocyte conversion process. Detailed tests were performed by the addition of DBCAMP to the cells in culture. An increase in lipoprotein lipase activity and adipocyte conversion was found. These results are compatible with either an effect of DBCAMP directly on the cell mechanism for adipocyte determination and lipoprotein lipase development, or a similar effect mediated via events associated with

contact inhibition of multiplication at confluence. In further experiments, culture in suspension media ruled out contact inhibition phenomena. Here DBCAMP did not increase lipoprotein lipase activity or adipocyte conversion in cells obtained essentially before confluence, and in cells obtained after confluence, an inhibition was seen. These results are compatible with an effect of DBCAMP, not directly on cell determination, but via events taking place at confluence associated with cell to cell interactions. This then is in accordance with similar effects of the cyclase system reported in other cell systems (20).

Development of functional characteristics

The striking increase of lipoprotein lipase was parallel in time with increases in triglyceride synthesis from glucose and triglyceride hydrolysis activity. The lipoprotein lipase activity "exploded" by a factor of 1000 times the activity of the precursor cells upon isolation, amounting to about 50 times the activity in mature fat cells. The glucose incorporation in triglycerides was much lower in relative terms and, furthermore, occurred mainly in the glycerol moiety of the triglyceride. It may well be that this increase was secondary to the large increase in lipoprotein lipase activity, which provides fatty acids for triglyceride formation within the cell (21). No doubt the development of the system for assimilation of exogenous fatty acids (lipoprotein lipase activity) is much more pronounced in these cells than the pathways for de novo fatty acid synthesis from glucose.

It is noticable that triglyceride hydrolysis became hormone-sensitive at the time of confluence. This indicates that the mechanism for hormonal regulation of triglyceride hydrolysis is created at this time-point. This also seems to be the case with insulin receptors.[3] These problems are currently being analyzed.

Several agents stimulating lipoprotein lipase activity thus seemed to be followed by cell conversion to adipocytes. This suggests a close connection between the formation of lipoprotein lipase and the conversion of cells to adipocytes. Furthermore, the tremendous increase in lipoprotein lipase activity, apparently beginning at the same time as the cells are starting to be converted to adipocytes, also suggests such an association.

Eckel, Fujimoto, and Brunzell (22) and Wise and Green (23) have recently studied lipoprotein lipase activity in the 3T3 lines collecting triglyceride. At confluence, activity began to develop and peaked at a level of about 40 times that measured before confluence. Insulin was necessary for its full development. Qualitatively these results then are in agreement with the present report. Quantitatively, however, the activity peak seems much more pronounced in the present work. This might be due either to the difference in cells or to the fact that the culture system used in the present work is saturated with lipid substrate. The pronounced dependence on the de novo fatty acid synthesis pathway for the adipose conversion of the 3T3-sublines also suggests differences in characteristics between these cells and those studied in the present work in primary cultures, where very little triglyceride fatty acids are accumulated from glucose. In this respect the cells studied here are more similar to the mature adipocyte (24).

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