Anchorage Dependency Effects on Difluoromethylornithine Cytotoxicity in Human Lung Carcinoma Cells

Gordon D. Luk² and Stephen B. Baylin

The Oncology Center and Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT

Difluoromethylornithine (DFMO), a specific, irreversible, enzyme-activated inhibitor of ornithine decarboxylase activity, the first and rate-limiting step in polyamine biosynthesis, has been shown to inhibit neo-

plastic cell proliferation in culture. In most cases, such inhibition is not accompanied by cell loss, with the exception of multiple cell lines of human small cell lung carcinoma (SCC), a human leukemia cell line (HL-

60), and possibly the B16 melanoma cell line. The first two cell types grow as anchorage-independent suspension cultures, the HL-60 as single cells and the SCC as multicellular spheroid aggregates. Moreover, in the spectrum of human lung carcinoma cells in culture, the SCC cells respond in a cytotoxic manner to DFMO, whereas the non-small cell lung carcinoma (non-SCC) cells, which are anchorage dependent, show only growth inhibition, without actual cell loss.

In the present study, we have investigated relationships between anchorage-dependent and -independent growth patterns of cells in culture and their response to DFMO treatment. Two non-SCC lung cancer cell lines, which normally grow as anchorage-dependent monolayers, show growth inhibition but no cell loss with the addition of DFMO. When these anchorage-dependent cells were forced to grow as multicellular aggregates, by coating the culture flask with Teflon, the cells developed an increased sensitivity to DFMO. They showed not only inhibition of cell proliferation but also cell death. Two SCC cell lines, which normally grow as anchorage-independent spheroids, developed adherence to the culture dishes coated with fibronectin. These cells, which show a cytotoxic response to DFMO during normal anchorage-independent growth, developed a decreased sensitivity to DFMO, showing only cell growth inhibition, but no cell death when treated during anchorage-dependent growth. Our data thus suggest that the state of anchorage dependence of lung cancer cells in culture is a critical factor in determining their response to polyamine depletion during treatment with DFMO.

INTRODUCTION

The importance of polyamines for cell proliferation has recently been documented in studies using a potent and specific inhibitor of polyamine biosynthesis, DFMO, developed at the Merrell Research Institute (1, 2). This drug irreversibly inhibits the conversion of ornithine to putrescine via the enzyme ornithine decarboxylase (EC 4.1.1.17), the first and rate-limiting step in polyamine formation (3-6). The apparent specificity of DFMO has thus provided a potent new tool for studying the role of polyamines in cellular growth processes (5-9).

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1 Recipient of a Faculty Research Award from the American Cancer Society and the American Gastroenterological Association/Robins Research Scholar Award. To whom requests for reprints should be addressed, at Room 2-127, The Oncology Center, The Johns Hopkins Hospital, 600 N. Wolfe Street, Baltimore, MD 21205.

2 The abbreviations used are: DFMO, difluoromethylornithine; SCC, small cell lung carcinoma.

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MATERIALS AND METHODS

Cell Cultures. We used the previously described human classic SCC cell line OH1, a variant SCC line H82, a squamous cell carcinoma cell line U1752, and a large cell carcinoma cell line H157 (16, 17, 20-22) for our studies. The documentation of nude mouse histology of all these established lines and their freedom from Mycoplasma infection and freedom from HeLa cells have been described previously (20, 21).

Culture conditions and experimental protocols are as previously described (16, 17). All cells were maintained in RPMI 1640 in the presence of 10% fetal calf serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50 µg/ml) (all from Grand Island Biological Co., Grand Island, NY). The SCC cells grew as multicell suspended aggregates with varying degrees of cell-cell adhesion and the non-SCC cultures, which normally grow as spheroids, so that they grew as anchored monolayer cells. We showed that the non-SCC anchorage-independent spheroids developed a cytotoxic response to DFMO, while the monolayer SCC cells now responded only with cytostasis.
of logarithmic growth was obtained for all cell lines used. Cell viability during growth curves was always assessed by trypan blue exclusion and during drug exposure by ability of the cell to proliferate when placed into drug-free media. In all results, cell number is expressed as viable cells. However, the results would be similar if expressed as total cells, because the percentage of viable cells remains relatively constant for each cell line under similar anchorage-dependent conditions, ranging from 60% in SCC cells to 95% in squamous cell carcinoma cells (16, 17).

Loss of Anchorage Dependency. The non-SCC cell lines U1752 and H157, which normally grow as monolayers, were detached by 2 mM EDTA and seeded as single-cell suspensions into nonconfluence (bacteriological) Petri dishes coated with Teflon. These non-SCC cells lost their anchorage dependency and grew as loose multicellular spheroid aggregates, without attachment to the substratum. Their growth characteristics as measured by doubling times and trypan blue viability did not change significantly, as detailed in “Results.” The cell lines continued to form xenografts when injected into athymic nude mice. When reseeded into untreated culture flasks, they resumed growth as anchorage-dependent monolayers.

Development of Anchorage Dependency. The SCC cell lines OH1 and H82, which normally grow as anchorage-independent spheroids, were triturated with a 1-ml pipet and seeded into cell culture flasks coated with fibronectin (Collaborative Research, Inc., Lexington, MA). The SCC cells began to grow as monolayers attached to the substratum. In general, these SCC monolayers were not as tightly attached as the non-SCC monolayers. The SCC monolayers were detached by 2 mM EDTA within 1 to 2 min, whereas the non-SCC monolayers often required up to 10 min for detachment to be complete. The doubling times and dopa decarboxylase activities did not change significantly. The viability of the OH1 cells, as measured by trypan blue exclusion, showed an apparent increase from 55% to 90% because trituration for breaking up the aggregates was no longer necessary. When trypsinized monolayer cells were trituated similarly, the viability decreased to the 60–70% range. The anchorage-dependent SCC cells continued to form xenografts in nude mice (23). They resumed growth as spheroids when seeded into untreated culture flasks.

Drug Studies. DFMO (MDL 71,782A; Merrell Dow Research Institute, Cincinnati, OH), at concentrations of 0.5 to 5 mM, was added directly to the culture media at the time of single-cell seeding and culture medium was changed every 3 days, as described previously (16, 17). For prevention of DFMO effect, putrescine dihydrochloride at a concentration of 10 μM was added to the culture media simultaneously with DFMO.

Enzyme Assays. l-Dopa decarboxylase activity was measured by quantitation of 14CO2 released by incubation of cell homogenates with the 1C-labeled substrate, l-dopa, as described previously (20, 24).

RESULTS

Growth Characteristics. The SCC and non-SCC anchorage-dependent monolayers, after seeding as single-cell suspensions, showed an initial lag phase of 1–2 days, with no change or only a small increase in cell number. This was followed by exponential-phase growth which lasted from 14–21 days. The doubling times ranged from approximately 56 to 86 h (Table 1).

When the SCC and non-SCC cells were grown as anchorage-independent spheroid aggregates in suspension, they began showing cell-cell adhesion within 4 to 8 h after seeding as single-cell suspensions. Small aggregates began forming within the first 12 h. By 24 h distinct multicellular aggregates of greater than 16 cells were evident. These aggregates began to enlarge to 60–80 μm in diameter (non-SCC and H82) and 400–600 μm in diameter (OH1) over the subsequent 14–21 days, with greater than 95% of total viable cells being found in the aggregates. The increase in viable trypan blue-excluding cell number and total cell number was exponential between days 2 and 21 after seeding, with doubling times ranging from 60 to 84 h (Table 1). The viability of OH1 cells as measured by trypan blue exclusion, was 55% when grown as spheroids and 90% when grown as monolayers. This was due to the trituration required to disaggregate the spheroids into single-cell suspensions for cell counting, as described under “Development of Anchorage Dependency.” l-Dopa decarboxylase activity was much higher in SCC cells as compared to SCC variants and non-SCC cells, as was described previously (20, 22, 25). Interestingly, the enzyme activities, which can be used as a marker of SCC spheroids, did not change significantly with the anchorage dependency status of the cells in culture (Table 2).

Response of SCC Cells to DFMO. The SCC cells, which normally grow as anchorage-independent multicell spheroids, showed marked cell loss and disruption of their aggregates during continuous treatment with DFMO. As established in previous studies of multiple SCC cell lines (16), DFMO at doses of 0.5 mM or greater produced inhibition of cell growth and subsequent cell death. Growth inhibition of OH1 and H82 cells produced by DFMO concentrations of 1 to 5 mM was similar and thus only results obtained in H82 cells with 5 mM DFMO are shown (Fig. 1). The marked cytotoxic effect of DFMO was completely abrogated by the simultaneous addition of 10 μM putrescine, suggesting that the cytotoxic effects observed were most probably due specifically to putrescine depletion. In addition, removal of DFMO resulted in a prompt resumption of exponential growth in 3–5 days.

When the SCC cells were grown as adherent monolayers on fibronectin-coated substratum, the marked cytotoxic response to DFMO was lost (Fig. 1), and the SCC cells showed the more commonly reported cytostatic response to DFMO at doses up to 5 mM. Over periods of exposure to DFMO for as long as 6 weeks, cell proliferation was inhibited, but no loss of cell number from culture was observed. This growth-inhibitory effect was prevented by the simultaneous addition of putrescine (Fig. 1, legend). Trypan blue exclusion studies showed 90–95% continued viability of these growth-inhibited anchorage-dependent SCC cells. In addition, removal of DFMO resulted in

### Table 1 Growth characteristics of human small cell and non-small cell lung cancer cells under anchorage-dependent and anchorage-independent conditions

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<td>OH1</td>
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<td>H82</td>
<td>68</td>
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<td>Non-small cell</td>
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<td>U1752</td>
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* For cell counts, these multicellular spheroids require trituration with pipets to obtain a single-cell suspension. The viability of the different cell types does not affect the results, because the results shown in Figs. 1 and 2 are similar whether expressed as viable cell number or total cell number.
cells resumed growth within 3-5 days. On fibronectin-coated substratum as monolayers (A), Cells were untreated controls (•, A) or treated with 5 mM DFMO alone (C, △). The growth curves of cells treated simultaneously with 5 mM DFMO and 10 μM putrescine were virtually superimposable upon those of untreated control cells and are thus not shown separately. Results are expressed as N/N₀, where N₀ is the number of viable (trypan blue-excluding) cells on day 0 and N is the number of viable cells on subsequent days. The results are similar if expressed as the number of total (trypan blue-excluding and trypan blue-staining) cells. Results are from at least 4 separate experiments. The SE is less than 15% of the mean for all data shown.

Response of non-SCC Cells to DFMO. In contrast to SCC spheroids in culture, non-SCC cells growing normally as anchorage-dependent monolayer cultures showed the more commonly reported cytostatic response to DFMO. Marked inhibition of cell growth was seen, but no cell loss from culture was apparent. Results for U1752 and H157 cells were similar, and only those for H157 cells are shown (Fig. 2). From 90-95% of the cells continued to exclude trypan blue. This DFMO-induced growth inhibition was prevented by putrescine (Fig. 2, legend). With removal of DFMO, these non-SCC adherent monolayer cells resumed growth within 3-5 days.

When these non-SCC cells were grown as anchorage-independent aggregates in Teflon-coated nonculture (bacteriologic) Petri dishes, they developed a marked cytotoxic response to DFMO at doses of 1 mM or greater (Fig. 2). They showed disruption of the aggregate morphology and marked cell loss (expressed either as total or viable cell number) from culture, beginning within 7-10 days after continuous DFMO exposure, similar to the results described in previous studies for SCC spheroidal aggregates (16, 17). Similar to SCC cells, these cells resumed exponential growth with removal of DFMO. Furthermore, this cytotoxic effect of DFMO was prevented by the simultaneous addition of putrescine, again showing that the observed effects were due to putrescine depletion.

DISCUSSION

Our current results document clearly the effect of anchorage dependence of human lung cancer cells on their response to DFMO. SCC and non-SCC cells grown as adherent monolayers are relatively resistant to DFMO, showing growth inhibition but little cell death. When both SCC and non-SCC cells are grown as spheroids, they develop marked cytotoxicity to DFMO, showing cell death and actual loss of cell number (total and viable) from culture. The “cytostatic” and “cytotoxic” effects of DFMO were prevented by the simultaneous addition of putrescine to the culture medium, similar to our previous findings and findings by other investigators (5, 8, 9, 13, 15-18). This suggests that putrescine (and polyamine) depletion plays a major role in the growth-inhibitory effect of DFMO.

Our previous studies had documented the exquisite sensitivity of a spectrum of human SCC cell lines to DFMO in culture (16, 17). DFMO was also effective in treating established human SCC xenografts in athymic mice (23). This exquisite sensitivity of SCC cells and their apparent “cytotoxicity” to DFMO is quite distinct from the usual “cytostatic” growth-inhibitory effect of DFMO in most other cell lines studied (5, 9, 13). A few other cell lines have been shown to share a similar cytotoxic response to DFMO. These include the human promyelocytic leukemia HL-60 cells (18) and possibly also the 9L rat brain tumor cells grown as spheroids (15, 26). Even in the 9L spheroids, the growth of spheroids was arrested in plateau phase, without actual decrease in size or disruption or disaggregation of the spheroids (15). Nevertheless, the few cell types shown to share the property of cytotoxic response to DFMO appear to share the characteristic of growth as anchorage-independent suspension cultures.

Several possibilities exist to account for the effects of anchorage dependency on the response of cells to DFMO cytotoxicity. First, multicellular spheroids approximate many characteristics of in vivo tumors that are usually not present in monolayer cultures or single-cell suspensions. These include three-dimensional intercellular contact, gradients across the cell layers of the aggregates in pH, oxygen tension, and nutrient levels, and the ability of the anchorage-independent cells to grow in culture for up to 4 weeks without trypsinization (26, 27). While these characteristics of multicellular spheroids could play a role in DFMO-induced cytotoxicity, it is unlikely that they alone account for the cell death. Cell loss in spheroids begins within the first week in culture when the multicellular aggregates

Fig. 1. The effect of anchorage dependence on DFMO growth inhibition of the human SCC cell line H82 in culture. Exponentially growing SCC spheroids were triturated to single-cell suspensions and seeded to grow as spheroids (•) or on fibronectin-coated substratum as monolayers (A). Cells were untreated controls (•, A) or treated with 5 mM DFMO alone (C, △). The growth curves of cells treated simultaneously with 5 mM DFMO and 10 μM putrescine were virtually superimposable upon those of untreated control cells and are thus not shown separately. Results are expressed as N/N₀, where N₀ is the number of viable (trypan blue-excluding) cells on day 0 and N is the number of viable cells on subsequent days. The results are similar if expressed as the number of total (trypan blue-excluding and trypan blue-staining) cells. Results are from at least 4 separate experiments. The SE is less than 15% of the mean for all data shown.

Fig. 2. The effect of anchorage dependence on DFMO growth inhibition of the human non-SCC cell line U1752 in culture. Exponentially growing non-SCC monolayer cells were trypsinized to single-cell suspensions and seeded to grow in Teflon-coated Petri dishes as spheroids (•) or as monolayers (A). Cells were untreated controls (•, A) or treated with 5 mM DFMO alone (C, △). The growth curves of cells treated simultaneously with 5 mM DFMO and 10 μM putrescine were virtually superimposable upon those of untreated control cells and are thus not shown separately. Results are expressed as N/N₀, where N₀ is the number of viable cells on day 0 and N is the number of viable cells on subsequent days. The results are similar if expressed as the number of total cells in culture. Results are from at least 4 separate experiments. The SE is less than 15% of the mean for all data shown.
aggregates are still very small, less than 30–50 μm in diameter, and at a time when differences in pH, oxygen tension, and nutrient should be minimal within different areas of the aggregates. Furthermore, as we reported previously, even large spheroids, in apparent plateau phase growth after 4 to 6 weeks in culture, demonstrated a cytotoxic response to DFMO (16).

Second, it is also possible that our SCC and non-SCC lines are heterogeneous cell populations, and anchorage dependence bestows a growth advantage to DFMO-resistant cells, whereas anchorage independence provides a growth advantage to DFMO-sensitive cells. The lack of change in the biological properties of the cell lines, as measured by doubling times and dopa decarboxylase activities, would argue against a heterogeneous cell population in our cultures, at least in terms of DFMO sensitivity.

Third, DFMO may have a marked effect on the aggregation of the multicellular spheroids. Tunicamycin, which inhibits the synthesis of cell surface glycoproteins, inhibits the initial aggregation of SCC spheroids and results in significant SCC cell death (28). This is unlikely to be a major cause of DFMO cytotoxicity in spheroids. DFMO treatment does not significantly affect the initial aggregation and enlargement of spheroids. Only after spheroids are well formed and have enlarged to a diameter of approximately 60 μm (non-SCC and H82) or 200 μm (OH1) do aggregates begin to lose their multicellular morphology, and cell death ensue. Furthermore, as discussed above, established spheroids in plateau phase growth after 4 to 6 weeks in culture still show a cytotoxic response to DFMO (16).

Fourth, the anchorage dependency may have an effect on, or be reflected in, the cytoskeletal structure of the lung cancer cells. Polyamine starvation in a polyamine- auxotrophic Chinese hamster ovary cell line caused marked disruption of actin filaments and microtubules (29). This interference with the fibrillar cytoskeletal network might be much more detrimental to cells in spheroids, which grow without anchorage dependency, than to cells in monolayer culture. However, we have now found that, in 3T3 cells, DFMO induction of polyamine depletion and growth inhibition had no significant effect on actin filaments or microtubules (30).

Fifth, it is conceivable that the differential cytotoxicity of DFMO may be due to a different degree of polyamine biosynthesis inhibition depending upon the culture conditions. It is possible that anchorage-independent spheroids manifest a more profound suppression of polyamine biosynthesis following DFMO treatment than anchorage-dependent monolayer cells. This appears unlikely because previous studies in 9L rat brain tumor cells grown as monolayers and spheroids did not demonstrate any difference in degree of polyamine depletion (13, 15). Nevertheless, the intracellular levels of polyamines and polyamine-bisynthetic enzymes under basal conditions and following DFMO treatment should be systemically studied and compared in the different cell types growing under different culture conditions.

Finally, anchorage dependency may affect the growth fraction of the cultured cells. SCC is known to have a very high \textit{in vivo} growth fraction (31). Even in the stationary (plateau) phase, SCC spheroids continue to be in a proliferative status, as shown by continued initiated thymidine uptake and incorporation (24). Thus, the rapidly proliferative SCC spheroids may do very poorly as resting nonproliferative cells. Our preliminary studies suggest that SCC and non-SCC spheroids have increased thymidine incorporation during plateau phase growth when compared with SCC and non-SCC monolayer cells.

Whatever mechanism(s) might be operative for our findings, the cell-cell interaction in the lung cancer cell spheroids must play a role in the marked cytotoxic response to DFMO. Cell kill and loss of cell number from culture in both SCC and non-SCC cells are seen at DFMO doses as low as 0.5 mM. Plasma levels in this range are achievable with tolerable doses of DFMO p.o. in Phase I studies (32). Recent studies with heterogeneous mixed-cell spheroids suggest that the cell-cell interaction occurring in spheroids may mimic that of tumors \textit{in situ} (26). Scanning electron microscopy have shown a high degree of cell-cell interaction in the SCC spheroids.\textsuperscript{4} Our results show that the spectrum of human lung cancer cells, from SCC to non-SCC cells, are markedly sensitive to DFMO. If the spheroid model is indeed a closer approximation to human tumors \textit{in vivo} than monolayer cell cultures, our results further suggest potential therapeutic efficacy of DFMO in human lung cancer.

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