Proc. Natl. Acad. Sci. USA
Vol. 78, No. 4, pp. 2355–2358, April 1981
Cell Biology

Polyamines are necessary for the survival of human small-cell lung carcinoma in culture

(ornithine decarboxylase/inhibitor/α-difluoromethylornithine/spheroids)

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Communicated by Victor A. McKusick, December 22, 1980

ABSTRACT **Many human small-cell lung carcinoma culture lines grow as multicellular aggregate spheroids, for which high L-dopa decarboxylase activity is a marker. During the initial cell aggregation and the exponential growth phase, there is a marked increase in ornithine decarboxylase activity and an accumulation of polyamines. α-Difluoromethylornithine, a specific enzyme-activated, irreversible ornithine decarboxylase inhibitor, blocks the increase in ornithine decarboxylase activity and in polyamines and inhibits human small-cell lung carcinoma cell growth. After the onset of a decreased proliferation rate, the multicellular spheroid aggregates become poorly formed, cell loss ensues, and there is a decrease in L-dopa decarboxylase activity. These findings support the hypothesis that ornithine decarboxylase and the polyamines play an essential role not only in the proliferative phase but also in the viability of human small-cell lung carcinoma cells in culture. The results suggest that α-difluoromethylornithine, a virtually nontoxic compound, may be potentially useful in the therapy of this human tumor.**

The products of the polyamine biosynthesis pathway—putrescine, spermidine, and spermine—have long been implicated in the initiation and maintenance of rapid cell growth (1–4). The importance of polyamines for such cellular processes has recently been emphasized in studies using a potent new specific inhibitor of polyamine biosynthesis. α-Difluoromethylornithine (F_2MeOrn; DFMO) irreversibly inhibits the conversion of ornithine to putrescine via the enzyme ornithine decarboxylase (OrnDase; L-ornithine carboxy-lyase, EC 4.1.1.17) (5, 6), the first and rate-limiting step in polyamine formation (1–4). *In vivo*, F_2MeOrn prevents the transient increase in OrnDase that occurs during the first few days of embryogenesis and results in complete inhibition of fetal development (7). F_2MeOrn also blocks multiplication of the parasite *Trypanosoma brucei brucei* in mice and eliminates the infection (8). We have found that F_2MeOrn prevents transient increases in OrnDase that accompany rat intestinal mucosal maturation and regeneration with a resultant marked delay in the progression of these processes (9). The apparent specificity and virtual nontoxicity of F_2MeOrn, coupled with its ease of use *in vivo*, thus provides a potent new tool for studying the role of polyamines in cellular growth processes.

The association of polyamines with rapid cellular growth has logically created much interest in the role of these compounds in neoplasia. Indeed, elevation of the polyamine levels in body fluids of patients with various types of cancer has now been documented (2).

Work with other inhibitors of various steps in polyamine biosynthesis has suggested that blockage of this pathway could retard or block the growth of some neoplasms (10, 11). Recently, F_2MeOrn has been shown to inhibit the growth of rat hepatoma cells, mouse mammary EMT6 sarcoma cells, mouse L1210 leukemia cells, and human prostate adenoma cells in tissue culture (12–15). *In vivo* F_2MeOrn prolongs the survival of mice bearing L1210 leukemia cells and markedly retards the growth of transplanted EMT6 cells in mice (14, 15). In all of these studies of the effects of F_2MeOrn on tumor growth, no reports of altered viability of tumor cells or change in morphology have been published. In the present study, we report a marked sensitivity to F_2MeOrn of human small (oat) cell lung carcinoma (SCC) cells growing in culture. Not only is the growth of these cells inhibited but their viability is profoundly reduced. The decreased viability is manifested by cell loss and disruption of the morphology of the cell aggregate system in which the cells grow. These effects appear to be entirely secondary to the inhibition of OrnDase activity and subsequent depletion of polyamines.

MATERIALS AND METHODS

The established line of SCC cells (O-H-1) used in the present study grows as a multicellular aggregate (16, 17). Its growth pattern is similar to that of other SCC lines (16, 17). The cells are maintained in RPMI 1640 in the presence of 16% fetal calf serum/penicillin at 50 units per ml/streptomycin at 50 μg/ml (all from G1BCO). For preparation of single cell suspensions to initiate cultures and to count cells in a hemocytometer, the aggregates are triturated with a 1-ml pipette. The O-H-1 cell line is characterized by the presence of a high activity of L-dopa decarboxylase (dopaDase; aromatic-L-amino-acid carboxy-lyase, EC 4.1.1.28) as are the other aggregate lines of small-cell carcinoma recently described (16).

F_2MeOrn (RMI 71,782, from Merrell Research Center, Cincinnati, OH) was added directly to the culture media at 0.05–5 mM, and the culture media were changed every 3 days as described (16). Putrescine (Sigma) was added at 10 μM in studies designed to test for reversal of the growth inhibitory effect of F_2MeOrn.

Activities of OrnDase, dopaDase, and S-adenosylmethionine decarboxylase (AdoMetDase; S-adenosyl-L-methionine carboxy-lyase, EC 4.1.1.50) were assayed in supernatants of cell sonicates by measuring the $^{14}CO_2$ liberated from DL-[1-^{14}C]ornithine hydrochloride, L-3,4-dihydroxyphenyl[1-^{14}C]-alanine (dopa), and S-adenosyl-L-[*carboxyl*-^{14}C]methionine (Amersham) as in previous studies (9, 18).

Aliquots of 5–10 × 10^6 cells were centrifuged, and the pellets were washed with cold phosphate-buffered saline and sonicated

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Abbreviations: SCC, small-cell lung carcinoma; dopa, L-3,4-dihydroxyphenylalanine; dopaDase, L-dopa decarboxylase; OrnDase, L-ornithine decarboxylase; AdoMetDase, S-adenosylmethionine decarboxylase; F_2MeOrn, α-difluoromethylornithine (DFMO).

in 0.5 ml of 0.1 M sodium phosphate buffer, pH 6.8/5 mM dithiothreitol. OrnDase activity was determined in the sonicating buffer supplemented with 0.2 mM pyridoxal phosphate/0.67 mM L-ornithine/8.6 nmol of DL-[1-^{14}C]ornithine (0.5 μCi; 1 Ci = 3.7×10^{10} becquerels). dopaDase activity was determined in the sonicating buffer supplemented with 0.2 mM pyridoxal phosphate/1 mM dopa/10.6 nmol of L-[1-^{14}C]dopa (0.08 μCi). AdoMetDase activity was determined in the sonicating buffer supplemented with 0.2 mM pyridoxal phosphate/2.5 mM putrescine/1.64 nmol of *S*-adenosyl-L-[*carboxyl*-^{14}C] methionine (0.1 μCi).

For the enzyme activity determinations, the microprocedure of Beaven *et al.* was used (19). Protosol (25 μl) (New England Nuclear) was placed in the bottom of a 20-ml screw-top polypropylene liquid scintillation vial. The incubation mixtures were placed in 1.5-ml polypropylene Eppendorf microtubes, which were left open and placed inside the liquid scintillation vials, which were then closed. The closed vials were incubated by shaking in a water bath at 37°C for 1 hr. Then, 100 μl of 0.2 M perchloric acid was added to the incubation mixture in the microtube, and incubation was continued for an additional 30 min. At the end of this second incubation, the microtubes were removed, 7.5 ml of Econofluor (New England Nuclear) was added directly to each scintillation vial, and the ^{14}C was assayed.

Putrescine, spermidine, and spermine were measured fluorometrically in acid extracts of cell sonicates as in previous studies (9).

RESULTS

The dose–response curve for the effects of F_2MeOrn on growth of O-H-1 cells is shown in Fig. 1. All doses having a final concentration >0.5 mM inhibited the progression of growth in these cells; differences from control were not noted until day 3 or day 4, when the treated cells ceased to proliferate. After the cessation of growth, an exponential loss of total cells from culture was seen, beginning at day 8.

The effects of F_2MeOrn on the morphology of the cell aggregates are shown in Fig. 2. Within 3 hr of seeding of a single-cell suspension, spontaneous aggregation of the cells occurred. Within 24 hr, multicellular spheroidal aggregates were present, and these aggregates continued to enlarge (Fig. 2 *A–D*). Studies of cell viability, as determined by trypan blue exclusion, showed that all viable cells were contained within the cell aggregates while floating single cells appeared to be nonviable and not participate in the cell aggregation process. In the presence of 5 mM F_2MeOrn, the initial phases of cell aggregation and the first 5 days of aggregation-enlargement were not affected by the drug. After 5–7 days, depending on the individual culture (i.e., after the cessation of growth as shown in Fig. 1), the cell aggregates treated with F_2MeOrn ceased to enlarge, began to deteriorate, and showed large areas of cell necrosis (Fig. 2 *E–G*). At late stationary phases of the culture (Fig. 2*H*), the cell aggregates had essentially lost all configuration and existed as amorphous clumps of dying or dead cells; cell loss was occurring during this period as shown in Fig. 1. The findings for all concentrations of F_2MeOrn greater than 0.5 mM were similar to those for 5 mM.

The specific biochemical events that appear to underly the effects of F_2MeOrn on O-H-1 cells are shown in Fig. 3. As also occurs in other cell culture systems, during the exponential growth phase, there was a marked but transient increase in OrnDase activity that was accompanied by a small increase in

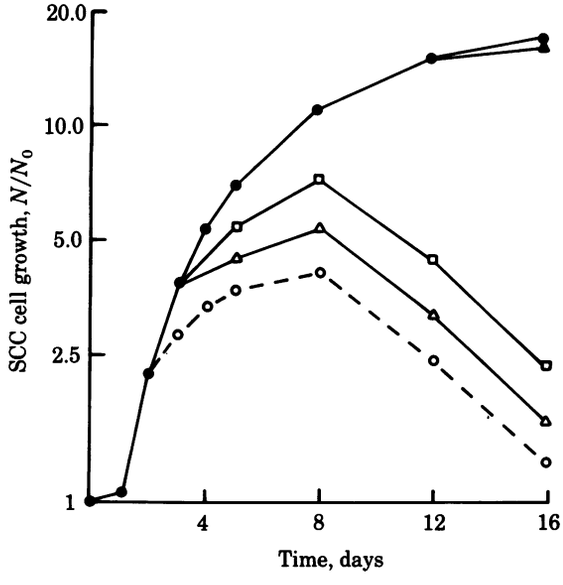

FIG. 1. Effect of concentration of F_2MeOrn on SCC growth in culture. ●, Control; ▲, 0.1 mM; □ 0.5 mM; △, 1mM; ○, 5 mM. Results are expressed as N/N_0, where N_0 is the number of cells per ml on day 0 and N is the number of cells per ml on subsequent days. Results are from six separate experiments: the SEM is less than 10% of the mean for all data shown.

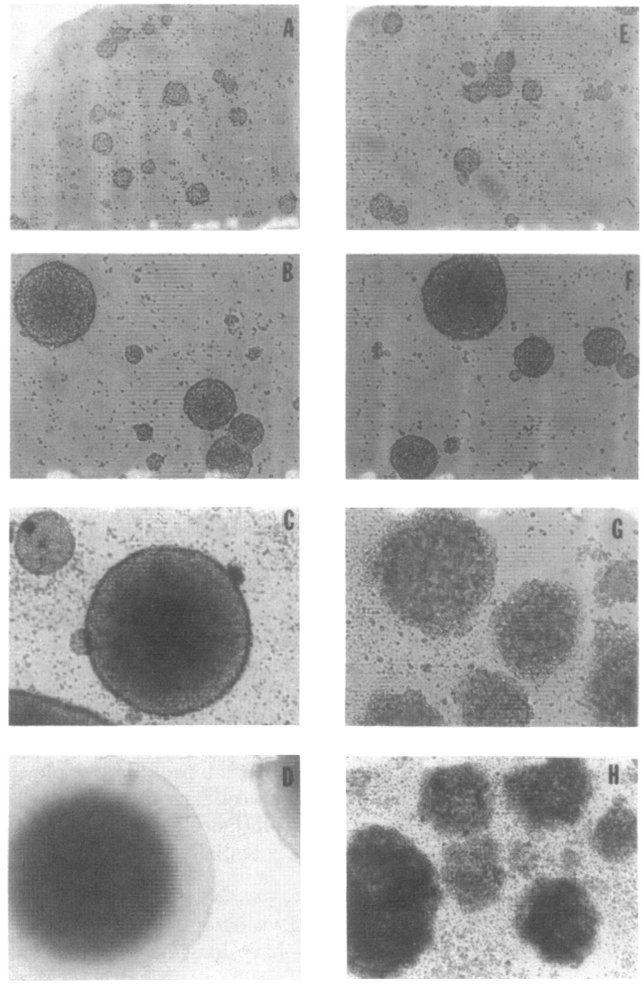

FIG. 2. Effect of 5 mM F_2MeOrn on SCC cell aggregate morphology in culture. (*A, B, C,* and *D*) Control cell cultures 24 hr, 3 days, 8 days, and 15 days after seeding. (*E, F, G,* and *H*) F_2MeOrn-treated cell cultures 24 hr, 3 days, 8 days, and 15 days after seeding. (×160.)

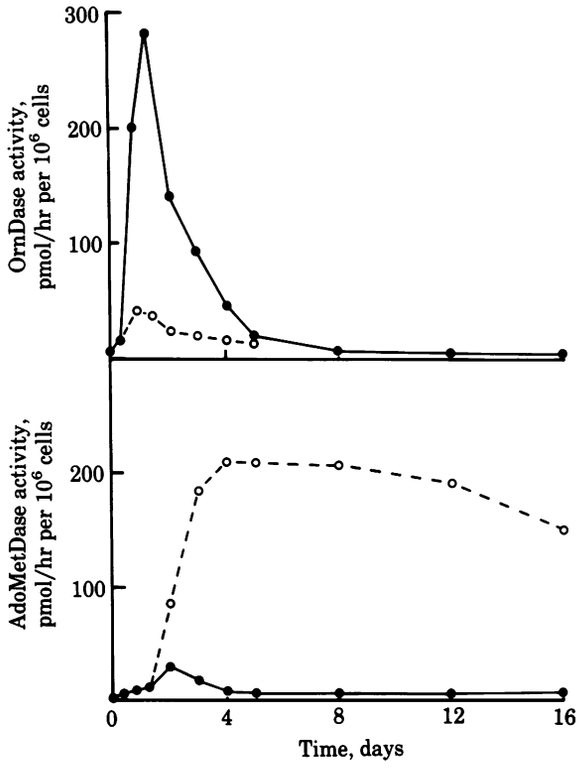

FIG. 3. Effect of 5 mM F_2MeOrn on SCC OrnDase and AdoMetDase activities in culture. ●, Control; ○, F_2MeOrn-treated cells. The SEM is less than 10% of the mean for all data shown.

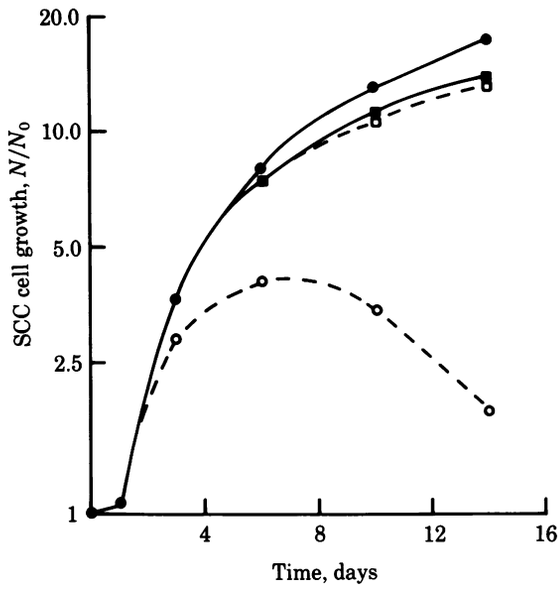

FIG. 4. Reversal by 10 μM putrescine of the inhibitory effect of 5 mM F_2MeOrn on SCC cell growth in culture. ●, Control; ■, putresine-treated cells; ○, F_2MeOrn-treated cells; □, cells treated with both F_2MeOrn and putrescine. The SEM is less than 10% of the mean for all data shown.

AdoMetDase activity. Concomitant with these increases of enzyme activity, there was an increase in all three polyamine products from their low basal levels. Putrescine increased 16-fold, spermidine increased 3-fold, and spermine doubled along a time course similar to the increases in OrnDase and AdoMetDase activities.

In cells treated with F_2MeOrn, there was virtually complete abolition of the OrnDase-activity spike seen in the control cells (see Fig. 3). The results were not changed when expressed as per mg of cell protein. Concomitantly, putrescine and spermidine levels failed to increase from their low basal values in the F_2MeOrn-treated cells, but the doubling in spermine was unaffected by F_2MeOrn, consistent with previous studies (7, 9, 10, 12, 13, 15). The F_2MeOrn-treated cells also had a marked decrease in the specific activity of dopaDase, the enzyme marker for the O-H-1 SCC cells. This 85% decrease in dopaDase activity from the level in untreated cells is compatible with a marked decrease in cell viability.

The activity of the second enzyme in the pathway, AdoMetDase, increased profoundly in F_2MeOrn-treated cells. This increase was maintained throughout the period of F_2MeOrn treatment (see Fig. 3), consistent with previous studies (7, 9, 10, 12, 13, 15).

The addition of 10 μM putrescine at day 0 to cell cultures treated with F_2MeOrn completely prevented all of the effects of F_2MeOrn on cell growth, multicellular spheroid aggregate morphology, and cell viabilility (Fig. 4). This concentration of putrescine alone had no effect on the growth of non-F_2MeOrn-treated SCC cells.

DISCUSSION

This paper presents the biological and biochemical effects of specific inhibition of OrnDase with F_2MeOrn on a human cancer in culture. For this important lung tumor—SCC—F_2MeOrn not only inhibits cell growth but also has a distinct effect on the morphology of the aggregate system in which this cell type spontaneously grows in culture. The effects seen are apparently not due to a direct or immediate toxic effect of F_2MeOrn on the cells. Evidence for this is the lack of change in cell growth and morphology during the first several days of exposure of the cells to the drug and the abolition of the inhibitory effect by the addition of putrescine, the product of OrnDase activity.

The increase in AdoMetDase activity may be a compensatory response in the polyamine biosynthesis pathway, as has been observed in other situations in which OrnDase inhibitors have been used and may be the cause of the unchanged levels of spermine in the treated cells (7, 9, 10, 12, 13, 15). This compensatory response may also account for the long delay (2–4 doubling times) in any apparent biological effect of F_2MeOrn on the cells in culture.

In other studies, F_2MeOrn has been shown to slow the growth of cells in culture but to not alter their viability (12–15). Several possibilities exist to account for our findings that F_2MeOrn can have an effect on parameters other than cell growth. First, we carried out our culture experiments for much longer than has generally been reported. Most investigators have looked at the first 4–8 days of the growth curve, whereas our effects on viability and morphology became profound only after 7 days but were progressive thereafter. Second, SCC is known to have a very high *in vivo* growth fraction relative to many other human tumors (20). It is thus possible that this tumor survives well both *in vivo* and *in vitro* only in a proliferative phase; resting cells may do quite poorly. If this is the case, once F_2MeOrn has depleted endogenous polyamines and caused a decrease in cell proliferative activity, the viability and morphology of the cells will be altered. The biologic growth patterns exhibited by our cells in culture and the existence of a biochemical marker for following the cells make an ideal situation to observe these changes in morphology and viability. Integrity of the multicellular aggregate apparently depends on continued proliferative activity, and the loss of cells is dramatically man-

ifested by a dissolution of the spheroids. The specific activity of dopaDase is normally well maintained throughout the growth curve for SCC in culture (16). Thus, changes in the activity of this enzyme can provide an additional index of cell viability and metabolism.

The significance of the effects of F_2MeOrn in these *in vitro* studies for *in vivo* responses to F_2MeOrn must await clarification. However, the possibility that depletion of polyamines by this nontoxic drug could produce a therapeutic effect on tumors such as human SCC is certainly raised. The sensitivity of this tumor to F_2MeOrn in culture makes imperative a series of *in vivo* studies to assess the effects of F_2MeOrn on the tumor. The athymic or *nude* mouse should offer an ideal setting for animal work in this regard because many lines of human SCC appear to grow well in this animal (16, 17). If effects similar to those currently reported are observed in such an animal model, then human clinical trials with F_2MeOrn in selected tumors such as SCC might be warranted.

We thank Drs. Albert Sjoerdsma and Peter McCann, Merrell Research Center, for the generous gift of F_2MeOrn; Drs. Thomas R. Hendrix and Albert H. Owens, Jr., for advice, encouragement, and support; and RoxAnna Thompson and Kathy Wieman for technical assistance. This work was supported in part by Grants 5 R01 CA18404, 1R01 AM27447, 5 T32 AM07192, RR-5378, CA15515, and CA13525, from the National Institutes of Health and Grant PDT-108 from the American Cancer Society. S.B.B. was also supported by Research Career Development Award CA-00027 and G.D.L. is supported by Clinical Investigator Award 1 K08 AM00774 from the National Institutes of Health.

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