Inhibition of Adipose Conversion in 3T3-L2 Cells by Retinoic Acid

Thomas Murray and Thomas R. Russell

Departments of Biochemistry and Medicine, University of Miami School of Medicine, Miami, Florida 33101

The role of retinoic acid in modulating the differentiation of 3T3-L2 fibroblasts into adipocytes has been examined. Results indicate that the retinoid is capable of effectively inhibiting the degree of adipose conversion which is brought about by treatment of preadipocytes with 1-methyl-3-isobutylxanthine plus dexamethasone. Morphological and enzymatic (fatty acid synthetase activity) expression of the adipose phenotype are both inhibited more than 90% by 10^{-6} M retinoic acid. The inhibition is concentration dependent with retinoic acid levels as low as 10^{-11} M capable of reducing adipose conversion by 20%. Retinoic acid must be administered simultaneously with the triggering agents to be effective. Exposure of nongrowing preadipocytes to retinoic acid does not alter the ability of the cells to differentiate in response to a subsequent treatment with methylisobutylxanthine plus dexamethasone. Further, the inhibition is reversible. Cultures in which methylisobutylxanthine plus dexamethasone triggered differentiation has been blocked by addition of retinoic acid (10^{-6} M) will readily undergo adipose conversion in response to a second treatment with methylisobutylxanthine plus dexamethasone in the absence of the retinoid. Similar inhibition of differentiation was found when cultures were treated with drugs in medium supplemented with either newborn calf serum or fetal calf serum. However, the extent to which methylisobutylxanthine plus dexamethasone are able to promote differentiation in these cells is considerably greater in medium containing fetal calf serum.

Key words: cytodifferentiation, dexamethasone, methylisobutylxanthine, fetal calf serum, retinoic acid, preadipocytes, adipose conversion

The conversion of 3T3-L2 fibroblasts into adipocytes in culture represents a convenient model system for the analysis of events which regulate cellular differentiation. The preadipocyte subclones isolated by Green and his associates [1—4] were derived from the original mouse embryo 3T3 fibroblast stock [5] and are similar to nondifferentiating 3T3 fibroblasts in many characteristics during exponential growth and approach to confluence. These include cell morphology, growth rate, sensitivity to density dependent growth inhibition and a high rate of collagen biosynthesis [2]. After preadipocyte cultures enter a

nongrowing state, a large proportion of the cells enter a differentiation program and exhibit a morphology characteristic of mammalian adipocytes. Expression of the new phenotype is marked by large increases in the synthesis and storage of triglyceride [3] as well as dramatic and coordinate increases in levels [6, 7] and activities [6—14] of numerous enzymes involved in lipogenesis and lipid metabolism. Adipose conversion is also accompanied by large increases in insulin receptor number [15—19] and the appearance of hormone sensitivities and responsiveness characteristic of adipocytes [3, 19—23].

Compounds which can alter the expression of the fatty phenotype can be divided into two classes based upon whether they regulate lipid accumulation in the differentiated cell or regulate a step in the differentiation process[1, 24]. Insulin falls into the first category as it stimulates the rate of lipid synthesis and accumulation in the differentiated cell [3]. Other examples include lipolytic agents such as L-epinephrine and N^6, $O^{2'}$-dibutyrylcyclic AMP [1] which effectively reduce the formation of intracellular lipid droplets. Also, if cultures are maintained under conditions where most of the fatty acids for storage must come from de novo synthesis and biotin is removed from the medium, little triglyceride accumulation takes place [12]. While these agents can have a dramatic influence on the amounts of lipid accumulated, they do not appear to be involved in the differentiation process which commits cells to adopt the new phenotype [1, 12]. Compounds of the second group are characterized by their ability to influence the proportion of cells undergoing adipose conversion in either a positive or a negative fashion. Kuri-Harcuch and Green [24] have shown that differentiation of 3T3 preadipocytes is dependent upon a nondialyzable serum factor which is present in cattle at highest concentrations before birth. Conversion of preadipocytes in the presence of the serum factor can be completely prevented if the cells are grown in medium containing 5-bromodeoxyuridine prior to reaching confluence [2, 25]. Short-term exposure of confluent cultures to prostaglandin $F_{2\alpha}$ (26), 1-methyl-3-isobutylxanthine (MIX) [25, 26], or MIX plus dexamethasone (MIX-DEX) [17] rapidly and irreversibly promotes the differentiation process, although little is known about the mechanisms through which they function.

One approach to studying the means by which agents like MIX and DEX influence the differentiation process is to examine the way in which other compounds alter the response to the triggering agents. Retinoids, including vitamin A alcohol and a variety of related compounds, are likely candidates for study in view of the established role they play in gene expression and differentiation in a variety of systems. They are essential for normal growth and development in several species of animals [27—29]. In rats, fetal requirements for vitamin A become especially critical from days 10 to 14 of gestation during a period of rapid tissue differentiation [29]. The mechanism of retinoid action remains yet uncertain, although much evidence suggests that interaction with specific cytosolic retinol or retinoic acid-binding proteins may be involved [30—32]. These two distinct proteins are present in nearly all embryonic tissues [30] and the pattern of organ distribution varies with the stage of development. Reports that the retinoid-binding protein complex is transported to the nucleus and interacts with DNA has led to the hypothesis that retinoids may be acting in a fashion analogous to steroid hormone action [31—36].

In view of the established influence of retinoids on differentiation in a variety of systems, we have tested retinoic acid for an effect on MIX-DEX promoted differentiation of 3T3-L2 fibroblasts into adipocytes. This article discusses the ability of low concentrations of retinoic acid to block the action of the triggering agents. The inhibition of differentiation observed with retinoic acid is seen only when it is administered together with MIX-DEX.

MATERIALS AND METHODS

Materials

1-Methyl-3-isobutylxanthine was purchased from Aldrich. Dexamethasone, crystalline bovine serum albumin (fatty acid-poor), malonyl-CoA, NADPH, dithiothreitol, cyclic AMP, and all-*trans* retinoic acid were obtained from Sigma. Acetyl-CoA was prepared from CoA (P-L Biochemicals) according to the method of Simon and Shemin [37]. [G-^{3}H] cAMP (25 Ci/mmol) was obtained from New England Nuclear and purified by chromatography on BioRad AG-50-X8.

Cell Culture

3T3-L2 Fibroblasts were provided by Dr. Howard Green (Massachusetts Institute of Technology). Cultures were inoculated with 2×10^4 cells/60-mm dish and grown to confluence (6–7 days) in Dulbecco's modified Eagle's medium (GIBCO). Growth medium was supplemented with 10% newborn calf serum (GIBCO), penicillin (50 Units/ml) and streptomycin (50 μg/ml). Cultures were fed three times weekly with 5 ml of medium and maintained at 37°C in a humidified 5% CO_2 atmosphere.

As cultures reached confluence, differentiation was promoted by feeding with fresh medium containing either 10% newborn calf serum or 10% fetal calf serum and supplemented with 0.5 mM MIX and 0.25 μM DEX [17]. Three days later the medium was aspirated and cells were washed three times with warm (37°C) phosphate-buffered saline (PBS). Cultures were refed and maintained thereafter on standard medium containing 10% newborn calf serum.

Retinoic acid was prepared as a 10 mM solution in absolute ethanol and stored under nitrogen at −70°C. Stock solutions were diluted in standard serum-containing medium and added to cultures as an aliquot equal to 1% of the medium volume. Ethanol concentrations in the culture medium were always 0.01% or less. Cultures were maintained in the dark and fed under subdued lighting conditions when retinoic acid was used.

Analysis of Morphological Adipose Conversion

Cells were grown on 60-mm culture dishes which had a 2-mm grid etched onto the bottom. Dishes were examined for microscopic adipose clusters by scanning the dishes ($\times$ 40 magnification) and scoring as positive those 2-mm squares which had at least one cluster containing five or more adipose cells (Fig. 1A and B). Confluent cultures of undifferentiated preadipocytes can be made to appear as a dark field through the use of the 4X objective with the 20X phase-contrast annular diaphragm on a Nikon inverted microscope (model MS). Cells containing lipid droplets are readily distinguished from this dark background due to the different refractive properties of the accumulated lipid. One hundred squares were examined per dish (approximately 20% of the total squares) at each time and numbers reported are the percent of positive squares. Microscopic monitoring of the differentiation in this fashion is perhaps the most sensitive way to quantitate low levels of conversion, as even single adipose cells are easily distinguishable from backgrounds of fibroblast-like cells.

As the differentiation becomes more extensive and many of the squares contain more than one small cluster, another criteria is used to reflect the degree of morphological differentiation. This is necessary since a square containing one cluster with five cells would otherwise be scored the same as a square containing 10 clusters with 100 cells each. There-
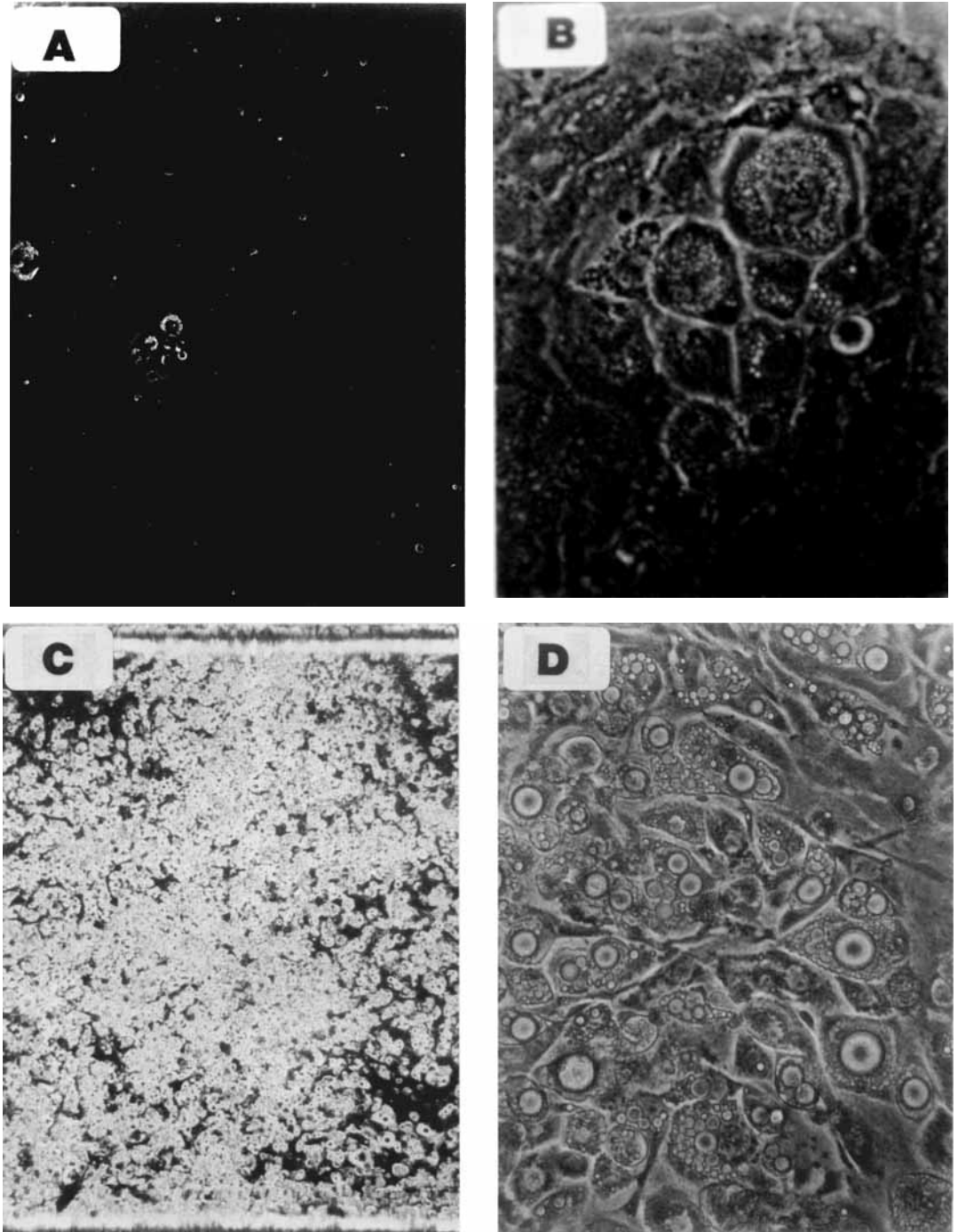
Fig. 1. Appearance of cytoplasmic lipid droplets during adipose conversion. Confluent monolayers were treated with MIX-DEX for 72 hr as described in Materials and Methods. Cultures were refed with fresh medium lacking drugs and expression of the differentiation was allowed to proceed. Examples of 2-mm squares which had either small adipose clusters (A,B) or more than 50% adipocytes (C,D) were photographed through phase-contrast optics at × 40 (A, C) and × 200 (B,D) magnification. A 4X objective was used with a 20X phase-contrast annular diaphragm to produce the dark background seen at × 40 magnification. The height of each × 40 photomicrograph depicts the height of one square on the 2-mm grid.

fore, when adipose conversion is more extensive at least 50% of the cells in a 2-mm square must contain lipid droplets for the square to be scored as positive (Fig. 1C and D).

Fatty Acid Synthetase Assay

Fatty acid synthetase activity was used as a biochemical indicator of the adipose conversion [6, 7]. Enzyme activity was determined using a modification of the spectrophotometric assay of Smith and Abraham [38] in which oxidation of NADPH is monitored. Cultures were rinsed three times with ice-cold phosphate buffered saline (pH 7.2) and once with 5 mM potassium phosphate buffer, pH 6.5, containing 6 mM EDTA. Cells were harvested in 0.4 ml of buffer using a Teflon scraper and disrupted manually in a Dounce homogenizer (B-pestle, 15 strokes). The homogenate was adjusted to 200 mM potassium phosphate (pH 6.5), 2 mM dithiothreitol and 6 mM EDTA, and centrifuged at 27,000g for 60 min. Supernatant fractions were assayed for synthetase activity in a reaction mixture containing 200 mM potassium phosphate buffer (pH 6.5), 2 mM dithiothreitol, 6 mM EDTA, 0.15 mM NADPH, 0.05 mM acetyl-CoA, 0.1 mM malonyl-CoA and 0.6 mg/ml of fatty acid-poor bovine serum albumin. Under these assay conditions the incorporation of 1 μmol of malonyl-CoA into fatty acids corresponds to oxidation of approximately 2 μmol of NADPH [7]. One unit of enzyme activity is the amount of protein catalyzing the incorporation of 1 μmol of malonyl-CoA into fatty acids/min at 37°C. Protein values were determined by the method of Bradford [39] with ovalbumin as standard.

Cyclic Nucleotide Phosphodiesterase Assay

The methods for preparation of cellular homogenates and determination of cyclic AMP phosphodiesterase activity were carried out as previously described [23].

RESULTS

The effects of MIX-DEX and retinoic acid on the adipose conversion have been examined using adipose cluster development as an indicator of the differentiation (as described in Materials and Methods). Cells were seeded into 60-mm dishes which had 2-mm grids etched onto the bottoms. Cultures of 3T3-L2 cells grown to confluence and maintained for 4 days in medium containing 10% newborn calf serum have very low levels of conversion as indicated by the absence of adipose clusters (Table I). Adipose conversion is known to be promoted by treating confluent cultures with 0.5 mM MIX plus 0.25 μM DEX. Under the conditions employed here, a 72 hr MIX-DEX treatment increased the percentage of 2-mm squares containing small adipose clusters from 0 to 65%. Addition of 10^{-6} M retinoic acid during the 72 hr MIX-DEX period inhibited the action of the triggering agents by over 95%. Retinoic acid appears to be blocking the action of MIX-DEX on differentiation rather than merely delaying in time the onset of the adipose conversion as cluster numbers remain nearly constant for at least 12 days following removal of the drugs (when cells are maintained in 10% newborn calf serum following drug treatment, the percents of squares containing adipose clusters on day 15 are, on the average, within 2.5% of the values determined on day 4). The same qualitative results are also seen when cultures are exposed to the drugs in fetal calf serum, although the degree of MIX-DEX promoted differentiation is much higher (Table I).

Adipose conversion in the absence of exogenously added drugs is known to be dependent upon a nondialyzable serum factor which, in cattle, is present in highest concentrations in fetal serum. Results here demonstrate that MIX-DEX triggered cytodiffer-

TABLE I. Inhibition of MIX-DEX Triggered Differentiation With Retinoic Acid*

Serum	Additions	% of squares with small adipose clusters	% of squares with more than 50% adipocytes
Newborn	None	0	0
	MIX-DEX	64.7 ± 1.5	0
	MIX-DEX + 10^{-6}M retinoic acid	2.3 ± 1.2	0
Fetal	None	0	0
	MIX-DEX	100.0 ± 0	36.3 ± 1.2
	MIX-DEX + 10^{-6} retinoic acid	8.3 ± 1.7	0

*Cultures of 3T3-L2 preadipocytes were treated with MIX-DEX in the presence or absence of 10^{-6}M retinoic acid for 72 hr. Cells were rinsed free of the drugs with warm PBS and examined 1 day later for microscopically detectable adipose clusters as described under Materials and Methods. Data reported are the mean ± SEM for n = 3.
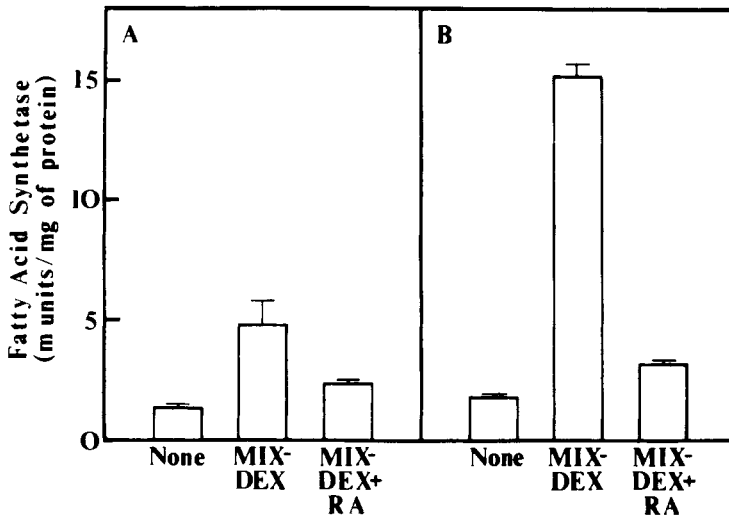

Fig. 2. Retinoic acid inhibition of changes in fatty acid synthetase activity during adipose conversion. Confluent cultures were pulsed with MIX-DEX or MIX-DEX plus 10^{-6}M retinoic acid (RA) for 72 hr in 10% newborn calf serum (A) or 10% fetal calf serum (B). Cells were then rinsed free of the drugs and 24 hr later cultures were harvested for fatty acid synthetase determinations. Data is from a typical assay and represents the mean ± SEM of three cultures.

entiation is also significantly enhanced by the presence of fetal serum (Table I). In these experiments cells were grown and maintained in 10% newborn calf serum and exposed to fetal serum only during the 72 hr MIX-DEX treatment. Within 24 hr after removal of the drugs extensive adipose cluster development is apparent. Although the differentiated cells contain only small lipid droplets at this point, evaluations of the extent of morphological differentiation can easily be made. Use of this procedure minimizes the requirements for fetal calf serum and yet high degrees of adipose conversion are maintained.
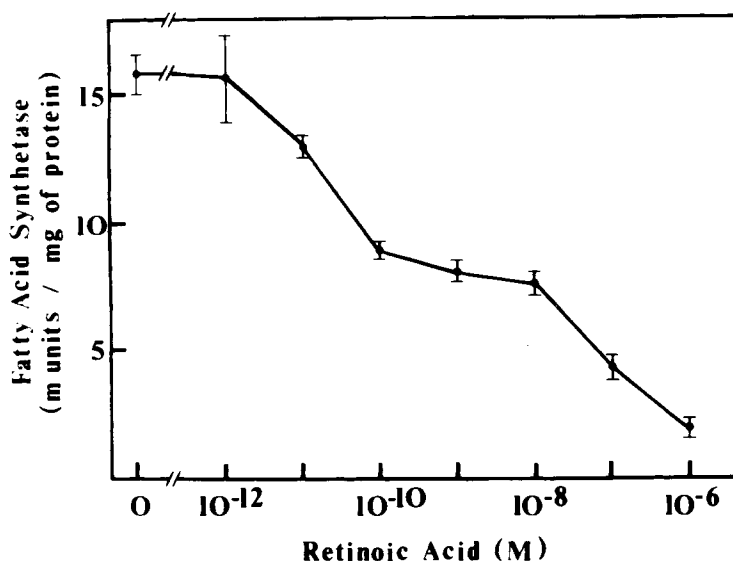
Fig. 3. Dose-response curve for retinoic acid inhibition of differentiation. Nongrowing cultures of preadipocytes received MIX-DEX plus the indicated concentration of retinoic acid for 72 hr in 10% fetal calf serum. Cultures were then refed with fresh medium containing 10% newborn calf serum for 24 hr before being harvested for fatty acid synthetase assays. Points are the mean ± SEM of triplicate determinations.

The appearance of lipid droplets in differentiating 3T3-L2 cells is known to be accompanied by large increases in lipogenic enzymes, including fatty acid synthetase [6, 7]. Confluent cultures of preadipocytes have low, but detectable levels of synthetase activity in the absence of adipose conversion (Fig. 2). MIX-DEX triggering (72 hr) of differentiation in newborn serum or fetal calf serum causes a 2.6- or 7.5-fold increase, respectively, in synthetase activity when measured 1 day following removal of the triggers. This increase can be blocked by 70% (newborn) or 90% (fetal) upon addition of 10^{-6} M retinoid acid with the triggering agents, thus confirming with an enzymatic parameter the effects of retinoic acid seen on morphological adipose conversion.

Dose-response curves for the inhibition of differentiation by retinoic acid indicate that the compound is effective over a wide range of concentrations (Fig. 3). At 10^{-6} M, retinoic acid inhibits MIX-DEX triggered adipose conversion (as measured by increases in fatty acid synthetase) by over 90% without any effects on cellular viability. Lower concentrations of the retinoid have reduced capacities for blocking differentiation, with 10^{-11} M producing about 20% inhibition. The nature of the dose response over the concentrations tested indicates that the effect of retinoic acid cannot be explained as a simple stoichiometric response. This same pattern of response is found when cultures are scored for adipose clusters as well.

Once it was established that retinoic acid could inhibit the ability of MIX-DEX to promote adipose conversion, studies were undertaken to determine when the retinoid was needed to exert its effects. Confluent cultures were fed with medium containing 10^{-7} M retinoic acid for 72 hr and then rinsed free of the drug. One day later a 72 hr MIX-DEX treatment was initiated in the absence of the retinoid. Cells were again rinsed free of drugs

TABLE II. Effect of Prior Retinoic Acid Treatment on Subsequent MIX-DEX Triggered Differentiation*

Additions		Adipose cluster development (% of squares with more than 50% adipocytes)	Fatty acid synthetase (mU/mg of protein)
Days 0−3	Days 4−7		
None	None	0	1.68 ± 0.2
10^{-7}M retinoic acid	None	0	1.59 ± 0.1
None	MIX-DEX	62.3 ± 5.0	23.46 ± 0.8
10^{-7}M retinoic acid	MIX-DEX	49.7 ± 4.4	21.12 ± 0.4

*At confluency (day 0), cultures were fed as indicated with medium containing 10% fetal calf serum for 3 days. All dishes were then rinsed three times with warm PBS. Cultures were refed with drug-free medium for 1 day before half of the cultures were given a 3-day pulse (days 4−7) with MIX-DEX. Dishes were again washed free of drugs with PBS and 1 day later cultures were scored for adipose cluster development and assayed for fatty acid synthetase activity. Means ± SEM of triplicate determinations are shown.

TABLE III. Reversal of Retinoic Acid Inhibited Differentiation*

Additions		Adipose cluster development		Fatty acid synthetase (mU/mg of protein)
		% of squares with small adipose clusters	% of squares with more than 50% adipocytes	
Days 0−3	Days 4−7			
None		0	0	1.61 ± 0.05
MIX-DEX		99.0 ± 0.6	10.0 ± 1.2	10.79 ± 0.18
MIX-DEX + 10^{-7}M retinoic acid		26.0 ± 2.0	0	3.33 ± 0.18
None	MIX-DEX	98.7 ± 0.6	9.0 ± 0.6	10.99 ± 0.50
MIX-DEX	MIX-DEX	100.0 ± 0	94.7 ± 1.5	22.41 ± 1.62
MIX-DEX + 10^{-7}M retinoic acid	MIX-DEX	100.0 ± 0	91.0 ± 2.3	22.37 ± 0.61

*Confluent cultures (day 0) were treated with MIX-DEX in the presence or absence of 10^{-7}M retinoic acid for 3 days. Drugs were removed by rinsing with warm PBS and cells were maintained on drug-free medium for 1 day. Half of the cultures were assayed for cluster development and fatty acid synthetase activity. The rest were treated with MIX-DEX for 3 days and refed for 1 day before being analyzed. Results of a typical experiment are reported (mean ± SEM of three determinations).

TABLE IV. Effect of Retinoic Acid on Induction of Cyclic Nucleotide Phosphodiesterase With MIX-DEX*

Additions	Phosphodiesterase activity
None	14.49 ± 0.21
10^{-6}M retinoic acid	16.61 ± 1.44
MIX-DEX	29.46 ± 3.31
MIX-DEX + 10^{-6}M retinoic acid	33.01 ± 2.91

*Confluent monolayers of preadipocytes were treated with the indicated agents for 72 hr before being homogenized and assayed for phosphodiesterase activity with 0.1 μM cyclic AMP as substrate. Activities are given as pmol cyclic AMP hydrolyzed/min/mg of protein and represent the mean ± SEM of triplicate determinations.

and 24 hr later cultures were analyzed for adipose cluster development and fatty acid synthetase activity. Pretreatment of nongrowing cells with retinoic acid had no significant effect on either of these indicators of adipose conversion (Table II). Similarly, growth to confluence in the presence of retinoic acid does not alter the degree of differentiation brought about by subsequent MIX-DEX treatment (fatty acid synthetase in control, 1.70 ± 0.1 mU/mg of protein; MIX-DEX, 19.85 ± 0.7 mU/mg of protein).

Experiments were conducted to determine if retinoic acid could alter the expression of the adipose phenotype in cultures which had already undergone adipose conversion. Confluent monolayers were treated with MIX-DEX for 4 days in 10% fetal serum and maintained an additional 6 days in drug-free medium containing 10% newborn serum. This protocol resulted in cultures composed predominantly of well developed adipocytes. Cells were treated with 10^{-6} M retinoic acid for 48 hr and assayed for fatty acid synthetase activity. Retinoic acid had no effect on the enzymatic activity under these conditions (11.3 ± 0.2 mU/mg of protein in retinoic acid treated cultures vs. 12.6 ± 0.6 mU/mg of protein in untreated controls).

It was also of considerable interest to investigate the extent to which retinoic acid inhibition of differentiation could be reversed. That is, could cells in which conversion had been blocked by including retinoic acid with MIX-DEX differentiate in response to a second MIX-DEX pulse? Nongrowing cultures were treated with MIX-DEX in the presence or absence of retinoic acid for 72 hr. Cells were washed free of the drugs and 1 day later were exposed to MIX-DEX alone for another 72 hr. Cultures respond to a single 72 hr pulse with MIX-DEX equally well during either period in terms of increases in both cluster development and fatty acid synthetase activity (Table II). A second 72-hr pulse with MIX-DEX further increases both of these parameters. Conversely, inclusion of retinoic acid with the MIX-DEX from days 0 to 3 retards the response to the triggers. However, when cells pulsed with MIX-DEX plus retinoic acid receive MIX-DEX alone from days 4 to 7, the effects of the prior retinoic acid exposure are completely overcome and levels comparable to two 72 hr pulses with MIX-DEX are seen. Thus, neither cellular viability nor sensitivity to the triggering agents is lost due to feeding with retinoic acid plus MIX-DEX.

Results presented thus far indicate that retinoic acid is blocking an event which occurs during the MIX-DEX pulse and has no lasting effects on the cells. One possible mechanism of action consistent with this data would be retinoid interference with the uptake of the triggering agents. However, this explanation seems unlikely in light of the following experiment. MIX [40, 41] and DEX [42] are known to alter the levels of intracellular cyclic nucleotide phosphodiesterase activity. Induction of the enzyme with MIX involves both transcriptional and translational events [40, 41] and requires the action of a cyclic AMP-dependent protein kinase [43]. Phosphodiesterase activities in control and MIX-DEX treated 3T3-L preadipocytes in the presence and absence of retinoic acid were determined (Table IV). No effects on basal nor MIX-DEX induced enzyme levels were found. Thus, retinoic acid does not appear to block MIX-DEX uptake nor does it block other cellular responses to MIX-DEX.

DISCUSSION

Identification of the intracellular events which result in the conversion of 3T3-L fibroblasts into adipocytes has been a matter of interest since the clones were first isolated by Green et al [1, 3]. Important advancements have been made in terms of identifying agents which can promote cytodifferentiation in this system, although their modes of

action remain undetermined. Adipose conversion in the absence of triggering agents has been shown to depend upon a serum factor which is present in relatively high concentrations in fetal calf serum [24]. It has also been established that some exogenously added agents, including MIX [26] and MIX-DEX [17], can rapidly promote expression of the differentiated program. The studies reported here illustrate two features of MIX-DEX action which may be important in determining the mechanism through which these agents influence regulation of the adipose conversion. First, the effectiveness of MIX-DEX in promoting differentiation is dramatically reduced when retinoic acid is added together with the triggering agents. And second, MIX-DEX-induced differentiation, like nontriggered adipose conversion, appears to be dependent upon a serum factor as indicated by the higher levels of conversion in fetal versus newborn calf serum.

Retinoid action on MIX-DEX-promoted differentiation has been demonstrated to be specific in terms of blocking events which occur during exposure of cultures to MIX-DEX, and thus may be very useful in discerning the mechanism of MIX-DEX action. Treatment of preadipocyte cultures with retinoic acid has no effect on subsequent MIX-DEX-induced differentiation, nor does the retinoid alter the enzymatic expression of the adipose phenotype when administered after differentiation has taken place. Also, retinoic acid has no permanent effects on cellular sensitivity to MIX-DEX in that its inhibitory action can be reversed by exposure of cells to MIX-DEX alone following removal of the retinoid from the cultures. This is in contrast to the inhibition of differentiation caused by bromodeoxyuridine, which requires growth in the absence of the nucleotide to bring about total reversal of the inhibition [2, 25].

Treatment of nongrowing cultures of 3T3-L preadipocytes with MIX-DEX has been used in this laboratory [23] and others [17, 18] as a convenient procedure for rapidly promoting adipose conversion. Typically cultures are pulsed for 48–72 hr with these agents. Results reported here indicate that two 72-hr pulses with MIX-DEX bring about a higher degree of differentiation (in terms of both morphological and enzymatic changes) than does a single drug exposure. This may be due in part to an increase in cell number in cultures treated with MIX-DEX [17]. Including 10^{-7} M retinoic acid during the first MIX-DEX pulse inhibits adipose conversion, but does not totally prevent it. Treating these cultures with a second MIX-DEX pulse in the absence of retinoic acid gives rise to cultures with adipose cluster numbers and synthetase levels comparable to cultures which did not receive the retinoid initially. Thus, the net increase during the second MIX-DEX pulse in cultures previously inhibited with retinoic acid during the first pulse is more than the change observed in cultures receiving only a single MIX-DEX pulse from days 4 to 7. Further, these increases observed after the second MIX-DEX pulse in the previously inhibited cultures are dependent upon cells receiving the triggers again in the absence of the retinoid. If cultures are maintained on newborn calf serum following treatment with MIX-DEX and retinoic acid from days 0 to 3, no changes in cluster numbers occur from day 4 through day 15. Retinoic acid, therefore, is not merely slowing down the rate at which the differentiated functions are expressed, but rather it is blocking an effect that MIX-DEX has while it is present in the cultures.

Dose-response curves of the type seen here with retinoic acid cannot be accounted for on the basis of a single compound interacting with a single site. Similar patterns of response are observed in negatively cooperative systems where there are multiple, interacting binding sites [44]. A complex response pattern such as the one seen here can also arise from there being more than one mode of action or more than one process which is affected

by the agent in question. The molecular basis of the apparent complexity in the retinoic acid dose-response pattern remains to be determined.

Retinoids have been demonstrated to be involved in several types of differentiation and a variety of systems have been used to study these effects. Tracheal explants from hamsters [32] and rats [45] and mouse prostate organ cultures [46] require some form of retinoid for maintenance and expression of differentiated functions. Low concentrations of retinoic acid stimulate the differentiation of F9 embryonal carcinoma cells into endoderm [47] and Nulli-SCC1 cells into both epitheliod and fibroblastic derivatives [48]. While in most cases retinoids have been shown to promote the expression of new cellular phenotypes, there are also reports of it inhibiting cytodifferentiation as well. In this sense, the effects of retinoic acid on inhibiting adipose conversion in 3T3-L cells is similar to its ability to block chondrogenesis [49, 50]. Prechondrogenic limb bud cells remain mesenchymal in appearance and do not differentiate into cartilage nodules when treated with retinoic acid. Like its action in 3T3-L2 cell adipose conversion, the timing of the retinoid treatment is important here as well. Inhibition occurs only when retinoic acid is administered during a susceptible period in the early stages of chondrogenesis [51]. The retinoid has no effects on the differentiation when administered before this susceptible period or after the cartilage rudiment is established. Thus, results from experiments with these two systems suggests that retinoic acid could play a physiological role in inhibiting early events in cytodifferentiation as well as functioning to promote it in other cell types.

The biochemical basis of retinoic acid effects on cellular differentiation is not completely understood, although recent developments have led to much interest in two areas. The isolation of cytoplasmic binding proteins for retinol and retinoic acid [30–33] which as complexes can bind to nuclear components [31–36], has led to the hypothesis that retinoids may be acting as hormones [31–33, 36]. Experiments which show that the binding affinities of various retinoid analogs for the binding proteins correlate well with their action on differentiation suggests that retinoid action may be mediated by these proteins [31, 52]. Alternatively, retinoids have been shown to alter cell surface characteristics in several different cell lines [51, 53, 54] and may function as lipid intermediates in glycoprotein synthesis [54]. It is hoped that further experimentation will indicate how retinoic acid is functioning to block MIX-DEX promoted adipose conversion of 3T3-L cells and thus shed some light on the mechanisms of MIX-DEX action in this process.

ACKNOWLEDGMENTS

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