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Adipose Conversion of 3T3-L1 Cells in a Serum-free Culture System Depends on Epidermal Growth Factor, Insulin-like Growth Factor I, Corticosterone, and Cyclic AMP*

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A culture system for 3T3-L1 preadipocytes based on a serum-free chemically defined medium containing fetuin, transferrin, and pantothenate is described. In this system, adipose conversion depends on the following conditions. 1) In the presence of high insulin concentrations (1 μM), addition of corticosterone together with 1-methyl-3-isobutylxanthine (MIX) for not more than the first 4 days after confluence to the culture medium induces maximal adipose conversion within 12–14 days. MIX may be replaced by forskolin or permeable analogues of cAMP, indicating that its effect is due to elevated cellular cAMP levels. 2) At low insulin concentrations (1 nM), adipose conversion is reduced. Growth hormone or insulin-like growth factor I together with epidermal growth factor have to be present as a medium supplement together with corticosterone and MIX to get maximal adipose conversion. 3) The induction of adipose conversion by corticosterone and MIX in the presence of either high insulin concentrations or insulin-like growth factor I together with epidermal growth factor is accompanied by postconfluent mitoses. Inhibitors of DNA replication markedly reduce adipose conversion. Fibroblast growth factor and platelet-derived growth factor, although acting as potent mitogens on 3T3-L1 cells, do not support adipose conversion induced by corticosterone and MIX.

The use of 3T3 preadipocytes originally cloned by Green and Kehinde (1, 3) and Meuth (2) for the study of adipocyte differentiation has been demonstrated repeatedly. One of the main features of this system is the dependence of the differentiation process, which includes the expression of a new adipocyte-specific set of proteins (4), upon the presence of "adipogenic" sera (5), mainly fetal calf serum, and high, supraphysiological insulin concentrations. Efforts to analyze the serum constituents responsible for its adipogenic action have shown that the differentiation process is influenced by certain hormones. GH[1] has a strong adipogenic activity in the 3T3-F442A (6–8) as well as in the 3T3-L1 (9) subclones. Glucocorticoids induce differentiation in 3T3-L1 cells (10–13), but seem to be rather inhibitory in 3T3-F442A cells (14). In addition, the cyclic nucleotide phosphodiesterase inhibitor MIX has been shown to potentiate the effects of glucocorticoids in 3T3-L1 preadipocytes (10–13). The elucidation of the molecular mechanisms of these hormones in inducing the differentiation of 3T3 preadipocytes has been hampered by the fact that 3T3 cells generally do not remain viable in serum-free culture systems. ob17 cells, another preadipocyte line, may be kept growing in a serum-free medium containing fetuin as a protein substitute (15). We have found that this medium not only supports growth of 3T3 preadipocytes, but is also suited for study of the influence of hormonal and growth factors on the differentiation process. Here, we report that in this serum-free culture system, adipose conversion is induced by the simultaneous presence of corticosterone and elevated cAMP concentrations. However, both hormonal factors are active exclusively during postconfluent mitoses, which have to be triggered by EGF together with IGF-I, and not by PDGF and FGF.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Induction of Adipose Conversion of 3T3-L1 Cells by 1-Methyl-3-isobutylxanthine and Corticosterone—Adipose conversion of 3T3-L1 preadipocytes kept in a chemically defined serum-free medium depends on the addition of a specific set of hormones to confluent cells (Table 1). When, in the presence of 1 μM insulin, corticosterone is added to cells together with MIX during the first 4 days after confluence adipose conversion is started. This process may be estimated by the observation of lipid accumulation or by determination of glycerophosphate dehydrogenase activity, which is a marker enzyme for lipogenesis (Table 2). MIX can be substituted with dibutyryl cyclic AMP or forskolin (Table 3).

Fig. 1 summarizes the time course of adipose conversion under these conditions. Glycerophosphate dehydrogenase activity rises after a lag phase of 4 days. At that time, corticosterone and MIX as the inducing agents have already been removed from the medium. The activity obtained after 14 days is ~80% of the maximal activity, which is reached after ~20 days (data not shown). Besides being able to induce differentiation of 3T3-L1 cells, corticosterone and MIX in combination have a mitogenic effect, as seen from the increase in protein and DNA levels. Again, both compounds are inef-

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Dedicated to Prof. Dr. O. H. Wieland on the occasion of his 70th birthday.

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[1] The abbreviations used are: GH, growth hormone; EGF, epidermal growth factor; IGF-I, insulin-like growth factor I; PDGF, platelet-derived growth factor; bFGF, bovine fibroblast growth factor; MIX, 1-methyl-3-isobutylxanthine; NCS, newborn calf serum; GPDH, glycerophosphate dehydrogenase; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 2 and 4, and Tables 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

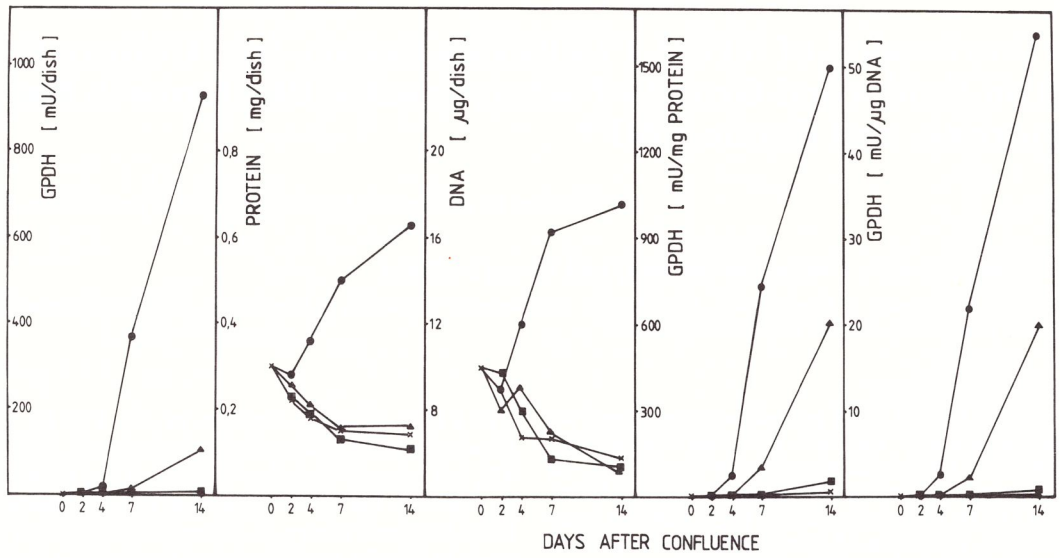

FIG. 1. **Development of glycerophosphate dehydrogenase activity and protein and DNA content in 3T3-L1 cells during adipose conversion.** 3T3-L1 fibroblasts were grown to confluence in NCS medium (see Miniprint). Adipose conversion was started after transfer to SFD medium containing 1 µM insulin. Glycerophosphate dehydrogenase (*GPDH*) activity and protein and DNA levels were measured as described under "Experimental Procedures." ×, control; ■, 0.1 µM corticosterone; ▲, 0.5 mM MIX; ●, 0.1 µM corticosterone and 0.5 mM MIX. Each point represents the mean of two to three experiments. Corticosterone and MIX were present only during the first 4 days after confluence.

fective when given alone. Under these conditions, during a culture period of 14 days, a decrease in cellular protein (28%) and DNA (31%) levels occurs, indicating some detachment of cells.

Role of Growth Factors in Adipose Conversion—Supraphysiological insulin concentrations are necessary for the induction of maximum adipose conversion in the presence of corticosterone and elevated cyclic AMP concentrations (Fig. 2). At physiological insulin concentrations, the conversion rate is greatly diminished, and growth hormone is able to relieve this reduction (Table 4). From this observation, one might suggest that mitogenic factors related to growth hormone are of importance for the conversion process. Fig. 3 gives the dose-response dependence of the adipogenic conversion on the presence of various growth factors added alone or in combination to the culture medium. Insulin (1 µM) could only be partially substituted with IGF-I in concentrations of 0.1–10 nM. EGF exhibited rather moderate ability in supporting adipose conversion. To obtain conversion rates comparable to those after treatment of cultures with corticosterone and MIX in the presence of high insulin concentrations, addition of EGF (0.8 nM) together with IGF-I is necessary. The maximal effective concentration of IGF-I is 5 nM.

Table 5 summarizes the effects of PDGF and bFGF on adipose conversion. In the presence of 1 nM insulin, neither PDGF nor bFGF was able to support adipose conversion when added to cultures instead of EGF and IGF-I. In addition, PDGF and bFGF had no influence on adipose conversion induced by corticosterone, MIX, and 1 µM insulin.

Besides being necessary for maximum adipose conversion, EGF was most useful in supporting optimum growth of 3T3-L1 preadipocytes in serum-free medium, even after repeated subcultures under serum-free conditions (Table 6).

Adipose Conversion and Postconfluent Mitoses—As in serum-containing media (23–25), postconfluent mitoses also occur during the initial stage of differentiation in a serum-free culture system (Fig. 4). The data summarized in Table 7 show that treatment of 3T3-L1 cells with aphidicolin during

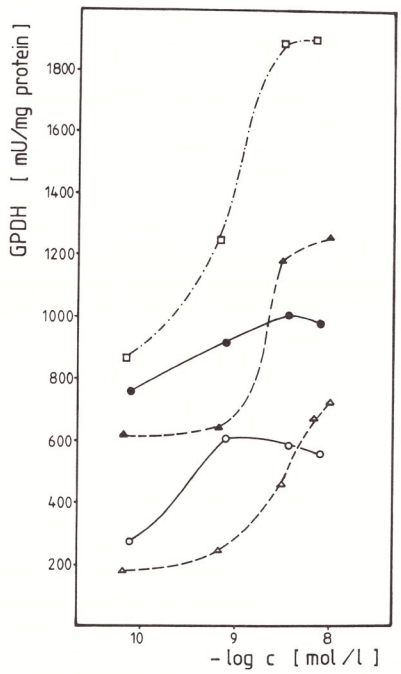

FIG. 3. **Dependence of adipose differentiation of 3T3-L1 cells on growth-promoting factors.** 3T3-L1 fibroblasts were grown to confluence in NCS medium. After 2 days in SFD medium without any additions, differentiation was started with corticosterone (0.1 µM) and MIX (0.5 mM) for the first 4 days. Growth factors were added at the same time at the concentrations indicated. △, IGF-I (days 0–4); ▲, IGF-I (days 0–4) and insulin (1 nM; days 0–10); ○, EGF (days 0–4); ●, EGF (days 0–4) and insulin (1 nM; days 0–10); □, IGF-I (days 0–4) and EGF (0.8 nM; days 0–10). Ten days after the start of adipose conversion, glycerophosphate dehydrogenase (*GPDH*) activities were measured. Glycerophosphate dehydrogenase activity measured under identical conditions, but with 1 µM insulin instead of growth factors, was in the range of 1400–1800 milliunits/mg of protein after 10 days.

TABLE 5

Influence of PDGF and bFGF on adipose conversion of 3T3-L1 cells

3T3-L1 cells grown to confluence in NCS medium were transferred to SFD medium containing either 1 nM or 1 μM insulin. Adipose conversion was started with 0.1 μM corticosterone and 0.5 mM 1-methyl-3-isobutylxanthine (CM) for 4 days in the presence or absence of PDGF or bFGF as indicated. Glycerophosphate dehydrogenase (GPDH) activity was determined 8 days after the start. Values represent the means of three independent experiments.

Additions	Specific GPDH activity
	milliunits/mg protein
1 nM insulin	
Control	
Only PDGF (0.7 nM)	36.3
Only bFGF (2.0 nM)	87.9
Only CM	217.1
CM + PDGF	
0.1 nM	356.4
0.7 nM	281.4
1.0 nM	183.9
CM + bFGF	
0.3 nM	108.2
2.0 nM	187.3
6.0 nM	315.5
1 μM insulin	
CM	1052.3
CM + PDGF (0.7 nM)	
Days 0–4	865.0
Days 0–8	873.1
CM + bFGF (20 nM)	
Days 0–4	1129.3
Days 0–8	876.7

TABLE 6

Adipose conversion of 3T3-L1 cells grown to confluence in serum-containing and serum-free media substituted with EGF

3T3-L1 cells were grown to confluence either in SFG medium (without serum) or in NCS medium (with serum). After transfer to SFD medium, adipose conversion was started as indicated. The concentrations used were: 0.1 μM corticosterone, 0.5 mM MIX, 0.8 nM EGF, and 7 nM IGF-I. 12 days after the start of adipose conversion, specific glycerophosphate dehydrogenase (GPDH) activity was determined. The ability of 3T3-L1 cells to undergo adipose conversion was retained for at least three subcultures in serum-free medium, with each subculture starting with a cell density of 1000–2000 cells/cm^2.

Additions	Specific GPDH activity in cells grown to confluence	
	Without serum	With serum
	milliunits/mg protein	
Corticosterone/MIX/insulin (1 μM)	1501.3	1571.0
EGF/insulin (1 nM)	47.4	32.9
Corticosterone/MIX/EGF/insulin (1 nM)	477.0	604.9
Corticosterone/MIX/EGF/IGF-I	630.3	1647.5
Corticosterone/MIX/EGF/IGF-I/insulin (1 nM)	1285.2	1542.5

the induction period by corticosterone and MIX not only abolishes [^{3}H]thymidine incorporation, but also markedly reduces adipose conversion as estimated from glycerophosphate dehydrogenase activity. When treatment of the cells with aphidicolin is performed before the addition of corticosterone and MIX, no inhibition of differentiation occurs. This indicates that aphidicolin under these conditions does not act by nonspecific toxicity. Similar results are obtained when, during treatment with corticosterone and MIX, replication is inhibited by hydroxyurea or deoxyadenosine.

Table 8 shows experiments carried out to obtain information on the relation between mitogenic activity of growth factors and their ability to support adipose conversion induced by corticosterone together with MIX. As expected, all growth factors were able to increase [^{3}H]thymidine incorporation when added to confluent cells which were made quiescent by culture in a serum-free medium without any hormones for 2 days. Addition of corticosterone and MIX as inducers of conversion led to an additional increase in [^{3}H]thymidine incorporation. However, this was observed only in cultures that had been treated with high insulin levels, IGF-I, or IGF-I together with EGF and that readily undergo adipose conversion (see Fig. 3). In the presence of either PDGF or FGF, corticosterone together with MIX were not only ineffective in

TABLE 7

Inhibition of glycerophosphate dehydrogenase development and [^{3}H]thymidine incorporation of differentiating 3T3-L1 cells by inhibitors of replication

3T3-L1 cells grown to confluence in NCS medium were transferred to SFD medium containing 1 μM insulin. After 2 days, adipose conversion was started by addition of corticosterone and MIX in the presence or absence of inhibitors of replication. 48 h later, medium was replaced by SFD medium containing insulin. Glycerophosphate dehydrogenase (GPDH) activity was determined 12 days after the start of conversion and is given as percent of the value obtained in cultures treated with insulin, corticosterone, and MIX. [^{3}H]Thymidine incorporation was measured 24 h after addition of corticosterone and MIX. Values represent the means of at least five independent experiments ± S.E. The following concentrations were used: 0.1 μM corticosterone, 0.5 mM MIX, 3 μM aphidicolin, 2 mM hydroxyurea, and 5 mM deoxyadenosine.

Addition	Corticosterone/MIX	GPDH activity	[^{3}H]Thymidine incorporation
		%	*cpm/dish*
None	−	6.8 ± 3.3	1113 ± 195
None	+	100	5505 ± 578
Aphidicolin	−	5.2 ± 1.6	265 ± 18
Aphidicolin	+	23.9 ± 6.8	398 ± 42
Aphidicolin[a]	+	82.3 ± 9.2	
Hydroxyurea	−	4.3 ± 0.7	125 ± 16
Hydroxyurea	+	15.6 ± 4.4	116 ± 22
Hydroxyurea[a]	+	84.5 ± 5.4	
dADo	−	4.2 ± 0.3	245 ± 31
dADo	+	10.4 ± 3.5	427 ± 103
dADo	+	84 ± 7.3	

[a] Cultures in which the inhibitors of replication were added during the first 48 h after confluence and then removed immediately before addition of corticosterone and MIX.

TABLE 8

Effect of corticosterone and MIX on [^{3}H]thymidine incorporation in 3T3-L1 cells stimulated with 1 μM insulin or growth-promoting factors

3T3-L1 cells grown to confluence in NCS medium were transferred to SFD medium. Two days later, the medium was replaced by SFD medium substituted as indicated. After 24 h [^{3}H]thymidine incorporation was measured as described under "Experimental Procedures."

Insulin	Growth factor	[^{3}H]Thymidine incorporation		
		No addition	With corticosterone/MIX	Difference
		cpm/dish		
1 μM	None	18,560	36,263	17,703
1 nM	None	3,324	5,578	2,254
1 nM	IGF-I	4,862	21,160	16,298
1 nM	EGF	4,789	6,470	1,681
1 nM	EGF, IGF-I	12,082	26,233	14,151
1 nM	PDGF	65,477	31,741	−33,736
1 nM	FGF	14,325	11,208	−3,117

inducing adipose conversion, but were also not able to increase [^{3}H]thymidine incorporation.

In the experiments shown in Fig. 5, confluent cells pretreated for 48 h with either high or low insulin concentrations together with EGF and IGF-I alone or in combination were used. Under these conditions, very low rates of [^{3}H]thymidine incorporation were observed, possibly due to down-regulation of receptors. Again, corticosterone and MIX were able to release postconfluent mitoses as measured from [^{3}H]thymidine incorporation. This increase was most prominent when conditions were chosen that supported adipose conversion most effectively.

DISCUSSION

As in the presence of serum (14), corticosterone together with MIX also very effectively induce adipose conversion in the serum-free culture system described here. However, as compared to serum-containing culture systems, much higher specific activities of glycerophosphate dehydrogenase are obtained within 12–14 days (Table 1), indicating that serum may also contain inhibitory "antiadipogenic" factors. As MIX can be replaced by forskolin or cyclic AMP analogues of high permeability (Table 3), it seems likely that its effect is due to its ability to elevate cellular cyclic AMP concentration. Neither corticosterone nor elevated cyclic AMP concentrations alone had any effect on differentiation. In addition, we were unable to induce differentiation by sequential addition of the inducing agents (Table 2). At least two different and simultaneous signals seem necessary to induce adipose conversion.

As in serum-containing culture systems, adipose conversion depends on the presence of insulin. To obtain optimum differentiation, insulin has to be added at a highly nonphysiological concentration. However, this is necessary only as long as conversion is induced by corticosterone and MIX (Fig. 2). During this time period, the events leading finally to differentiation take place. They include the changes in gene expression typical for adipocytes and force the cells to pass through further divisions which last approximately until day 8 or 10 after the start of conversion (Fig. 1).

The dependence of the conversion process on supraphysiological insulin concentrations could be explained by the assumption that insulin in high concentrations is acting via IGF-I receptors (26) and that differentiation depends on IGF-I. However, IGF-I alone had only a moderate effect on differentiation. A rate of adipose conversion comparable to that with high insulin concentrations was achieved only with IGF-I in combination with EGF (Fig. 3). It is not clear whether EGF acts only by improving the viability of 3T3 cells in serum-free culture or whether it has an additive effect on adipose conversion. From the data shown in Fig. 3, one may argue in favor of the latter assumption.

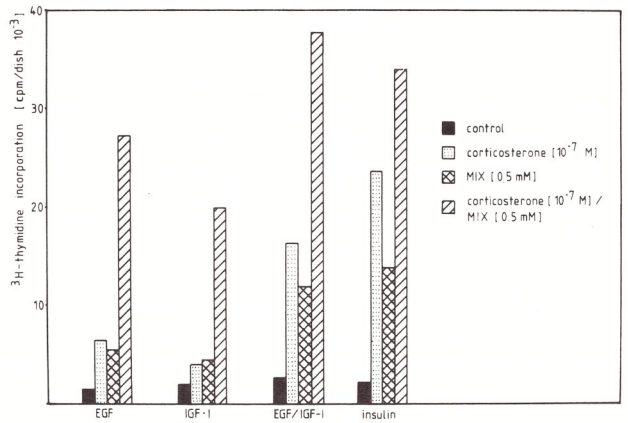

FIG. 5. **[^{3}H]Thymidine incorporation into 3T3-L1 cells pretreated with growth-promoting factors or insulin.** 3T3-L1 cells grown to confluence in NCS medium were transferred to SFD medium substituted with EGF (0.8 nM), IGF-I (7 nM), EGF and IGF-I, or insulin (1 μM). Two days later, the medium was replaced by fresh medium containing growth factors or insulin as described before plus corticosterone or MIX or both as indicated. [^{3}H]Thymidine incorporation was measured after 24 h as described under "Experimental Procedures." Each bar represents the mean of two independent experiments.

In addition, EGF was most effective in rendering possible a completely serum-free culture system for 3T3 cells. From a variety of serum constituents tested, it was by far the most potent not only in supporting the exponential growth phase necessary to obtain a confluent state of the cells, but also in preserving the ability of 3T3 cells to undergo adipose conversion (Table 6). These data strongly indicate that differentiation really depends on only defined hormonal factors. Any as yet unknown adipogenic compounds possibly introduced by previous treatment of cells with serum should be diluted completely after at least three subcultures.

GH induces the production of IGF-I in a variety of cells including ob17 preadipocytes (27, 28). If this were the case also in 3T3-L1 preadipocytes, one could explain the observation that GH promotes adipose conversion in serum-free culture only in the presence of low insulin concentrations and is totally ineffective at the high insulin concentrations able to activate the IGF-I receptor (Table 4). However, a direct action of GH on adipose conversion (8) as suggested in Refs. 29 and 30 cannot be ruled out.

Hayashi *et al.* (31) have demonstrated that in 3T3-F442A cells cultured with fetal calf serum, FGF as well as PDGF have a strong inhibitory action on adipose conversion. Similar results were reported with 10T½ cells (32, 33). Pretreatment of ob17 preadipocytes with serum-containing medium and with FGF inhibits later adipose conversion (15). In contrast to these reports, in the serum-free culture system described here, neither PDGF nor FGF had any effect on adipose conversion of 3T3-L1 cells. At low insulin concentrations, both growth factors were unable to induce differentiation in a manner similar to that of IGF-I, EGF, or a combination of both. At high insulin concentrations, no inhibition of the conversion was detectable (Table 5). Since in serum-containing cultures FGF and PDGF had only a weak effect, if any, it seems possible that adipose conversion of 3T3-L1 cells is not sensitive to both factors.

Smith *et al.* (26) have recently published an analysis of the effects of IGF-I on adipose conversion of 3T3-L1 cells. In cultures carried out in the presence of sera pretreated with an anion-exchange resin to remove insulin, IGF-I, and growth hormone, they clearly demonstrated that nanomolar amounts of IGF-I are equally potent in supporting adipose conversion as supraphysiological amounts of insulin and that adipose conversion does not depend on growth hormone. In contrast to our results, in their system, IGF-I alone was sufficient to support adipose conversion induced by dexamethasone and MIX. This discrepancy could be explained by the assumption that the resin treatment of fetal calf serum did not completely eliminate EGF.

Results reported by Guller *et al.* (34) are not in agreement with our data and those of Smith *et al.* (26). They presented evidence that in 3T3-F442A cells, growth hormone also has an adipogenic action in the presence of very high insulin and EGF concentrations. IGF-I was active as an adipogenic agent only in supraphysiological amounts, suggesting that GH is able to induce differentiation by a mechanism independent of

IGF-I. One could speculate that this discrepancy is due either to differences in the cell lines used (3T3-L1 *versus* 3T3-F442A cells) or to the pretreatment of the cells with cat serum during the exponential growth phase. In our hands, cat serum has a potent antiadiopogenic activity, which indeed can be relieved by growth hormone.

Until recently, it has been a matter of some controversy whether postconfluent mitoses are a necessary prerequisite for preadipocytes to undergo adipose conversion (16, 23–25, 36). The use of a serum-free culture system offers the possibility of answering this question more clearly. The observation that inhibition of DNA replication by various methods uniformly leads to reduction of adipose conversion suggests that the differentiation of 3T3-L1 cells is not only accompanied by postconfluent mitoses, but actually depends on these events (Table 7).

The three experiments shown in Figs. 4 and 5 and Table 8 corroborate this suggestion. They demonstrate that corticosterone and MIX in combination markedly increase [^{3}H]thymidine incorporation and that this effect depends on the presence of either high insulin concentrations or IGF-I together with EGF. This is true for cells studied during the decline of [^{3}H]thymidine incorporation immediately after transfer to SFD medium (see Miniprint) (Fig. 4) as well as for cells made quiescent either by treatment with low insulin concentrations or with IGF-I and EGF alone or in combination (Fig. 5 and Table 8).

The action of two other mitogens, PDGF and bFGF, is quite different. Both compounds have a strong mitogenic effect on confluent 3T3-L1 cells, but are not able to support adipose conversion induced by corticosterone and MIX. In addition, corticosterone and MIX are not able to enhance the mitogenic action of PDGF and bFGF. One may therefore speculate that besides DNA replication, some of the events related to the signal transmission mechanisms of mitogens may be important for adipose conversion. More recent reports have indeed shown that in 3T3-L1 cells, protein kinase C-dependent and -independent signal transmission pathways occur which are under the control of growth factors (37–40) and may possibly involve prostaglandins as suggested (35, 41). The use of the serum-free culture system described here should help to obtain more information on the mechanisms involved.

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Adipose Conversion of 3T3-L1 Cells in a Serum-free Medium

Supplemental Material to

ADIPOSE CONVERSION OF 3T3-L1 CELLS IN A SERUMFREE CULTURE SYSTEM
DEPENDS ON EGF, IGF-I, CORTICOSTERONE AND CYCLIC AMP

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EXPERIMENTAL PROCEDURES

Materials - IGF-I (rek., human), EGF (nat., mouse), bFGF (nat., bovine) were from Boehringer, Mannheim; PDGF bb was a gift from Prof. Hoppe (University of Würzburg). Insulin (bovine) and human growth hormone were kindly provided by Hormon Chemie, Munich; corticosterone, transferrin, fetuin, pantothenate, DME/Ham F12-medium were from Sigma, Munich; MIX and biotin were from Serva, Heidelberg;

Cell Culture Conditions and Media - 3T3-L1 cells were obtained from ATCC (Rockville, MD) and inoculated at 2 x 10^3 cells/cm^2 in 35-mm plastic dishes (Nunc, Roskilde, Denmark) in a humidified atmosphere of 5% CO_2 at 37° C. They were grown to confluence either in NCS-medium (DMEM containing 10% NCS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) or in SFG-medium (DMEM / Ham F 12 (3:1), penicillin (100 U/ml), streptomycin (0.1 mg/ml), fetuin (300 µg/ml), transferrin (2 µg/ml), pantothenate (17 µM), biotin (1 µM), EGF (0.8 nM) and insulin (1 nM). The medium was changed three times weekly.
Differentiation was induced as indicated in SFD-medium (DMEM / Ham F 12 (3:1), penicillin (100 U/ml), streptomycin (0.1 mg/ml), fetuin (300 µg/ml), transferrin (2 µg/ml), pantothenate (17 µM), biotin (1 µM).

Methods - GPDH activity was assayed spectrophotometrically (16, 17). 1 mU GPDH corresponds to the reduction of 1 nmole dihydroxyacetone phosphate/min. Protein was determined by a modified Lowry procedure (18) based on the results of Bensadoun and Weinstein (19) and Hess et al. (20). A BSA solution was used as standard. The DNA content of the cultures was determined fluorometrically (21, 22).
For the measurement of ^{3}H-thymidine incorporation into DNA, ^{3}H-thymidine was added in a concentration of 1 µCi/ml for 2 hrs. After removal of the medium cells were washed with 1.5 ml ice-cold phosphate-buffered saline. 400 µl thymidine solution (0.5 mM) were added and cells were lysed with 50 µl NaOH (1 M). After transfer into Eppendorf-tubes, DNA was precipitated with 500 µl ice-cold TCA (20% w/v). Labeled DNA was attached to cellulose nitrate filters and washed with TCA (10% w/v) and ethanol. After drying of filters in vacuum, radioactivity was measured.

RESULTS

Adipose conversion in a chemically defined, serumfree medium - When cultured in media supplemented with FCS and high insulin concentrations confluent 3T3-L1 preadipocytes undergo adipose conversion within 8-10 days. GPDH as a measure of differentiation usually reaches specific activities of 600-700mU/mg protein. When NCS is used instead of FCS adipogenic differentiation depends on a defined hormonal treatment of confluent cells (table 1). In the presence of 5% newborn calf serum and 1 µM insulin corticosterone effectively induces adipose conversion as estimated by its ability to increase GPDH-activity in differentiating cultures. MIX, which alone has only a limited influence on this process markedly enhances the effect of corticosterone. As has been shown repeatedly (6,7,8,9), GH is able to induce adipose conversion in cultures kept in media containing serum. There are a number of differences, when similar experiments are repeated with cells grown to confluence in NCS-medium and then transferred to serumfree SFD-medium (table 1, right column). GH, corticosterone as well as MIX alone are completely ineffective or have a rather modest effect. The conversion process is induced maximally only when corticosterone and MIX are added in combination. Under these conditions GPDH reaches specific activities of more than 1500 mU/mg protein. This value is significantly higher as compared to cultures differentiated in the presence of serum. It corresponds to values obtained with 3T3-F442A cells (6,7) and to the activities found in mature adipocytes. In cultures differentiated in the absence of serum, differentiated cells are homogeneously distributed. This is in contrast to cultures kept in the presence of serum, which exhibit clusters of adipocytes suggesting a clonal expansion of differentiating cells.
A maximal adipose conversion is obtained already, when corticosterone and MIX are present together for only 4 days after the cells have reached a confluent state (table 2). Reduction of this period to 1 or 2 days or successive additions of corticosterone and MIX result in markedly diminished conversion rates. The maximal effective concentration of corticosterone is 0.1 µM. Forskolin as well as permeable analogues of cAMP have the same efficiency in promoting adipose conversion in the presence of corticosterone as has MIX (table 3).
The induction of adipose conversion by the combination of corticosterone and MIX as described in table 2 and figure 1 depends on the presence of insulin in a supraphysiological concentration, which however has to be present in the culture medium only during the period, when corticosterone and MIX are present (figure 2). During later periods of differentiation high insulin is without effect on adipose conversion. In the presence of 1 nM insulin, adipose conversion as measured by induction of GPDH is considerably diminished.

The role of growth hormone for adipose conversion - As may be seen from the experiments shown in table 4 growth hormone has no adipogenic activity in cells stimulated to differentiate in the presence of 1 µM insulin. However, it is able to relieve the reduction of adipose conversion occuring when the differentiation is induced in the presence of low insulin concentrations.

Adipose conversion and postconfluent mitoses - In order to get more information on the events during the early stages of adipose conversion induced by corticosterone and MIX, ^{3}H-thymidine incorporation into DNA was measured (figure 4). After having reached a confluent state cells were transferred to SFD-medium containing 1 µM insulin. During the next 48 hrs. ^{3}H-thymidine incorporation decreased markedly. This reduction was relieved in cultures treated with corticosterone and MIX in addition to high insulin. When the incorporation rates obtained in SFD medium containing 1 µM insulin were subtracted from the incorporation rates in the presence of corticosterone/MIX, the curve shown in figure 4B is obtained, which suggests, that at least one round of postconfluent mitoses accompanies the induction of adipose conversion.

Table 1: Adipose conversion of 3T3-L1 cells in serum containing and serum free medium

Additions	Newborn calf serum (5%)	SFD-medium
--	18.9 ± 5.4 (7)	32.9 ± 8.1 (5)
corticosterone	286.1 ± 42.1 (7)	43.6 ± 4.7 (5)
human growth hormone	269.7 ± 69.3 (6)	54.7 ± 15.1 (4)
MIX	29.3 ± 7.2 (5)	115.5 ± 30.6 (4)
corticosterone/MIX	350.3 ± 58.0 (5)	856.8 ± 67.0 (9)

3T3-L1 cells grown to confluence in NCS-medium were transferred to either DME-medium substituted with 5% newborn calf serum or to SFD-medium each containing 1 µM insulin. Adipose conversion was started by addition of 0.1 µM corticosterone, 1 nM human growth hormone and 0.5 mM MIX for the first 4 days alone or in combination as indicated. GPDH activity was measured 8 days later. Values in mU GPDH/dish ± SEM. The figures in brackets give the number of individual experiments. The specific GPDH-activities obtained in the presence of corticosterone and MIX were 562.4 ±62.7 mU/mg protein in the presence of NCS and 1432.3 ± 124.8 mU/mg protein in serumfree cultures repectively.

Table 2: Influence of corticosterone and MIX alone or in combination on adipose conversion of 3T3-L1 cells

Additions [in days] MIX [0.5 mM]	Corticosterone [0.1 µM]	GPDH-activity [mU/mg protein]
------	------	53.6
------	0 - 14	92.0
0 - 2	------	135.8
0 - 2	0 - 2	962.8
0 - 4	------	412.5
0 - 4	4 - 14	460.0
0 - 4	0 - 4	1289.5
0 - 4	0 - 14	1360.9
4 - 6	------	85.7
4 - 6	0 - 4	390.0
4 - 6	0 - 14	920.3
0 - 14	0 - 4	1076.7

3T3-L1 cells grown to confluence in NCS-medium were transferred to SFD-medium containing 1 µM insulin. Corticosterone and MIX were added as indicated. 14 days after start of adipose conversion the specific activity of GPDH was determined. Values represent the mean of three individual experiments.

Table 3: Influence of agents increasing cellular cyclic AMP levels on adipose conversion of 3T3-L1 cells

Additions	---	corticosterone [0.1 µM]
----	58.2	130.9
MIX 0.5 mM	302.4	1002.0
Forskolin 1 µM	166.6	907.7
Forskolin 10 µM	193.7	960.9
db-cAMP 1 mM	318.9	1011.7

3T3-L1 cells grown to confluence in NCS-medium were transferred to SFD-medium containing 1 µM insulin. Additions were made as indicated during the first 4 days after confluence, GPDH activity was measured 8 days after confluence. Values represent the mean of 3 independent experiments and are given in mU GPDH/mg protein.

Table 4: Effect of human growth hormone on adipose conversion of 3T3-L1 cells

Additions	Insulin [1 µM]	[1 nM]
--	145.5 ± 60.7	51.8 ± 15.2
corticosterone/MIX	950.0 ± 88.6	485.0 ± 54.0 *
GH	113.0 ± 22.5	61.3 ± 18.7
corticosterone/MIX GH	1013.0 ± 157.0	887.2 ± 144.0 *

3T3-L1 cells grown to confluence in NCS-medium were transferred to SFD-medium containing hormones and MIX as indicated. GPDH activity was measured 8 days later. Values represent the mean of at least 5 independent experiments and are given in mU GPDH/mg protein ± SEM.
* difference statistically significant ($p < 0.05$)

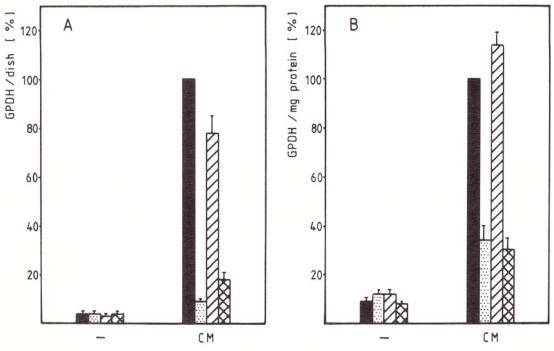

Figure 2: Dependence of adipose conversion of 3T3-cells on the medium insulin concentration.

Cells were grown to confluence in NCS-medium. After washing the cells with SFD-medium differentiation was started on day 0 by addition of 0.1 μM corticosterone and 0.5 mM MIX for the first 4 days (CM). In control cultures (-) corticosterone and MIX were omitted. Insulin (1 μM or 1 nM) was added during different time intervals as indicated. 14 days after induction, GPDH activities and protein content were measured. GPDH activity per dish (A) and specific GPDH activity (B) are given in % of the values obtained in cultures treated with corticosterone and MIX in the presence of 1 μM insulin. Each bar represents the mean of six independent cultures ± SEM. Black bars: insulin 1 μM, day 0-14; dotted bars: insulin 1 nM, day 0-14; hatched bars: insulin 1 μM, day 0-4 and insulin 1 nM, day 4-14; crossed bars insulin 1 nM, day 0-4 and insulin 1 μM, day 4-14.

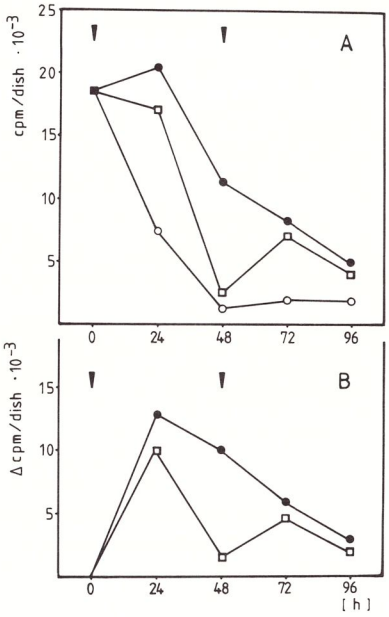

Figure 4: ^{3}H-thymidine incorporation in 3T3-L1 cells after start of adipose conversion.

Confluent 3T3-L1 cells grown in NCS-medium were transferred to SFD-medium and further additions were made as indicated. At the times indicated ^{3}H-thymidine incorporation was measured as given in methods. (○) insulin 1 μM (control); (□) insulin 1 μM, corticosterone 0.1 μM; (●) insulin 1 μM, corticosterone 0.1 μM, MIX 0.5 mM. Each point represents the mean of 3 independent experiments. Arrowheads mark medium changes. A: absolute values in cultures; B: difference to control cultures.