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POLYAMINES ARE NEEDED FOR THE DIFFERENTIATION OF 3T3-L1 FIBROBLASTS INTO ADIPOSE CELLS

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SUMMARY When non-proliferating 3T3-L1 fibroblasts were stimulated to differentiate into adipose cells, there was a dramatic increase in the intracellular level of the polyamine, spermidine. Addition of α-difluoromethylornithine, an inhibitor of polyamine biosynthesis, depleted the cellular polyamines and prevented triglyceride accumulation and differentiation. The inhibitory effect of α-difluoromethylornithine was completely abolished by provision of spermidine or putrescine. This suggests that polyamines are needed in the processes of differentiation as well as their established requirement for cell growth.

When 3T3 fibroblasts are maintained in their density-inhibited state for an extended period of time, a proportion of the cells begin to accumulate lipid droplets in their cytoplasm and undergo morphological changes characteristic of adipose cells. A subline, 3T3-L1 cells have a greater tendency to differentiate into adipose cells (1). This conversion is further enhanced by treatment of the cells with a number of compounds including 20-30% serum, insulin, 1-methyl-3-isobutylxanthine (MIX[2]), prostaglandins, d-biotin and dexamethazone (2-5). Most of these compounds were known to affect polyamine metabolism in other systems (6,7). The role of polyamines in mammalian cells has been examined in several recent experiments by using inhibitors of polyamine biosynthetic enzymes to prevent polyamine accumulation (8-15). The possible impor-

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[2]Abbreviations used; DFMO, α-difluoromethylornithine; MIX, 3-isobutyl-1-methylxanthine.

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tance of polyamines in differentiation of 3T3-L1 cells has, therefore, been tested by measuring intracellular polyamine levels and by using the specific inhibitor of polyamine biosynthesis, α-difluoromethylornithine (DFMO[2]). This compound is an enzyme-activated suicide inhibitor of ornithine decarboxylase (16), the first enzyme in the mammalian polyamine biosynthetic pathway, and is known to prevent the accumulation of putrescine and spermidine in a variety of mammalian cells (8,15,17) including 3T3 fibroblasts (12).

METHODS AND MATERIALS

Cell culture. Cultured 3T3-L1 cells (American Type Culture Collection, ATCC CCL 92.1) were maintained as described (3). Culture dishes (100 mm) were seeded with 1 x 10^3 cells in 10 ml Dulbecco's medium with 10% calf serum. In some experiments 60 mm dishes were seeded with 3 x 10^4 cells in 5 ml medium. After the cells reached confluence, all cultures were stimulated to differentiate by a four day exposure to medium containing 0.5 mM MIX and 1 μg/ml insulin. Following this treatment, the cells were fed every other day with medium containing 1 μg/ml insulin. Dishes were fixed and stained with Oil Red-O and triglyceride containing colonies were counted using an American Opitcal dissection microscope (3). Results were expressed as the mean number of colonies in 21 cm^2 ± standard error of 4-6 separate determinations.
DFMO was added to the cultures as indicated in the Tables at a final concentration of 5 mM at 24 h prior to MIX. This concentration of DFMO greatly reduces the rate of growth of mouse fibroblasts after a lag period during which polyamine depletion occurs but has no cytotoxic effects (12). When spermidine was added to reverse the effects of DFMO, the culture medium contained 1 mM aminoguanidine to prevent oxidation by the serum. This drug did not affect intracellular polyamine levels nor cell growth at this concentration (12). DNA synthesis in the cultures was determined by measuring the incorporation of radioactivity into trichloroacetic acid insoluble material after exposure of the cells to 1 μCi/dish of [^{3}H]-thymidine for 4 h.

Polyamine determination. Cultures were rinsed twice with phosphate buffered saline and polyamines extracted in 5% (w/v) trichloroacetic acid, separated on an amino acid analyzer and quantitated using a fluorescence detection system (12). Results were expressed as fmol of polyamine/cell and given as mean ± S.D. for three separate determinations at each time point.

Other methods and materials. DFMO was a generous gift from Merrell Research Center, Cincinnati, OH. [^{3}H]Thymidine (21.5 Ci/mmol) was purchased from NEN. All other biochemicals were obtained from Sigma.

RESULTS AND DISCUSSION.

The accumulation of triglyceride in 3T3-L1 cells stimulated to differentiate with insulin and MIX was investigated by staining with Oil Red-O (1,3). A significant number of such areas were present in the control cultures, but there were none in the plates also exposed to DFMO (Table 1). DFMO also prevented the other morphological changes (2) observed in the control dif-

Table 1. Effect of α-Difluoromethylornithine on Conversion of 3T3-L1 Fibroblasts into Adipose Cells

Treatment	Putrescine addition time	Number of lipid-containing colonies when measured	
		9 days after MIX removal	16 days after MIX removal
Control	None	23 ± 3	87 ± 8
5 mM DFMO	None	0	0
5 mM DFMO	24 h before MIX	58 ± 11	133 ± 17
5 mM DFMO	at MIX removal	5 ± 1	116 ± 11
5 mM DFMO	9 days after MIX removal	-	10 ± 4

Putrescine was added at a concentration of 50 μM. DFMO was added 24 prior to MIX.

ferentiating cells. Addition of 50 μM putrescine completely reversed the effects of DFMO when given 24 h prior to MIX and somewhat enhanced differentiation above that seen in the controls. Putrescine was also effective in reversing the effect of DFMO when added at the time of MIX removal although differentiation was slowed. Some conversion to adipocytes was produced when putrescine addition was delayed for a further 9 days but this was much less than in the controls not receiving DFMO (Table 1).

As shown in Table 2, spermidine could also completely reverse the inhibitory effects of DFMO on the appearance of lipid containing cells. In this experiment spermidine and putrescine were tested at concentrations of 10 μM and at this concentration neither affected the differentiation in the controls but both restored the process when DFMO was present.

It is now fully documented that depletion of polyamines in mammalian cells by DFMO and other inhibitors greatly reduces the rate of growth (8, 12, 15, 17) and putrescine acts as a growth factor for some cultured cells (18). However, the present effect of DFMO on differentiation does not appear to be mediated via an effect on cell growth. The effects of DFMO on cell growth rate become apparent only after a time needed to deplete endogenous polyamines

Table 2. Reversal by Spermidine of Inhibition of Differentiation by α-Difluoromethylornithine.

Treatment	Number of lipid-containing colonies
Control	243 ± 10
Control + 10 μM putrescine	258 ± 10
Control + 10 μM spermidine	245 ± 5
5 mM DFMO	0
5 mM DFMO + 10 μM putrescine	248 ± 9
5 mM DFMO + 10 μM spermidine	242 ± 29

This experiment was carried out with cells seeded in 60 mm dishes and lipid-containing colonies were determined 11 days after the removal of MIX. Putrescine or spermidine was added 24 h prior to MIX. The difference between the control in this experiment and that of Table 1 may reflect the effect of saturation density on the rate of lipid accumulation (2).

(8, 12, 15, 17). In the present experiments, DFMO was not added until the cells were approaching confluence and there was no difference in the cell number between the DFMO-treated and the control cultures harvested at confluence. Also, the incorporation of [^{3}H]-thymidine into the trichloracetic acid insoluble fraction was compared at different stages of growth and differentiation. Exponentially growing 3T3-L1 cells showed a [^{3}H]-thymidine incorporation of 1400 cpm/μg protein. When measured at confluence there was no difference in [^{3}H]-thymidine incorporation between control cultures and those treated with 5 mM DFMO. During treatment with MIX and the period of differentiation following that treatment, the incorporation of [^{3}H]-thymidine was only 1-5% of exponential incorporation in all cultures: control, DFMO treated and DFMO plus putrescine. The absence of any differences in the [^{3}H]-thymidine incorporation between those cultures exposed to DFMO and controls indicates the mechanism of action was not one of inhibition of cell division and suggests that polyamines are needed for a critical step in the differentiation.

Table 3. Polyamine Levels in Differentiating Fibroblast Cultures Treated with α-Difluoromethylornithine.

Treatment	Polyamines (fmol/cell) Putrescine	Spermidine	Spermine
	Measured at Confluence		
Control	0.13 ± .01	0.35 ± .07	0.77 ± .03
5 mM DFMO	0.02	0.22 ± .05	0.66 ± .20
	Measured 9 days after MIX		
Control	0.07 ± .01	1.64 ± .27	0.73 ± .15
5 mM DFMO	N.D.	0.05 ± .02	1.62 ± .12
5 mM DFMO + 50 μM Putrescine (added 24 h before MIX)	0.54 ± .12	1.80 ± .43	0.73 ± .10
5 mM DFMO + 50 μM Putrescine (added at MIX removal)	0.49 ± .18	2.44 ± 1.25	0.81 ± .39
	Measured 16 days after MIX		
Control	0.19 ± .03	4.03 ± .75	1.72 ± .48
5 mM DFMO	N.D.	0.04 ± .02	1.62 ± .07
5 mM DFMO + 50 μM Putrescine (added 24 h before MIX)	1.20 ± .18	2.84 ± .52	1.32 ± .27
5 mM DFMO + 50 μM Putrescine (added at MIX removal)	1.09 ± .12	2.57 ± .51	1.35 ± .15
5 mM DFMO + 50 μM Putrescine (added 9 days after MIX removal)	1.62 ± .51	2.56 ± .64	1.08 ± .27

Cells were treated as in Table 1 and polyamine levels determined at the times shown. N.D. was below the limit of detection (<0.02 fmol/cell).

Table 3 shows the actual polyamine levels in the 3T3-L1 cells. The confluent cells contain quite low amounts of putrescine and spermidine compared to growing cells (8, 12). The exposure to DFMO for 24 h reduced putrescine almost completely and spermidine slightly in the confluent cells. Polyamine levels did not change significantly in confluent cells when maintained without a stimulus to differentiation (results not shown). No change in putrescine occurred at the times measured during differentiation, but spermidine levels increased progressively reaching a 10-fold increase

after 16 days. Spermine changed only 2-fold at this time. Treatment with DFMO over the period of differentiation reduced putrescine and spermidine to very low levels, but did not deplete spermine. Addition of putrescine to the DFMO-treated cells led to a substantial increase in putrescine above the control levels and restored the spermidine to at least 60% of that seen in the controls at 16 days and more than 100% at 9 days.

It, therefore, appears that the increased accumulation of spermidine is needed for the differentiation of the 3T3-L1 fibroblasts into adipocytes. The effects of DFMO are not due to the toxicity of the inhibitor since they can be reversed by addition of small amounts of putrescine or spermidine. Furthermore, other inhibitors of spermidine production (19) also inhibit differentiation whilst DFMO has no effect on the process when added after the treatment with MIX (unpublished observations). The present results, therefore, indicate an important role for polyamines which is distinct from their role in cell growth. The generality of this phenomenon remains to be determined. There is some indication that polyamines may be needed for differentiation in Friend erythroleukemia cells although polyamine levels were not measured in these experiments which used inhibitors less specific and potent than DFMO (13). There is also evidence that putrescine plays an important role in embryonic development which may be distinct from its requirement for cell division (14).

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