Vol. 140, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1986 Pages 66-73

α-DIFLUOROMETHYLORNITHINE INDUCES DIFFERENTIATION OF A HUMAN EMBRYONAL CARCINOMA CELL LINE IN VITRO

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Received August 19, 1986

Human embryonal carcinoma cells could serve as a useful model system for analysis of early human development. A limited number of human embryonal carcinoma cell lines have been generated from *in vivo* tumors. We report here that α-difluoromethylornithine, a specific enzyme-activated inhibitor of ornithine decarboxylase activity, can induce differentiation in human embryonal carcinoma cells. The differentiated phenotype could be distinguished from undifferentiated cells by altered cellular morphology, biochemical and cell surface antigenic properties. These results suggest that alterations in the intracellular levels of polyamines may play a role in human embryonal carcinoma cell differentiation, and possibly human embryogenesis.

Murine embryonal carcinoma (EC) cells, the malignant stem cells of teratocarcinomas, have been compared to inner cell mass cells of the mouse blastocyst (1). Cell lines directly isolated from murine tumors have proven useful for the study of both cellular differentiation and early mammalian embryogenesis (2). In the hope of identifying comparable human cells that would enhance our ability to directly address both the study of human cellular differentiation and early human embryogenesis, several EC cell lines have been isolated from human tumors and grown in monolayer culture (3,4). Their usefulness as a model system to study cellular differentiation has, however, been less successful than murine cells, primarily because these human EC cell lines lack the capacity to exhibit extensive differentiation *in vitro*.

Abbreviations used are EC, embryonal carcinoma; RA, retinoic acid; ODCase, ornithine decarboxylase; DFMO, α-difluoromethylornithine; cDME, complete Dulbecco's Modified Eagles Medium; SSEA-1, stage specific embryonic antigen 1; PBS, phosphate buffered saline.

0006-291X/86 $1.50

Recently, several cloned human EC cell lines have been induced to differentiate by retinoic acid (RA;5,6), a potent inducer of murine EC cell differentiation (7,8).

Inhibition of ornithine decarboxylase (L-ornithine carboxy-lyase EC 4.1.1.7, ODCase), the first enzyme involved in polyamine biosynthesis, induces murine EC cell differentiation in several cell lines (9). This has been accomplished with α-difluoromethylornithine (DFMO), a specific enzyme-activated inhibitor of ODCase (10). Since several human EC cell lines do respond to one known inducer of murine EC cell differentiation (RA;5,6), we asked whether inhibition of ODCase activity with DFMO could also induce human EC cell differentiation.

MATERIALS AND METHODS

Cell Culture. Human EC cell line Tera-1, originally isolated by Dr. Jorgen Fogh (3), and kindly provided by Dr. Peter McCue, Emory University, was exclusively used for these studies. Cells from this cell line were grown in Dulbecco's Modified Eagles medium containing 100U/ml each of penicillin and kanamycin, 100 μg/ml streptomycin, 15% (v/v) heat-inactivated (56°C, 20 min.) fetal calf serum and supplemented with extra glucose and glutamine (cDME;8). Cells were passaged at high density (>10^6 cells/25 cm^2 flask) to insure that the cultures were maintained as EC cells. α-difluoromethylornithine [(DFMO), Merrell Dow Research Center, Cincinnati, Ohio] was prepared as a stock solution in cDME and stored at 4°C, where it is stable for up to 3 months. All-trans retinoic acid [(RA), Hoffman-LaRoche, Nutley, N.J.] was prepared as a stock solution in 100% ethanol and stored at -70°C, where it is stable for 2 weeks. Cell cultures treated with either DFMO or RA were allowed to grow for 12 hrs. prior to the addition of either compound to insure that viable cells would have sufficient time to attach and divide. Media was changed every 24 hrs.

Immunofluorescence Assays. Control and DFMO-treated cells were analyzed by indirect immunofluorescence for the expression of SSEA-1, cellular actin, intermediate filaments, fibronectin and laminin *in situ* in tissue culture dishes. Cells were washed with PBS and permeabilized with cold 100% methanol for 5 minutes. Prior to permeabilization, cells analyzed for cellular actin and TROMA-1 were fixed with 3.7% formaldehyde in PBS. Following a 20 minute incubation with appropriate normal serum and subsequent washing with PBS, cells were exposed to 80 μl of specific antibody for 30 minutes. A mouse monoclonal antibody against SSEA-1 (kindly provided by Dr. D. Solter) and cellular actin (kindly provided by Dr. J. Lessard) were used at a dilution of 1:100 and 5 μg/ml respectively. A rat monoclonal antibody against trophectoderm specific intermediate filaments (TROMA-1, kindly provided by Dr. R. Kemmler) was used undiluted. Antibodies against fibronectin and laminin (BRL) were used at a 1:20 dilution. Following incubation, cells were washed with PBS and exposed to a 1:20 dilution of the appropriate fluorescein-conjugated second antisera for 45 minutes. Stained cells were washed with PBS, mounted with glycerol:PBS (9:1), and observed with a Zeiss IM-35 inverted phase contrast photomicroscope equipped with epi-fluorescence illumination and an FITC filter set.

Polyamine Determination. Cultured cells were washed with PBS and removed by trypsinization. Following removal of an aliquot for protein determination (Bio-Rad assay), cells were recovered by centrifugation, resuspended in 0.4M perchloric acid and extracted overnight at 4°C. Precipitated material was removed by centrifugation. The supernatant was filtered through a 0.22 μm Gelman polysulfone filter and analyzed in an amino acid analyzer to determine the quantities of polyamines present (11).

ODCase Activity. Cells were recovered as described above for polyamine determinations. Cell pellets were resuspended in disrupting buffer (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 (12).

RESULTS AND DISCUSSION

TERA-1 human EC cells were incubated in cDME containing several different concentrations of DFMO, and cultures were inspected for overt changes in cell morphology. Untreated control cells exhibited a characteristic EC morphology with small, round shapes and a large nucleus-to-cytoplasm ratio (Figure 1A). The DFMO treated cells had an altered morphology (Figure 1B) that was endoderm-like. The treated cultures were characterized by large, flat cells with discrete boundaries between them. The addition of exogenous putrescine at the same time as DFMO prevented any morphological changes (Figure 1C), suggesting that DFMO induces differentiation by interfering with polyamine biosynthesis. Alterations in cell morphology were observed over a 100-fold concentration range of DFMO (0.21 mM to 21 mM). Since Tera-1 EC cells exhibit anomalous behavior when grown at low cell densities, high initial densities

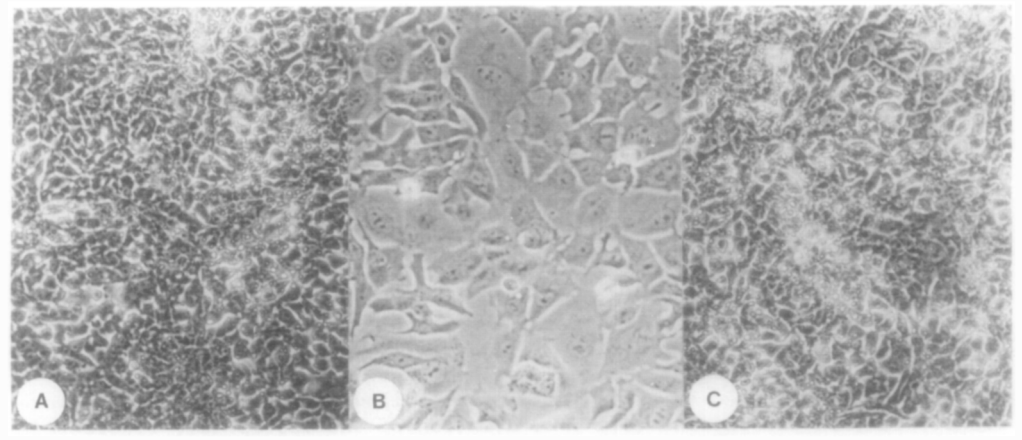

Figure 1. Effect of DFMO on Tera-1 EC cell morphology. A. untreated Tera-1 cells. B. Tera-1 cells cultured for 96 hours in the presence of 2.1 mM DFMO. C. Tera-1 cells cultured for 96 hours in the presence of 2.1 mM DFMO and 100 μM putrescine. All cells were photographed at the same magnification (X160).

were always maintained. To the extent that those densities were changed (from $3.5X10^5$ to 10^6 cells/60 mm dish), no differences in responsiveness to DFMO were noted. The concentrations of DFMO needed to induce differentiation in these human EC cells were identical to those necessary for the induction of murine EC cell differentiation (9), and were comparable to levels that elicit biological responses in other experimental systems (13,14).

Morphological differentiation was induced in Tera-1 EC cells during continuous exposure to DFMO. We attempted to determine if shorter exposure times could be equally sufficient for the induction of differentiation. To avoid possible ambiguities resulting from changes in cell growth parameters, these studies were performed with confluent monolayer cultures of cells. Such cells were exposed to DFMO (2.1 mM) for either 24, 48, 72 or 96 hours, after which time the inhibitor was removed and replaced with fresh cDME. The cultures were subsequently scanned for morphological differentiation. The results indicated that Tera-1 cells require a minimum exposure of 48 hours to DFMO in order to differentiate. This observation, identical to that seen in murine EC cells (9), suggests that polyamine levels must remain below some threshold for a minimum period of time before differentiation can occur. Once differentiation does occur, DFMO is not required to maintain the differentiated phenotype.

Exposure of Tera-1 EC cells to DFMO not only induces morphological differentiation, but also influences cell growth. Figure 2 shows the growth kinetics of Tera-1 cells treated with either RA or various concentrations of DFMO. Panel 2B demonstrates that at the highest DFMO concentration used, cell growth stops almost immediately, while at lower concentrations, cessation is far more gradual. While it is difficult to establish if the induction of differentiation simply results from decreased growth, or if the observed decrease in growth is characteristic of the newly differentiated phenotype, our experiments with RA suggest the latter. Panel 2A shows that treatment of Tera-1 EC cells with RA causes a reduction in growth rate, but fails to induce differentiation, consistent with a previous report on the effect of RA on

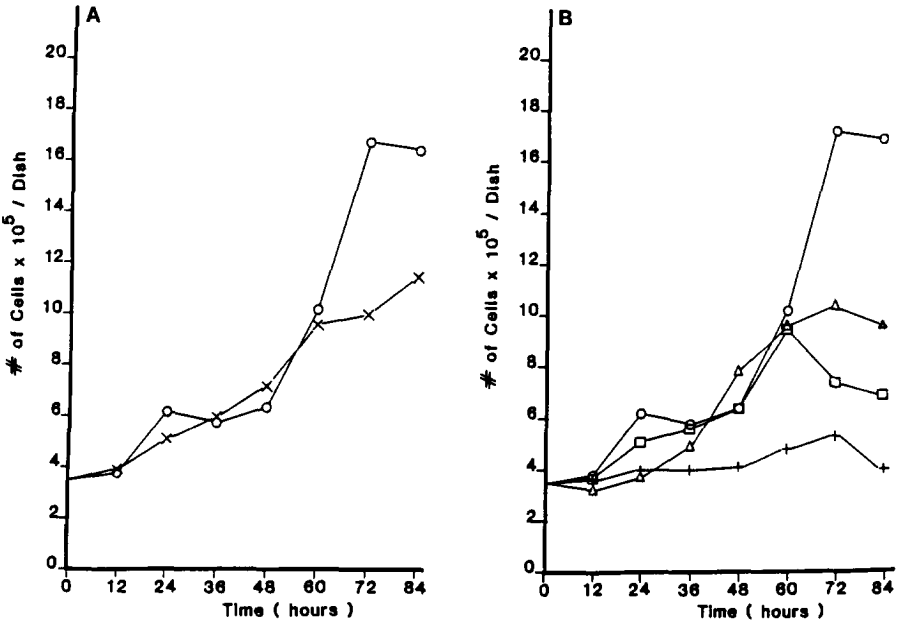

Figure 2. Effect of RA or DFMO on cell growth. Cells from human EC cell line Tera-1 were grown as described in Materials and Methods. Twelve hours after initial innoculation, either RA or DFMO were added to the cell cultures. At times indicated, cells were harvested by trypsinization and counted in a haemocytometer. Cell death caused by either RA or DFMO, as determined by subsequent cloning of treated cells, was minimal (10%). Panel A: untreated controls, 0-0; 10^{-5}MRA treated cells, X-X. Panel B: untreated controls, 0-0; 2.1 mM DFMO treated cells, Δ-Δ; 21 mM DFMO treated cells, □-□; 42 mM DFMO treated cells, +-+.

Tera-1 cells (15). These observations taken together suggest that the induction of differentiation by DFMO is not simply the result of a cessation in growth rate, but is the result of lower intracellular polyamine levels.

Table 1. Phenotypic characterization of Tera-1 cells exposed to DFMO

Condition		Analyzed Characteristics				
		TROMA-1	SSEA-1	Laminin	Cellular Actin	Fibronectin
Untreated Controls	Expt. 1	8%	7%	22%	21%	56%
	Expt. 2	6%	9%	16%	19%	50%
2.1 mM DFMO	Expt. 1	62%	92%	59%	58%	7%
	Expt. 2	57%	87%	66%	52%	6%

Cells from human EC cell line Tera-1 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 are expressed as a percentage of cells exhibiting a positive reaction. Each experiment had duplicate dishes of each sample, and a minimum of 200 cells per dish were counted.

To further characterize the cells that showed morphological changes following exposure to DFMO, we analyzed the expression of several antigenic and biochemical parameters that change during human EC cell differentiation (16). The results are summarized in Table 1. TROMA-1, a trophectoderm specific antigen (17) is minimally expressed in untreated cells, but increases dramatically (>8 fold) in DFMO treated cells. Even more dramatic (>10 fold) is the increased expression in SSEA-1. This observation is in direct contrast to murine EC cells, which show a dramatic decrease in SSEA-1 expression following differentiation (8,9). Laminin expression, as well as the expression of cellular actin, both increase following exposure to DFMO, but not to the extent seen with the specific antigens noted above. Interestingly, cellular actin expression changes qualitatively as well as quantitatively. In untreated cells, actin is distributed intracellularly in a diffuse manner. DFMO treatment not only increases the extent of actin expression, but also induces polymerization to form easily identifiable cables within the cells. Finally, fibronectin expression is dramatically reduced (>8 fold) following exposure to DFMO.

To confirm that DFMO does directly effect polyamine and ODCase levels in the Tera-1 EC cells, their levels were determined in both treated and untreated cell cultures. Results shown in Table 2 show that ODCase enzyme activity is reduced by greater than 80% in Tera-1 cells exposed to 2.1 mM DFMO for 96 hrs. in culture. Table 3 demonstrates that levels of putrescine and spermidine are reduced in DFMO treated Tera-1 cells as compared to untreated

Table 2. ODCase enzyme activity in Tera-1 human EC cells

Condition	Enzyme activity (nmoles/mg protein/hour)
Extract from Tera-1 cells cultured for 96 hrs.	0.496
Extract from Tera-1 cells cultured in 2.1 mM DFMO for 96 hrs.	0.019

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

Table 3. Polyamine levels in Tera-1 human EC cells

Condition	Polyamines (nmol/mg protein)		
	Putrescine	Spermidine	Spermine
Untreated Controls	0.247	5.02	30.94
2.1 mM DFMO	0.023	0.609	41.22

Cells from Human EC cell line Tera-1 were grown as described in Materials and Methods. Ninety-six hours after the addition of DFMO, cells were harvested and polyamine levels determined.

controls. Spermine levels are not reduced, consistent with reports on DFMO treatment in other mammalian cells (18).

The exact role of polyamines in cellular differentiation is not clear. Studies in several different experimental systems have demonstrated that, depending on the specific experimental system employed, either increases (14) or decreases (8) in polyamine biosynthesis are necessary for differentiation to proceed normally. Studies on murine EC cells (9,19,20) have shown that decreased polyamine levels accompany cellular differentiation. In the studies reported here, we demonstrate for the first time that cells from human EC cell line Tera-1 can be induced to differentiate *in vitro*, and that this induction, like that reported for murine EC cells, results from perturbation of polyamine biosynthesis. The ability to induce extensive differentiation in this cell system provides an excellent tool for the study of human embryonic cellular differentiation.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Peter P. McCann for providing the polyamine analysis.

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