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Conversion of 3T3 fibroblasts into adipose cells: Triggering of differentiation by prostaglandin $F_{2\alpha}$ and 1-methyl-3-isobutyl xanthine

(triglycerides/3′:5′-cyclic AMP)

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ABSTRACT **Green and Kehinde [(1974) *Cell* 1, 113–116] have isolated clones of Swiss 3T3 fibroblasts that are able to convert to adipose cells. In this paper we report on two chemicals (prostaglandin $F_{2\alpha}$, 0.1 μg/ml, and 1-methyl-3-isobutyl xanthine, 0.5 mM) that are able to rapidly and irreversibly program the fibroblasts to differentiate into adipose cells. Confluent cultures treated with prostaglandin $F_{2\alpha}$ and insulin for 3–5 days, followed by insulin alone for 7–48 hr, yield numerous adipocyte colonies compared to control dishes and dishes treated with insulin alone. Cells treated with prostaglandin $F_{2\alpha}$ or 1-methyl-3-isobutyl xanthine alone, rinsed, and then exposed to insulin gave similar results, indicating that the continuous presence of the triggering agent is not required to elicit the conversion of the fibroblasts to adipocytes. Agents that raise intracellular levels of 3′:5′-cyclic AMP (dibutyryl cyclic AMP, 1.0 mM; 8-bromo-cyclic AMP, 0.5 mM; and prostaglandin E_1, 0.1 μg/ml) do not trigger the conversion process, suggesting that cyclic AMP may not be the mediator of differentiation in these cells. 8-Bromo-cyclic AMP, however, does induce the cyclic AMP phosphodiesterase (3′:5′-cyclic-nucleotide phosphodiesterase; 3′:5′-cyclic nucleotide 5′-nucleotidohydrolase; EC 3.1.4.17) in these cells; the induction appears to be mediated by increases in intracellular cyclic AMP levels. These results indicate that this cell line might offer a system for studying the regulation of a type of cellular differentiation.**

Green and his associates (1–3) have isolated a number of clones from the original stock of Swiss 3T3 fibroblasts that convert to adipose cells under defined culture conditions. This conversion is a type of cellular differentiation which occurs after the cells stop growing, at which time there is an increased incorporation of fatty acid precursors into triglycerides. The conversion is blocked by treatment of growing cultures with bromodeoxyuridine. The differentiated phenotype appears, by electron microscopy, to be morphologically similar to normal adipose cells (2). The conversion involves the formation of numerous fat vacuoles, which coalesce into one large fat vacuole with time. The mature adipocyte does not divide. Insulin stimulates the uptake of glucose and the incorporation of radioactively labeled glucose, acetate, and palmitate into triglycerides, while epinephrine and dibutyryl cyclic AMP decrease the triglyceride content (3). These 3T3 fibroblasts appear to offer an excellent model system for the study of a differentiation process using cell culture.

In this paper we report the ability of a natural compound (prostaglandin $F_{2\alpha}$)($PGF_{2\alpha}$) and a synthetic compound (1-methyl-3-isobutyl xanthine) (MeiBu-Xan) to trigger the differentiation process by rapidly and irreversibly programming the fibroblasts to differentiate into adipose cells.

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; MeiBu-Xan 1-methyl-3-isobutyl xanthine; $PGF_{2\alpha}$ and PGE_1, prostaglandins $F_{2\alpha}$ and E_1, respectively.

MATERIALS AND METHODS

The 3T3-L1 fibroblasts were obtained from Dr. H. Green, Massachusetts Institute of Technology, Cambridge, Mass., and were grown in Dulbecco-Vogt's modified Eagle's medium supplemented with 10% calf serum, penicillin (50 μnits/ml), and streptomycin (50 μg/ml). The cells were grown at 37° in a humidified 5% CO_2 atmosphere. Cells were fed every other day.

To promote the synthesis of triglycerides in the adipose cells, we treated confluent cultures with insulin (1 μg/ml) for 1–3 weeks. To test the effects of various agents on the adiose conversion we performed two types of experiments. In the first, confluent cells were treated with the agent plus insulin for various times (1–5 days) followed by removal of the agent and addition of insulin alone for 3 hr to 5 days. In the second type of experiment, confluent cells were treated with the agent alone for various times, the agent was removed, and insulin was added for various times.

To assay for conversion of 3T3-L1 fibroblasts into adipose cells, we rinsed the cells twice with cold phosphate-buffered saline, fixed them with a glutaraldehyde (2.45%)-sucrose (0.2 M) solution, and stained them for triglyceride using Oil-Red-O (1, 4). Visible colonies were counted. All experiments involved duplicate dishes. The dishes were photographed in a dark room by placing the dish on photographic paper and shining light directly onto the dish. Thus, the dark red adipocyte colonies appear as the light areas on the photograph.

Hydrolysis of 3′:5′-cyclic AMP (cAMP) was measured by the method of Wells *et al.* (5). Protein was determined by the method of Lowry *et al.* (6), with bovine serum albumin as standard. cAMP phosphodiesterase (3′:5′-cyclic-nucleotide phosphodiesterase; 3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase; EC 3.1.4.17) was induced as described by Russell and Pastan (7). Confluent dishes of 3T3-L1 were treated with either MeiBu-Xan or 8-bromo-cAMP for various times. The cells were homogenized and cAMP phosphodiesterase activity was determined.

Prostaglandins E_1 and $F_{2\alpha}$ were the kind gift of Dr. J. E. Pike, The Upjohn Co., Kalamazoo, Mich. MeiBu-Xan was obtained from Aldrich Chemical Co. and purified by recrystallization in ethanol. 8-Bromo-cAMP, dibutyryl cAMP, 8-bromo-cGMP, and dibutyryl cGMP were purchased from Boehringer-Mannheim Corp. Crystalline insulin was from Schwarz-Mann (24.5 units/mg). [G-^{3}H]cAMP (38 Ci/mmol) was obtained from New England Nuclear Corp. and purified by chromatography on AG 50-X8.

RESULTS

Formation of Adipocytes. 3T3-L1 fibroblasts appear morphologically similar to any clone of 3T3 fibroblasts before

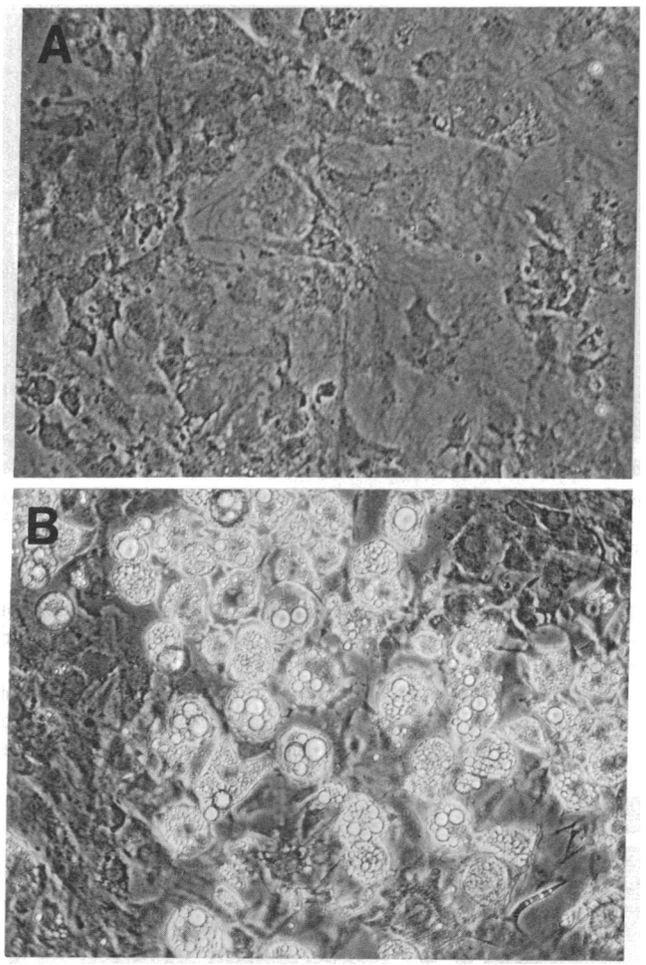

FIG. 1. (A) Phase contrast micrograph of a confluent dish of 3T3-L1 before conversion to adipocytes. Magnification ×155. (B) Phase contrast micrograph of a developing adipocyte colony. Magnification ×155.

conversion to adipose cells (1) (Fig. 1A). The cells exhibit density-dependent inhibition of growth which appears to be a requirement for the conversion into adipocytes (2). After treatment of the confluent cells with insulin for 2–3 weeks, numerous clusters of adipose cells develop (Fig. 1B) surrounded by fibroblasts. The colonial nature of the conversion is always quite evident. Initially one or two adipose cells can be seen in a given field, and a few days later a whole field will be comprised of adipose cells (1–3) (Fig. 1B).

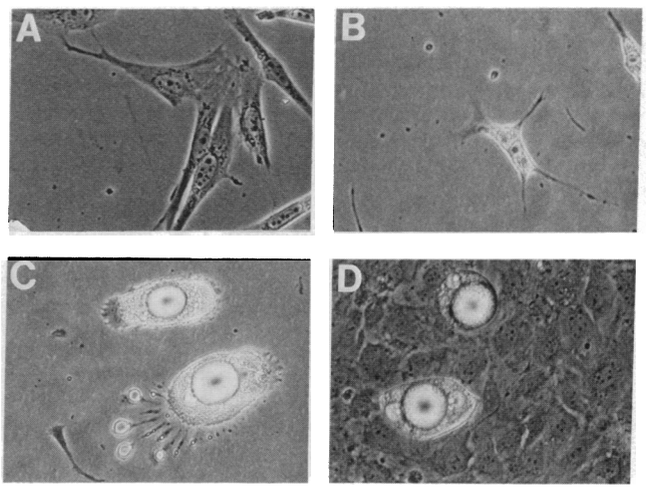

FIG. 3. Phase contrast micrograph of replated floating cells obtained by treatment of a confluent dish of 3T3-L1 with MeiBu-Xan (0.5 mM) plus insulin (1.0 μg/ml) for 6 days. Three types of cells are apparent: (A) fibroblasts; (B) newly differentiated adipose cells; (C) mature adipose cells. (D) Same dish after 6 days of feeding with serum (10%) plus insulin (1.0 μg/ml).

Effect of Cyclic Nucleotides on Adipocyte Formation. In an attempt to determine the effect of agents that alter intracellular cyclic nucleotide levels on the adipose conversion, cells were treated with insulin plus MeiBu-Xan. MeiBu-Xan is a potent inhibitor of cyclic nucleotide phosphodiesterase and can elevate intracellular cAMP and cGMP levels. These initial observations indicated that in the presence of MeiBu-Xan numerous adipocyte colonies developed within 8 days, while very few colonies were evident in dishes treated with insulin alone (Fig. 2A–C). In the early stages of the conversion the cells contain many small vacuoles of triglycerides. MeiBu-Xan at 0.5 mM is very effective in promoting the adipocyte conversion (Fig. 2C).

In dishes treated with MeiBu-Xan plus insulin for more than 4 days, cells began to detach from the dish. The floating cells could be replated by pouring the medium onto a new culture dish. Three populations of cells were evident in the replated dish: fibroblasts (Fig. 3A); newly differentiated adipose cells (Fig. 3B), which still retain a fibroblast appearance but contain many small fat vacuoles (1); and mature adipose cells (Fig. 3C),

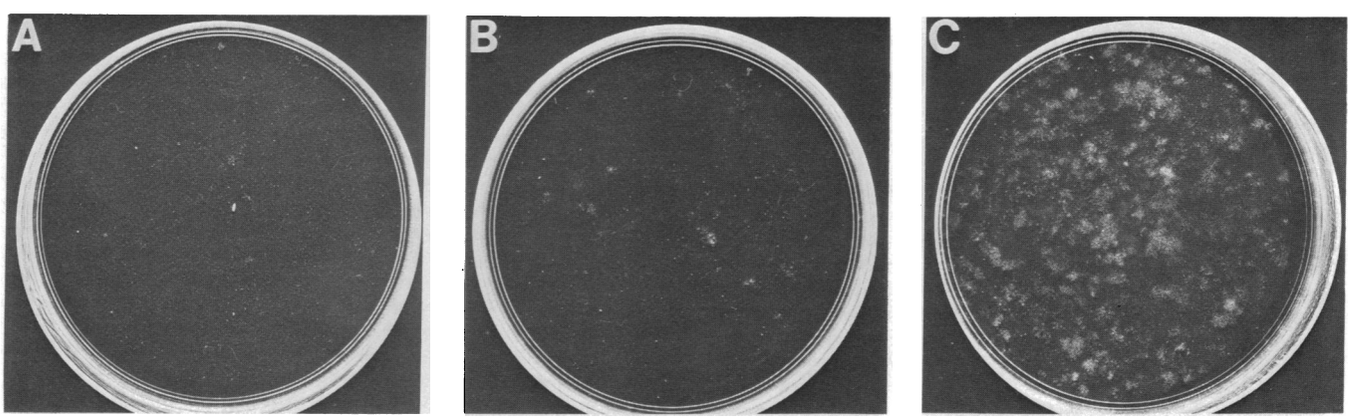

FIG. 2. Effect of MeiBu-Xan on the adipose conversion. Confluent dishes were treated with medium (A), insulin (1.0 μg/ml) (B), and insulin (1.0 μg/ml) plus MeiBu-Xan (0.5 mM) (C) for 8 days. Dishes were stained with Oil-Red-O and photographed. The dark areas appear as the light areas by this method.

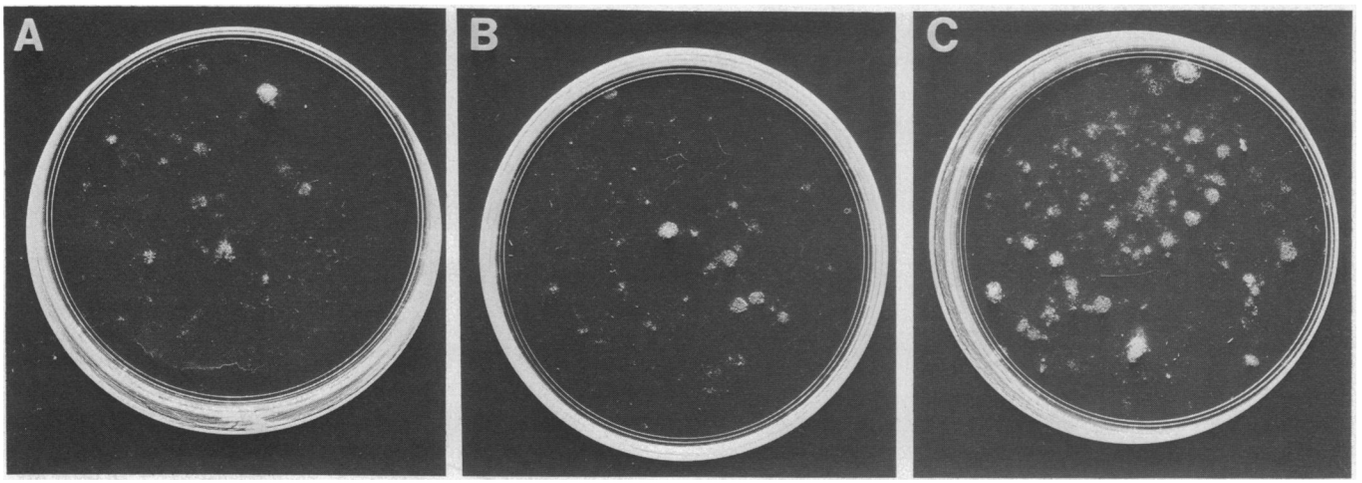

FIG. 4. Effect of $PGF_{2\alpha}$ on the adipose conversion. Confluent dishes were treated with insulin (A), insulin plus $PGF_{2\alpha}$ (0.01 μg/ml) (B), and insulin plus $PGF_{2\alpha}$ (0.1 μg/ml) (C) for 3 days, at which time all cells were fed with insulin alone for 6 days. Insulin is 1.0 μg/ml. Dishes were stained with Oil-Red-O and photographed. The dark spots appear as the light areas by this method.

which contain large fat vacuoles (1). Within a week, the fibroblasts grew to completely cover the dish surrounding the adipose cells (Fig. 3D). Floating cells were not evident in dishes treated with insulin alone. The reason for the floating phenomenon is not clear, but it appears to require at least a 4-day treatment with MeiBu-Xan plus insulin. To avoid this phenomenon, cells were treated with MeiBu-Xan plus insulin for 3 days, MeiBu-Xan was removed, and insulin was added alone.

Since MeiBu-Xan appeared to potentiate the conversion of 3T3-L1 fibroblasts to adipose cells, a number of other agents that have an effect on levels of intracellular cyclic nucleotides

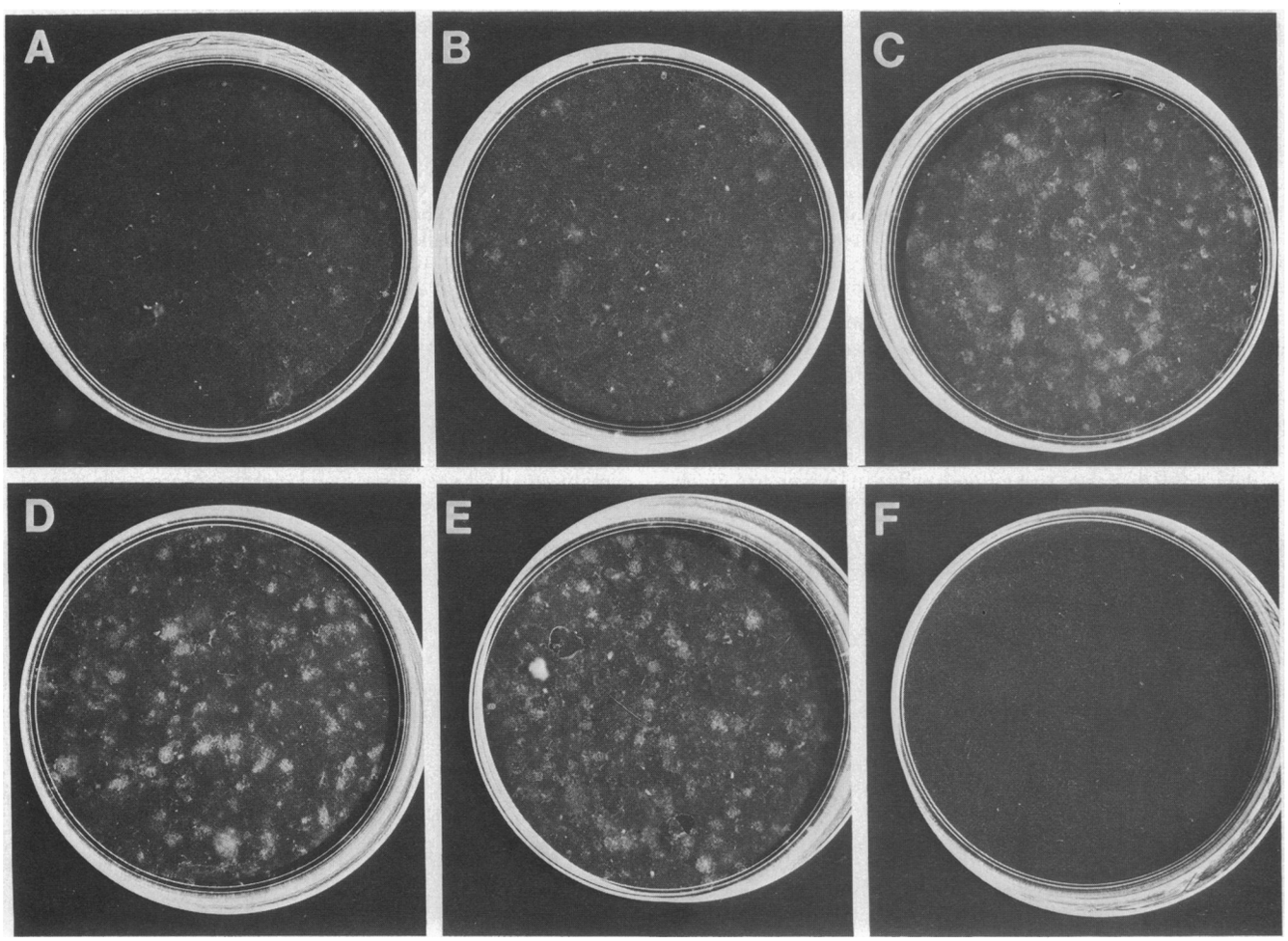

FIG. 5. Cells were treated with $PGF_{2\alpha}$ (10 μg/ml) plus insulin for 4 days. Medium was removed and medium with insulin alone was added ($t = 0$). (A) $t = 0$; (B) $t = 3.5$ hr; (C) $t = 7$ hr; (D) $t = 24$ hr; (E) $t = 48$ hr; (F) insulin alone at $t = 0$.

Table 1. Effect of various compounds on the conversion of 3T3-L1 to adipose cells

Compound	Concentration (mM)	No. of colonies*
None†	—	14
Dibutyryl cAMP	1.0	
+ caffeine	1.0	11
8-Bromo-cAMP	0.5	13
Dibutyryl cGMP	1.0	
+ caffeine	1.0	11
8-Bromo-cGMP	0.5	17
cGMP	0.17	16
MeiBu-Xan	0.5	73

* Average of duplicate dishes.

† All dishes contained insulin at 1.0 μg/ml. Cells were treated with the agent plus insulin for 4 days followed by insulin alone for 2 days.

were tested. These included dibutyryl cAMP, dibutyryl cGMP, 8-bromo-cAMP, 8-bromo-cGMP, cGMP, and caffeine. Results of these experiments are seen in Table 1. None of the agents tested appeared to effect the adipose conversion, with the exception of MeiBu-Xan. One possible explanation is that MeiBu-Xan is able to enter the cell and the other agents cannot. This was directly testable because MeiBu-Xan induces an increase in total cAMP phosphodiesterase activity (7–9). This effect of MeiBu-Xan is presumably due to its ability to inhibit the phosphodiesterase, which results in increased levels of cAMP. The resulting increased cAMP levels cause induction of the cAMP phosphodiesterase, most likely at the transcriptional level (7). The induction of the enzyme requires an active cAMP-dependent protein kinase (9). 3T3-L1 cells were treated with MeiBu-Xan (0.5 mM) or 8-bromo-cAMP (0.5 mM) for 5 days. The cells were homogenized and the whole homogenate was assayed for cAMP phosphodiesterase activity. Both MeiBu-Xan and 8-bromo-cAMP induced the enzyme 3.5-fold, indicating that 8-bromo-cAMP can increase the total activity of cAMP phosphodiesterase.

Effect of Prostaglandins on Adipocyte Formation. The effect of prostaglandin E_1 (PGE_1) and $PGF_{2\alpha}$ on the conversion of 3T3-L1 fibroblasts to adipose cells was investigated. Confluent cultures were treated with insulin plus $PGF_{2\alpha}$ for 3 days and then fed with medium plus insulin alone for 6 days (Fig. 4). The results indicate $PGF_{2\alpha}$ (100 ng/ml) is effective in promoting the adipose conversion. PGE_1 does not appear to be effective at 100 ng/ml. It is, however, effective at much higher concentrations (10,000 ng/ml, data not shown). Further studies with $PGF_{2\alpha}$ indicate that treatment with $PGF_{2\alpha}$ plus insulin for up to 100 hr does not produce a significant number of adipocyte colonies. However, numerous colonies develop over the next 48 hr with insulin alone (Fig. 5) or with insulin in the presence of $PGF_{2\alpha}$ (data not shown). The conversion appears to follow a sigmoidal rather than a hyperbolic response.

Irreversible Triggering of Differentiation. If MeiBu-Xan and $PGF_{2\alpha}$ are regulating differentiation by promoting the conversion of a fibroblast into an adipocyte, then both agents should be able to irreversibly trigger differentiation. To test this, we treated cells with $PGF_{2\alpha}$ at 0.1 and 1.0 μg/ml for 24 hr; the medium was removed and new medium plus insulin was added. Numerous adipocyte colonies developed in the dish that had been treated for 1 day with $PGF_{2\alpha}$ compared to a control, thus indicating that the continued presence of $PGF_{2\alpha}$ is not required to elicit the response (Fig. 6B and C). PGE_1 does not appear to have an effect (Fig. 6D and E). An irreversible triggering is also seen when cells are treated in this manner with MeiBu-Xan for

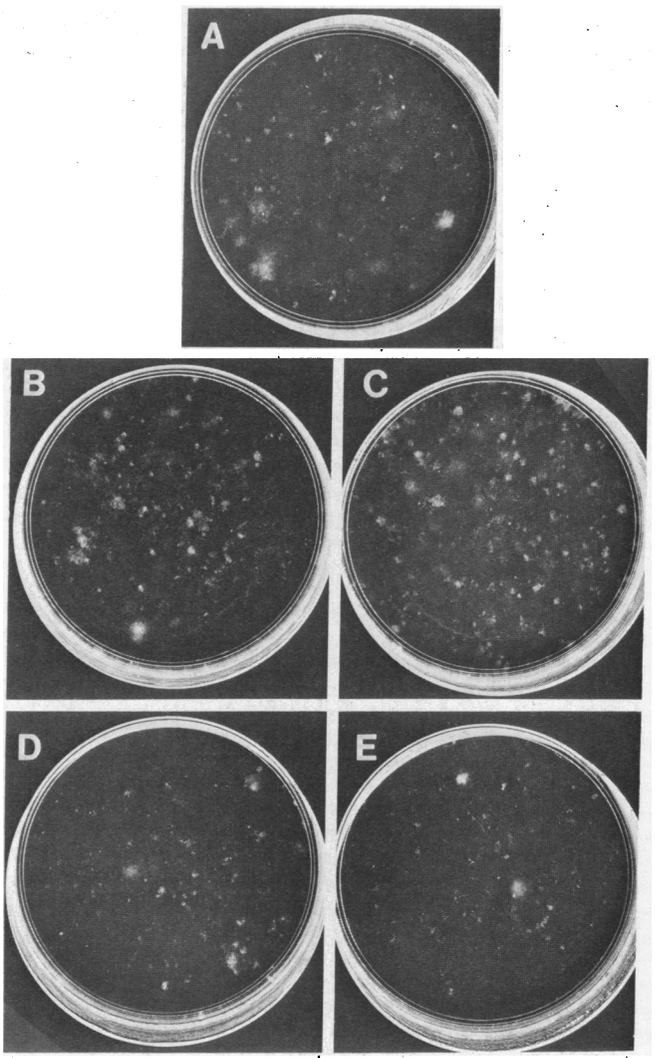

FIG. 6. Confluent dishes were treated with (A) medium; (B) $PGF_{2\alpha}$, 1.0 μg/ml; (C) $PGF_{2\alpha}$, 0.1 μg/ml; (D) PGE_1, 1.0 μg/ml; (E) PGE_1 0.1 μg/ml for 24 hr. The medium was then removed and the dishes were treated with insulin alone (1.0 μg/ml) for 100 hr. The cells were then fixed, stained, and photographed.

2 days (data not shown). Simple rinsing is sufficient to remove MeiBu-Xan from the cells, as the induced level of cAMP phosphodiesterase decays with a half-life of 70–80 min upon removal of MeiBu-Xan from the medium (7).

DISCUSSION

The fibroblast cell line isolated by Green and his associates (1–3) appears to offer an excellent experimental system for the study of a differentiation process under defined culture conditions. The adipose cells derived from fibroblasts in culture resemble normal adipose cells in many respects. Insulin stimulates triglyceride synthesis in the adipose cells in culture at concentrations approaching physiological levels (10 ng/ml) (3). Epinephrine and dibutyryl cAMP decrease the content of the triglycerides, and the cells are morphologically similar to normal adipose cells.

$PGF_{2\alpha}$ and MeiBu-Xan appear to irreversibly trigger the conversion of 3T3-L1 fibroblasts into adipose-like cells via a sigmoidal rather than hyperbolic mechanism. In the case of $PGF_{2\alpha}$, treatment with $PGF_{2\alpha}$ for 1 day or continued treatment with the trigger yields very few adipose cells for the first 100

hr, at which time adipose cells begin to appear. A maximal response is then seen within 48 hr. Similar results are seen in cells treated with MeiBu-Xan. The fact that neither MeiBu-Xan nor $PGF_{2\alpha}$ is continuously required for conversion indicates that these agents are able to promote a stable change in the phenotypic expression of these cells. The reason that PGE_1 is effective at 100 times the concentration of $PGF_{2\alpha}$ is not clear. Conceivably, the difference is due to the fact that PGE_1 can bind to the $PGF_{2\alpha}$ receptor, but at a much lower affinity (10).

The triggering of differentiation in this system may not be mediated by increases in intracellular cAMP levels. 8-Bromo-cAMP, dibutyryl cAMP plus caffeine, and PGE_1 do not appear to promote the adipose conversion. 8-Bromo-cAMP, however, does induce an increase in total cAMP phosphodiesterase activity. Induction of cAMP phosphodiesterase occurs upon increasing intracellular cAMP levels, and the induction requires an active cAMP-dependent protein kinase (7–9). Since $PGF_{2\alpha}$ has been reported to raise cGMP levels in fibroblasts (11), cGMP might be involved in the differentiation process. However, cGMP, 8-bromo-cGMP, and dibutyryl cGMP do not promote adipocyte formation at the concentrations tested. It is possible that MeiBu-Xan and $PGF_{2\alpha}$ are functioning entirely independent of the cyclic nucleotides. Further studies are required to ascertain the mechanism of action of MeiBu-Xan and $PGF_{2\alpha}$ in the conversion of 3T3-L1 into adipose cells.

When fibroblasts are treated with MeiBu-Xan plus insulin for more than 4 days, adipose cells begin to detach from the substratum. Recently, Green and Kehinde (12) have shown that prior to the adipose conversion the differentiating cells have highly extended cellular processes. As the adipose cells mature, the processes are retracted. If a mixture of MeiBu-Xan and insulin promotes this retraction process, then it is possible that the cells are stimulated to such an extent in the continued presence of MeiBu-Xan plus insulin that they easily detach from the substratum. The floating phenomenon is not seen with $PGF_{2\alpha}$, indicating that the detachment phenomenon is not a common feature of these triggering agents.

The fibroblasts isolated by Green (1–3) can be triggered to differentiate into adipose cells. Thus, this experimental system should be an aid in delineating the molecular mechanisms involved in a type of cellular differentiation.

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