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INHIBITION OF ORNITHINE DECARBOXYLASE INDUCES EMBRYONAL CARCINOMA CELL DIFFERENTIATION

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SUMMARY. Murine embryonal carcinoma cells can be induced to differentiate *in vitro* by various physical and chemical means. We report here that inhibition of ornithine decarboxylase activity with a specific enzyme-activated inhibitor, α-difluoromethylornithine, can induce differentiation in embryonal carcinoma cells. The differentiated phenotype can be distinguished from undifferentiated embryonal carcinoma cells by altered cellular morphology, biochemical and cell surface antigenic properties. These results suggest that alterations in the levels of cellular polyamines may play a role in embryonal carcinoma cell differentiation.

Murine embryonal carcinoma cells (EC) cells, the malignant stem cells of teratocarcinomas, have successfully been cultured from tumors to generate a large number of *in vitro* cell lines (1,2). The undifferentiated cells from these many independently derived cell lines share a common cellular morphology yet differ in their ability to respond to a number of known inducers of differentiation (2).

The polyamines (putrescine, spermidine and spermine) are involved in the regulation of cell growth and tumor promotion (3-5), and recent evidence suggests that they may play a role in the control of cell differentiation (6-10). Ornithine decarboxylase (L-ornithine carboxy-lyase EC 4.1.1.7)(ODCase), which catalyzes the formation of putrescine, is the first enzyme in the polyamine biosynthetic pathway (11).

The differentiation of EC cells to specific differentiated derivatives could involve alterations in polyamine levels. To investigate this possibility, we asked whether changes in polyamine levels caused by inhibition

Abbreviations used are EC, embryonal carcinoma; ODCase, ornithine decarboxylase; cDME, complete Dulbecco's Modified Eagles Medium; DFMO, α-difluoromethylornithine.

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of ODCase activity with α-difluoromethylornithine (DFMO)(12), a specific enzyme-activated inhibitor of ODCase activity, induce EC cells to differentiate in vitro.

MATERIALS AND METHODS.

Cell Culture. EC cell line PCC4azalR (13) was used for these studies. Cells from this cell line were grown in Dulbecco's Modified Eagles medium containing 100 U/ml each of penicillin and kanamycin, 100 µg/ml streptomycin, 10% (v/v) heat-inactivated (56°C, 20 min.) fetal calf serum and supplemented with extra glucose and glutamine (cDME). α-difluoromethylornithine [(DFMO), Merrell Dow Research Center, Cincinnati, Ohio], was prepared as a stock solution in cDME and stored at -20°C, where it is stable for up to 3 months. Cultures to contain DFMO were allowed to grow for 24 hrs. prior to the addition of the inhibitor to insure that viable cells would have a sufficient time to attach and divide. Culture medium was changed every 24 hrs..

Polyamine Determination. Cells were washed twice with phosphate buffered saline and removed from the culture dishes by trypsinization. An aliquot was taken to determine protein concentration (Bio-Rad assay) and the cells were recovered by centrifugation. Cell pellets were resuspended in 0.4M perchloric acid and extracted overnight at 4°C. The precipitated cellular material was removed by centrifugation and the recovered supernatant filtered through a 0.22 µm Gelman polysulfone filter. This filtered supernatant was analyzed in an amino acid analyzer to determine the quantities of polyamine present (14). Results are the average of three separate determinations.

ODCase Activity. Cells were washed twice with phosphate buffered saline, removed from culture dishes by trypsinization, sonicated in a disrupting buffer containing 30 mM sodium phosphate buffer pH 7.2, 0.1 mM pyridoxal 5'-phosphate, 5 mM dithiothreitol, and 0.1 mM EDTA, and assayed for the release of $^{14}CO_2$ from ^{14}C-ornithine as previously described (15).

Immunofluorescence Assays. Both stage specific embryonic antigen (SSEA) and cellular actin were analyzed by indirect immunofluorescence on cells grown in situ in tissue culture dishes. Cells were washed with Hank's Balanced Salt Solution containing 0.2% (w/v) bovine serum albumin and permeabilized by incubating with cold methanol for 10 minutes. Following a 20 minute incubation with appropriate normal serum and subsequent washing with PBS, cells were exposed to specific antisera for 45 minutes. A mouse monoclonal antibody against SSEA-1, a surface antigen specific to EC cells (16) was kindly provided by Dr. D. Solter and used at a dilution of 1/250. A mouse monoclonal antibody against cellular actin was kindly provided by Dr. J. Lessard and used at a concentration of 10 µg/ml. After incubation with the specific antisera, cells were washed with PBS and incubated for 45 minutes with the appropriate fluorescein-conjugated second antisera. Cells were washed with PBS, mounted in a glycerol:PBS (9:1) solution and viewed with a Zeiss Invertoscope equipped with Epi-fluorescence illumination and an FITC filter set.

RESULTS AND DISCUSSION.

Cells from EC cell line PCC4azalR were incubated in medium containing DFMO at several concentrations, and cultures were inspected for overt changes in cellular morphology. When compared with untreated control EC cells, which exhibit their characteristic small, round shape and large nucleus-to-cytoplasm

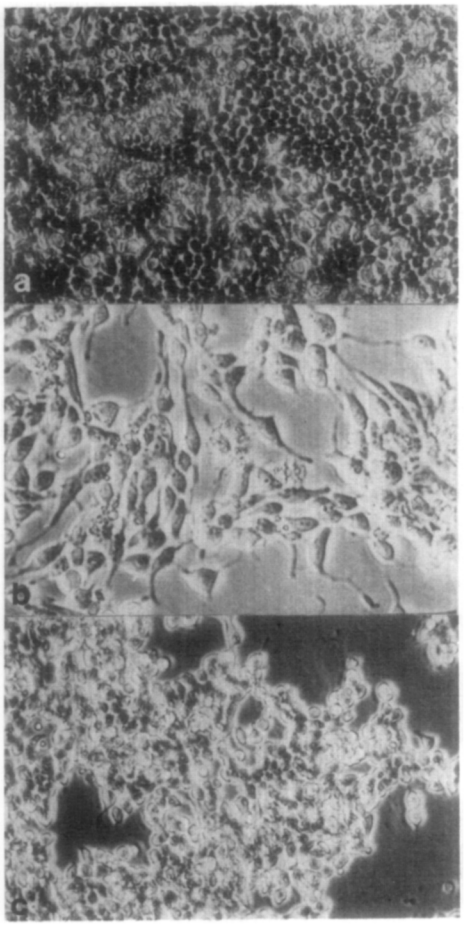

Figure 1: Effect of DFMO on EC cell morphology. a. untreated PCC4aza1R controls. b. PCC4aza1R cells cultured for 96 hours in the presence of 2.1 mM DFMO. c. PCC4aza1R cells cultured for 96 hours in the presence of 2.1 mM DFMO and 100 μM putrescine. All cells were photographed at the same magnification (128X).

ratio (Figure 1a), cells cultured in the presence of DFMO had an altered morphology (Figure 1b). They appeared differentiated, exhibiting a fibroblast-like morphology with extensive cellular polarity. They were flatter with discrete boundaries between cells. When putrescine, the product of ODCase catalysis, was added to the culture medium at the same time as DFMO, no overt morphological differentiation was observed (Figure 1c). The prevention of differentiation by exogenously supplied putrescine suggests that DFMO promotes differentiation by interfering with polyamine biosynthesis, as predicted, probably resulting in perturbed intracellular polyamine levels. The overt changes in cellular morphology were observed over a 200-fold concentration

range of DFMO (0.21 mM to 42 mM), but the timing at which these changes occurred varied depending on inhibitor concentration. Low concentrations of the inhibitor (0.21 mM), produced little morphologic differentiation before 48 hours, while increasing concentrations of DFMO resulted in an earlier appearance of morphologically differentiated cells. The levels of DFMO necessary to induce EC cell differentiation are well within the levels required for other chemical inducers of EC cell differentiation (17), and are equivalent to levels that produce biological effects in other experimental systems (8,9). Differences in initial cell densities had no effect on the response to DFMO. At any given DFMO concentration, cultures extending over a 15-fold range of cell densities responded identically.

The induction of morphological differentiation in PCC4azalR EC cells was observed with DFMO continuously in the culture media. We were interested in determining if shorter exposures to the inhibitor were sufficient to induce differentiation. In order to avoid potential ambiguities resulting from undifferentiated cells overgrowing cultures containing differentiated cells after the removal of DFMO or the addition of putrescine, these studies were carried out at low initial cell densities (10^5cells/60 mm dish) and high DFMO concentrations. Cells were incubated with 2.1 mM DFMO for either 24, 36, or 48 hours, after which time either the inhibitor was removed or 100 μM putrescine was added to the culture media. At various times following the removal of DFMO or the addition of putrescine, the cultures were scanned for morphologically differentiated cells. PCC4azalR cells require a minimum exposure of 24 hrs. to DFMO in order to become induced to differentiate. If 100 μM putrescine is added to DFMO-induced cell cultures at the times indicated above, 36 hrs. are required for irreversible induction of differentiation to occur. This observation suggests that polyamine levels must remain below some threshold level for a minimum period of time in order for differentiation to occur. If DFMO is removed from the culture, the EC cells can begin to resynthesize polyamines with newly synthesized ODCase. Therefore, the polyamine levels must be sufficiently low when DFMO is removed to be assured there are still

Table 1. Phenotypic characterization of PCC4azalR cells exposed to DFMO

Condition	Analyzed Characteristics		
		SSEA	Cellular Actin
PCC4azalR - untreated controls	Experiment 1	100%	8%
	Experiment 2	97%	10%
PCC4azalR - 2.1 mM DFMO	Experiment 1	4%	91%
	Experiment 2	3%	84%

Cells from EC cell line PCC4azalR were incubated as described in Materials and Methods. Ninety-six hours after the addition of DFMO, cells were analyzed for expression of the characteristics identified. Data is expressed as a percentage of cells exhibiting a positive reaction. Each experiment had triplicate dishes of each sample, and a minimum of 200 cells per dish were counted.

subthreshold levels for the time period necessary to induce differentiation. Since the addition of putrescine can restore polyamine levels faster than cells can resynthesize their own, such reversal experiments require cells to be exposed longer to DFMO to initially decrease polyamines to even lower levels. Once differentiation occurs, the differentiated phenotype can be maintained in culture both in the absence of DFMO or in the presence of DFMO + putrescine.

To confirm that the cells which exhibited morphological changes were in fact differentiated, we tested these cells for a number of biochemical and immunological markers which exhibit changes following the differentiation of PCC4azalR cells. Table 1 summarizes the results of such studies. SSEA-1, an antigen expressed on undifferentiated EC cells but not their differentiated derivatives (16), decreases dramatically (90%) in cells induced with DFMO. Consistent with a previous report that cellular actin increases in differentiated EC cells (18), DFMO produces an increase in actin concentration in PCCazalR cells induced to differentiate.

Together with the ability to induce differentiation in cells from cell line PCC4azalR, DFMO has a significant effect on cell growth. Figure 2 shows the growth curves of PCC4azalR cells in the presence of various concentrations of DFMO. At the highest concentrations of the inhibitor, cell growth ceases abruptly, while at lower concentrations, cessation of growth is much more gradual. EC cells cultured in the presence of DFMO and putrescine exhibited no cessation in their rate of growth. It is difficult to establish if the

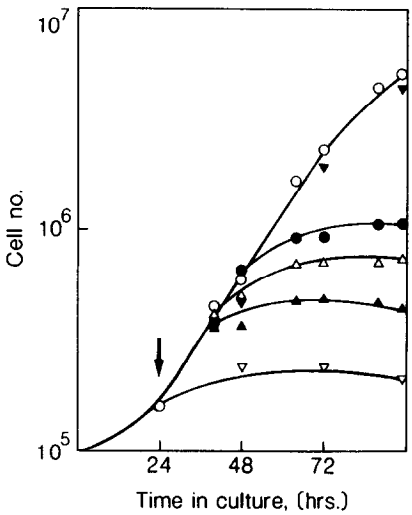

Figure 2: Effect of various concentratons of DFMO on cell growth. Cells from EC cell line PCC4azalR were grown as described in Materials and Methods. Twenty four hours after initial innoculaton of cells, DFMO at varous concentrations was added to the cell cultures (arrow). At times indicated, cells were harvested by trypsinization and counted in a haemocytometer. Cell death caused by DFMO, as determined by subsequent cloning of treated cells, was minimal (10%). Untreated controls,○-○; 0.21 mM DFMO,●-●; 2.1 mM DFMO, △-△ ; 21 mM DFMO, ▲-▲ ; 42 mM DFMO, ▽-▽ ; 2.1 mM DFMO + 100 μM putrescine, ▼-▼ .

observed differentiation is a result of a decreased rate of growth, or if the decreased rate of growth is due to the differentiation of EC cells into cells with longer doubling times. However, the observation that EC cells cultured in 50mM spermine exhibited a lower rate of growth than untreated controls, but failed to exhibit any morphological differentiation, suggests that differentiation is the result of lower levels of polyamines and is not simply due to a cessation in the growth rate of the cells in culture.

In order to insure that DFMO directly affects polyamine and ODCase levels in the EC cell cultures, their levels were determined in DFMO induced and uninduced cell cultures. Table 2 indicates that ODCase levels are reduced by almost 75% in PCC4azalR cells exposed to 2.1 mM DFMO for 96 hrs. in culture. Additionally, an *in vitro* control demonstrates that DFMO inhibits more than 90% of ODCase activity in a sonicated cell extract. Table 3 indicates that PCC4azalR cells exposed to DFMO contain significantly less putrescine and spermidine compared to untreated control cells or cells exposed to both DFMO and putrescine. The level of spermine in DFMO treated cells is only slightly

Table 2. ODCase enzyme activities in PCC4azalR embryonal carcinoma cells

Condition	Enzyme activity (nmoles/mg protein/hr)
Extract from PCC4azalR cells cultured for 96 hrs.	11.28
Extract from PCC4azalR cells cultured in 2.1 mM DFMO for 96 hrs.	2.75
Extract from PCC4azalR cells cultured for 96 hrs plus 2.1 mM DFMO added to the *in vitro* reaction mixture	0.88

Cells were grown as described in Materials and Methods. Extracts were prepared by sonication of harvested cells and enzyme activity was determined.

reduced compared with control cells. This observation is consistent with that observed in other mammalian cells following exposure to DFMO (9,11).

The inhibitor DFMO has unique specificity for ornithine decarboxylase. In a recent symposium, Heby et al., (19) reported preliminary observations that continuous exposure of EC cells from cell line PCC3 to DFMO induced morphological change in cells from that cell line. We here confirm that observation with cells from EC cell line PCC4azalR and extend the study to show that: 1) morphological changes can be correlated with biochemical and antigenic changes known to occur during EC cell differentiation; 2) the cellular response to DFMO is dose dependent and does not require continuous exposure in order to be effective; 3) DFMO directly effects ODCase enzyme activity from EC cells *in vitro* and lowers the enzyme activity of cells exposed to DFMO in culture; and 4) levels of putrescine and spermidine decrease in cells exposed to DFMO in culture.

It is clear that the role of polyamines in cell differentiation is complex. In some cases, differentiation may require cell proliferation and polyamine

Table 3. Polyamine levels in PCC4azalR embryonal carcinoma cells

Condition	Polyamines (nmol/mg protein)		
	Putrescine	Spermidine	Spermine
Untreated controls	0.626	12.74	15.95
2.1 mM DFMO	0.069	0.0895	12.285
2.1 mM DFMO + 100 μM putrescine	8.9	41.3	64.4

Cells from EC cell line PCC4azalR were grown as described in Materials and Methods. 96 hours after the addition of DFMO or DFMO + putrescine, cells were harvested and polyamine levels determined.

biosynthesis [differentiation of adipocytes from fibroblasts (9)], while in other cases differentiation may occur in the absence of cell proliferation and/or new DNA synthesis [HL-60 cells (20)]. The studies reported here argue strongly that inhibition of ODCase activity and consequent perturbation of polyamine levels results in the induction of EC cell differentiation.

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