Tumor Angiogenesis and Polyamines: α-Difluoromethylornithine, an Irreversible Inhibitor of Ornithine Decarboxylase, Inhibits B16 Melanoma-induced Angiogenesis in Ovo and the Proliferation of Vascular Endothelial Cells in Vitro

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ABSTRACT

α-Difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, inhibited B16 melanoma-induced angiogenesis in chick embryo chorioallantoic membrane and subsequently the growth of the tumor on the chorioallantoic membrane. These inhibitions were mediated by inhibition of polyamine biosynthesis. DFMO strongly inhibited DNA synthesis and proliferation of bovine pulmonary artery endothelial (BPAE) cells in culture and decreased their ornithine decarboxylase activity and intracellular polyamine concentrations. Addition of putrescine to the culture medium of DFMO-treated BPAE cells restored their intracellular putrescine and spermidine concentrations and their DNA synthesis and proliferation. Addition of spermidine to cultures of DFMO-treated BPAE cells restored their intracellular spermidine concentration and their DNA synthesis and proliferation. DFMO inhibited the proliferation of B16 melanoma cells in culture but the inhibitory effect was much less than that on BPAE cells. When one-half of the monolayer of confluent cultures of BPAE cells had been peeled off, addition of DFMO to the cultures inhibited the proliferation and extension of the BPAE cells into the vacant area but had no effect on stationary cells in the remaining half of the monolayer, suggesting that it inhibited induction of proliferation of endothelial cells. These findings suggest that the antitumor activity of DFMO against solid tumors is probably due more to its inhibition of tumor-induced angiogenesis by inhibition of proliferation of endothelial cells induced by polyamine depletion than to a direct effect on tumor cell proliferation.

INTRODUCTION

Growth of solid tumors has been distinguished into two stages, avascular and vascular (1, 2). In the avascular phase, spheroidal tumors grow slowly and do not generally exceed a diameter of a few mm, because of limited supplies of oxygen and nutrients. However, when new capillaries develop in the host and reach the tumors, the tumors grow rapidly. Therefore, inhibition of angiogenesis might be a rational approach to preventing tumor growth (2-4).

The polyamines putrescine, spermidine, and spermine are known to be associated with cellular growth and differentiation (5-10). ODC is the first and rate-limiting enzyme in the pathway of polyamine biosynthesis in animals (5-10). DFMO is a specific, enzyme-activated, irreversible inhibitor of ornithine decarboxylase (11) and has been shown to decrease the concentrations of polyamines in tumors (12-17) and to retard tumor growth in vivo (12-18). Because DFMO also decreases the intracellular concentrations of polyamines in various tumor cells in culture (19-21) and inhibits their in vitro proliferation (19-22), its inhibitory effect on growth of tumors in vivo has been thought to be caused by inhibition of polyamine synthesis by the tumor cells. This speculation might be in part correct, but it is also possible that DFMO inhibits angiogenesis, thereby inhibiting the growth of tumors. To examine the latter possibility, in the present study, we investigated the effects of DFMO on tumor-induced angiogenesis in ovo and compared the effects of DFMO on the proliferations of endothelial cells and tumor cells in vitro.

MATERIALS AND METHODS

Animals and Cells. Single-Comb white leghorn eggs were purchased from Nihon Animals Co., Osaka, Japan. BPAE cells which are registered in the American Type Culture Collection as CPAE cells, Swiss 3T3 fibroblasts, and normal rat kidney cells were purchased from Dainippon Pharmaceutical Co., Suita, Japan. B16 melanoma cells were kindly supplied by Dr. H. Tanaka, Center for Adult Diseases, Osaka, Japan.

Chemicals. DFMO hydrochloride was supplied by Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH. When determined by thin-layer chromatography, no detectable impurity was present in the preparation of DFMO. [6-3H]Thymidine (5 Ci/mmol) and [1-14C]ornithine (50.3 mCi/mmol) were purchased from New England Nuclear, Boston, MA. Putrescine dihydrochloride and spermidine trihydrochloride were from Sigma Chemical Co., St. Louis, MO.

Assay of Angiogenesis Activity. Inhibitory activity of DFMO against tumor-induced angiogenesis was assayed on CAM as described previously (3, 4). Briefly, B16 melanomas maintained in vivo (3, 4) were excised, cut into pieces of 10 mg, and placed on sterile Whatman GF/B glass fiber filter discs (6 mm in diameter; Reeve-Angel, Clifton, NJ) to which DFMO and/or polyamines dissolved in 20 µl of PBS had been added. The same volume of PBS was added to control filters. They were placed upside down on the CAM of 10-day-old chick embryos, incubated at 38°C and 60% relative humidity for 3 or 5 days, and then killed by injection of 10% formalin in PBS. The CAM was excised, fixed in 10% formalin in PBS, inverted, and examined under a stereomicroscope. Capillaries penetrating into tumors on the glass fiber filters were counted and scored by their thickness; thick, middle-sized and small, thin capillaries were assigned 3, 2, and 1 points, respectively, and 1 point was assigned for every 5 minute capillaries. The diameters of tumors on filters were measured in three dimensions with a slide caliper and the tumor weight was calculated as (π/6)abc (a, b, and c are length, width and height) (3).

Inhibitory activity of DFMO against normal neovascularization was assayed on the chick embryo yolk sac vascular membrane as described previously (23) with some modifications. Fertilized embryos were placed in Petri dishes on day 3 and cultured in a humidified incubator in 5% CO2. On day 4, 20 µl of DFMO and/or polyamines dissolved in PBS were mixed with 20 µl of 1% (w/v) saline solution of methyleth
lulose (Tokyo Kasei, Tokyo, Japan) and were introduced into a sterilized silicon ring (6 mm outside diameter, 4 mm inside diameter, 3 mm thick) placed on the yolk sac membrane. Embryos in dishes were further cultured and their photographs were taken 48 h after the administration of DFMO. Angiogenesis was assayed by the density of vessels inside the ring. The response of the yolk sac membrane was scored qualitatively as +++ (complete loss of capillaries), ++ (obvious decrease in the density of capillaries, but not complete loss), + (slight decrease), or − (no effect). The double blind method was used to determine the angiogenesis activity both by CAM assay and by yolk sac assay.

Cell Culture. BPAE cells were cultured in Eagle’s minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 20% FBS (GIBCO) and used between the 12th and 16th passages. B16 melanoma cells, Swiss 3T3 cells, normal rat kidney cells were maintained in serial passages in minimum essential medium containing 10% FBS every 5 days. For experiments, BPAE cells were plated at a density of 6–10 × 10^3 cells/cm² into 24-well plates (16 mm diameter; Corning), 6-well plates (35 mm diameter; Corning), or plastic dishes (35 or 60 mm diameter; Falcon). Other cells were plated at a density of 5–10 × 10^3 cells/cm². All cells were incubated at 37°C under 5% CO2 in air. DFMO dissolved in a small volume of PBS was added to cultures 24 h after cell inoculation, and the same volume of PBS was added to control cultures. The medium was changed on day 3 and then every other day. DFMO was added at the time of each medium change.

DNA Synthesis. The cells were labeled with 1 μCi/ml of [6-3H]-thymidine for 2 h on day 6, when they were proliferating rapidly. [3H]-Thymidine incorporation into acid-precipitable material was determined as described previously (24).

Determination of DNA Content. The cell layers were dissolved in 0.01 N NaOH containing 0.2% Triton X-100. The DNA in the resultant solution was assayed by the method of Kissane and Robins (25), with calf thymus DNA as standard.

Assay of ODC. The cell layer was washed 5 times with ice-cold PBS, collected with a rubber policeman in 0.8 ml of standard buffer, sonicated at 20 kHz for 60 s (two 30-s periods), and centrifuged at 15,000 × g for 15 min at 4°C, as described previously (10). The activity of ODC in the resultant supernatant was determined by measuring the rate of liberation of 14CO2 from [1-l4C]ornithine under the conditions described previously (10).

Determination of Intracellular Polyamine Levels. Samples for assay of intracellular polyamine levels were prepared as described previously (10). Individual polyamines were quantitated by dansylation, separation of the dansyl derivatives by thin-layer chromatography, and measurement of the fluorescent intensity of eluates of the individual derivatives (10, 15, 16).

Other Methods. Protein concentration was determined by the dye binding method of Bradford (26) with bovine serum albumin as a standard. Monolayers of BPAE cells were fixed in formalin and stained with 0.025% Coomasie blue in methanol:acetate-distilled water (5:1, v/v) for 5 h.

RESULTS

Inhibition by DFMO of B16 Melanoma-induced Angiogenesis in CAM. When the B16 melanoma was transplanted onto CAM, it induced capillaries in 5 days (Table 1; Fig. 1A). DFMO, applied to the filter disc, inhibited this angiogenesis dose dependently (Table 1; Fig. 1B). Because the background level of angiogenesis was approximately 2.0, the results in Table 1 indicate that 2 mg/egg of DFMO almost completely inhibited the angiogenesis. At this dose DFMO inhibited growth of the embryos themselves only slightly: the body weight of untreated embryos was 12.4 ± 1.4 g, and that of DFMO-treated embryos was 10.1 ± 1.0 g. DFMO did not cause death of embryos at all. DFMO also inhibited growth of the tumor on CAM, but this inhibition was only about 50% (Table 2). When CAM was harvested 3 days after transplantation of B16 melanoma, marked angiogenesis was already observable, but the tumor had grown only slightly (Table 2). At this time, DFMO inhibited only angiogenesis and had no effect on the tumor weight.

Reversal by Exogenous Polyamines of DFMO-inhibited Angiogenesis. To determine whether the effect of DFMO resulted from depletion of polyamines, we next examined the effect of putrescine and spermidine on DFMO-inhibited angiogenesis (Fig. 1; Table 2). Addition of 6 μmol of putrescine to the filter with DFMO resulted in the reappearance of marked angiogenesis 3 and 5 days after inoculation of the B16 melanoma, and even addition of 2.4 μmol/egg of putrescine caused slight but significant reversal. Spermidine also reversed the effect of DFMO on angiogenesis after 3 and 5 days (Table 2); a dose of 0.6 μmol/egg caused significant reversal; and 1.2 μmol/egg restored angiogenesis to the control level. A higher dose (6 μmol/egg) of spermidine resulted in less reversal effect (data not shown), suggesting its cytotoxicity.

Both putrescine and spermidine also reversed the inhibition by DFMO of increase in tumor weight observed after 5 days (Fig. 1; Table 2). Like their effects on angiogenesis, 6 μmol of putrescine and 1.2 μmol of spermidine restored tumor growth to the control level. At these doses, neither putrescine nor spermidine alone had any effect on melanoma-induced angiogenesis (data not shown).

Comparison of Inhibitory Effects of DFMO on DNA Synthesis and Proliferation of Endothelial Cells and B16 Melanoma Cells in Culture. Addition of DFMO to cultures of BPAE cells in the logarithmic growth phase inhibited DNA synthesis dose dependently (Fig. 2); DFMO caused 50% inhibition at a concentration of 0.2 mM and more than 80% inhibition at 2 mM. On the other hand, DNA synthesis in B16 melanoma cells was not inhibited much by DFMO; even 5 mM DFMO caused only 50% inhibition (Fig. 2). DFMO also inhibited DNA synthesis of 3T3 fibroblasts and normal rat kidney cells. Its inhibition of DNA synthesis in these cells was stronger than that in B16 melanoma cells but less than that in BPAE cells (Fig. 2).

Addition of 2 mM DFMO also strongly inhibited proliferation of BPAE cells, causing about 80% inhibition on day 9 (Fig. 3). In contrast, addition of 2 mM DFMO inhibited proliferation of B16 melanoma cells but the inhibition was only about 30% on days 7 and 9 (Fig. 3).

Decreases by DFMO in ODC Activity and Intracellular Polyamine Concentrations in BPAE cells. As shown in Table 3, ODC activity in BPAE cells treated with 2 mM DFMO for 2 days decreased from 5.8 ± 0.8 fU/egg to 4.7 ± 0.6 fU/egg (Table 3). These results show that DFMO inhibited the activity of ODC in BPAE cells. On the other hand, intracellular spermidine concentrations decreased from 4.3 ± 0.7 fM/egg to 2.4 ± 0.5 fM/egg (Table 3). These results also show that DFMO inhibited the concentration of spermidine in BPAE cells. Decreases in ODC activity and intracellular polyamine concentrations were observed in BPAE cells treated with 2 mM DFMO for 2 days.

| Table 1 Effect of DFMO on B16 melanoma-induced angiogenesis in chick embryo CAM |
|---------------------------------|-----------------|-----------------|
| Treatment | DFMO administered (mg/egg) | Angiogenesis activity a | % inhibition of induction a |
| Control | 0.1 | 5.8 ± 0.8 fU/egg | 29 |
| DFMO | 0.5 | 4.7 ± 0.6 fU/egg | 39 |
| DFMO | 2.0 | 2.4 ± 0.5 fU/egg | 89 |
| a The sum of the values scored as described in “Materials and Methods” was divided by the number of eggs examined and this quantitative value was expressed as the angiogenesis activity. |
| b The percentage of inhibition of induction was determined as Value for control eggs — value for DFMO-treated egg × 100 |
| p < 0.2, smaller versus control. |
| p < 0.01, smaller versus control. |

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Fig. 1. Effects of DFMO on B16 melanoma-induced angiogenesis in chick embryo CAM and growth of the tumors on CAM. Experimental procedures were as described for Table 1. Photographs were taken 5 days after the inoculation of B16 melanoma. A, control CAM with PBS. Many capillaries are introduced into the tumor, which has grown in three dimensions. B, CAM with PBS containing 2 mg DFMO. Few capillaries are seen and the tumor is flat. C, CAM with 2 mg DFMO and 6 μmol putrescine. Many capillaries are again introduced into the tumor, which has grown in three dimensions. D, CAM with 2 mg DFMO and 1.2 μmol spermidine. Marked angiogenesis and marked growth of the tumor are observed as in C (×6.5).

Table 2 Effects of DFMO on B16 melanoma-induced angiogenesis in chick embryo CAM and growth of the tumors on CAM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose of polyamines (μmol/egg)</th>
<th>Angiogenesis activity*</th>
<th>Tumor wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Day 5</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>6.0 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.4 ± 0.6</td>
</tr>
<tr>
<td>DFMO</td>
<td></td>
<td>2.0 ± 0.3&lt;sup&gt;b&lt;/sup&gt; (100)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.3 ± 0.5&lt;sup&gt;f&lt;/sup&gt; (94)</td>
</tr>
<tr>
<td>+ putrescine</td>
<td>2.4</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.4 ± 0.6&lt;sup&gt;f&lt;/sup&gt; (59)</td>
</tr>
<tr>
<td>+ spermidine</td>
<td>6.0</td>
<td>5.6 ± 0.6 (10)</td>
<td>7.5 ± 0.7 (−2)</td>
</tr>
<tr>
<td>DFMO</td>
<td></td>
<td>0.6</td>
<td>ND</td>
</tr>
<tr>
<td>+ putrescine</td>
<td></td>
<td>1.2</td>
<td>5.1 ± 0.6 (23)</td>
</tr>
<tr>
<td>+ spermidine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated as the sum of the values scored as described in "Materials and Methods" divided by the number of eggs.
<sup>b</sup> Days after transplantation.
<sup>c</sup> Mean ± SEM for 16 to 28 eggs in 4 experiments.
<sup>d</sup> p < 0.01, smaller versus control.
<sup>e</sup> Numbers in parentheses, percentage of inhibitions of induction of angiogenesis and of increase in tumor weight, calculated as described in the legend to Table 1.
<sup>f</sup> p < 0.001, smaller versus control.
<sup>g</sup> p < 0.05, smaller versus control.
<sup>h</sup> ND, not determined.
<sup>i</sup> p < 0.05, larger versus DFMO alone.

Days and 6 h from day 1 was only 10% that of control cells (Table 3). Moreover, determination of intracellular polyamine levels in BPAE cells after similar treatments showed that DFMO decreased the putrescine level by 61% and the spermidine level by 75% but had no effect on the spermine level (Table 3).
Additions of polyamines both caused dose-dependent reversal of inhibition of DNA synthesis by DFMO (Table 4). Addition of 10 μM putrescine, which completely restored the intracellular putrescine and spermidine levels, increased the DFMO-inhibited DNA synthesis to the level in control cultures. Addition of 1 μM spermidine, which restored the intracellular spermidine level to the control level, also restored DNA synthesis completely.

In addition to DNA synthesis, the accumulation of BPAE cells per culture was also strongly inhibited by 2 mM DFMO, and putrescine and spermidine reversed this inhibition almost completely (Table 4), but spermine caused only partial reversal,

### Table 3 Effects of DFMO on the activity of ODC and the levels of polyamines in BPAE cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ODC activity (nmol/mg protein)</th>
<th>Putrescine (nmol/mg DNA)</th>
<th>Spermidine (nmol/mg DNA)</th>
<th>Spermine (nmol/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.5 ± 0.3*</td>
<td>79 ± 16</td>
<td>289 ± 68</td>
<td>191 ± 27</td>
</tr>
<tr>
<td>DFMO (2 mM)</td>
<td>0.4 ± 0.1</td>
<td>31 ± 15</td>
<td>72 ± 43</td>
<td>190 ± 64</td>
</tr>
<tr>
<td>+ putrescine (1 mM)</td>
<td>ND*</td>
<td>44 ± 19</td>
<td>180 ± 75</td>
<td>246 ± 44</td>
</tr>
<tr>
<td>+ putrescine (10 μM)</td>
<td>ND*</td>
<td>150 ± 39</td>
<td>362 ± 149</td>
<td>282 ± 76</td>
</tr>
<tr>
<td>+ spermidine (1 mM)</td>
<td>ND*</td>
<td>44 ± 28</td>
<td>266 ± 71</td>
<td>187 ± 75</td>
</tr>
<tr>
<td>+ spermidine (10 μM)</td>
<td>ND*</td>
<td>51 ± 16</td>
<td>386 ± 133</td>
<td>269 ± 65</td>
</tr>
</tbody>
</table>

* Mean ± SD for values in four cultures.

### Table 4 Effects of DFMO on DNA synthesis and proliferation of BPAE cells

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Conc. of polyamines (μM)</th>
<th>DNA synthesis x 10^-3 cpm/well</th>
<th>Cell no. x 10^4/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>24.1 ± 2.7*</td>
<td>100 ± 0.4*</td>
</tr>
<tr>
<td>DFMO (2 mM)</td>
<td></td>
<td>5.3 ± 0.8</td>
<td>22 ± 0.5</td>
</tr>
<tr>
<td>Putrescine</td>
<td>10</td>
<td>21.2 ± 0.8</td>
<td>88 ND*</td>
</tr>
<tr>
<td>Spermidine</td>
<td>1</td>
<td>25.1 ± 0.6</td>
<td>104 ND</td>
</tr>
<tr>
<td>DFMO (2 mM)</td>
<td>+ putrescine (1 μM)</td>
<td>7.5 ± 0.6</td>
<td>31 ± 0.2</td>
</tr>
<tr>
<td>+ putrescine (10 μM)</td>
<td>ND*</td>
<td>17.4 ± 0.6</td>
<td>72 ± 0.8</td>
</tr>
<tr>
<td>+ spermidine (1 μM)</td>
<td>ND*</td>
<td>20.9 ± 0.9</td>
<td>87 ± 1.1</td>
</tr>
<tr>
<td>+ spermidine (10 μM)</td>
<td>ND*</td>
<td>20.0 ± 1.3</td>
<td>83 ± 1.1</td>
</tr>
<tr>
<td>DFMO (2 mM)</td>
<td>+ spermidine (1 mM)</td>
<td>12.8 ± 0.2</td>
<td>53 ± 1.5</td>
</tr>
<tr>
<td>+ spermidine (5 μM)</td>
<td>ND*</td>
<td>17.4 ± 0.3</td>
<td>72 ± 0.8</td>
</tr>
<tr>
<td>+ spermidine (1 μM)</td>
<td>ND*</td>
<td>19.6 ± 0.9</td>
<td>81 ± 1.7</td>
</tr>
<tr>
<td>+ spermidine (3 μM)</td>
<td>ND*</td>
<td>20.3 ± 0.4</td>
<td>84 ± 0.5</td>
</tr>
<tr>
<td>+ spermidine (10 μM)</td>
<td>ND*</td>
<td>17.2 ± 0.5</td>
<td>71 ± 0.2</td>
</tr>
</tbody>
</table>

* Mean ± SD for values in four cultures.

### Table 5 Effect of DFMO on proliferation of bovine carotid artery endothelial cells in culture

Experimental procedures were as described for Table 1, except that bovine carotid artery endothelial cells were used instead of BPAE cells. On day 6, the cells were harvested for determination of the DNA content and cell number. The cell number and DNA content were measured in separate experiments.

<table>
<thead>
<tr>
<th>Cell no.</th>
<th>DNA content %</th>
<th>µg DNA/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.3 ± 1.4±</td>
<td>100</td>
</tr>
<tr>
<td>DFMO (2 mM)</td>
<td>1.9 ± 0.8</td>
<td>107</td>
</tr>
<tr>
<td>Putrescine (10 μM)</td>
<td>8.9 ± 0.8</td>
<td>86 ± 0.1</td>
</tr>
<tr>
<td>Spermidine (1 μM)</td>
<td>7.9 ± 1.6</td>
<td>ND</td>
</tr>
<tr>
<td>DFMO (2 mM) + putrescine (10 μM)</td>
<td>6.6 ± 1.2</td>
<td>86 ± 0.2</td>
</tr>
<tr>
<td>+ spermidine (1 μM)</td>
<td>6.6 ± 0.4</td>
<td>88 ± 0.1</td>
</tr>
</tbody>
</table>

* Mean ± SD for 4 wells.

**Restoration by Polyamines of Proliferation of BPAE Cells Inhibited by DFMO.** To confirm that the growth inhibition of endothelial cells in culture by DFMO was due to depletion of the cells of putrescine and spermidine, we next investigated the effects of exogenous polyamines on intracellular polyamine levels, DNA synthesis, and proliferation of DFMO-treated BPAE cells (Tables 3 and 4). Addition of 10 μM putrescine to the culture medium of DFMO-treated cells resulted in increase in the intracellular putrescine concentration to more than the control level. Addition of putrescine also increased the intracellular spermidine concentration, indicating that incorporated putrescine was effectively converted to spermidine in the cells. Addition of 1 μM spermidine increased the intracellular spermidine concentration to that of control cells but did not affect the putrescine level.

Additions of putrescine and spermidine both caused dose-dependent reversal of inhibition of DNA synthesis by DFMO.
Fig. 4. Effects of DFMO on quiescent BPAE cells and BPAE cells stimulated to proliferate. BPAE cells were inoculated at a density of $1.5 \times 10^5$ cells/35-mm-diameter well of 6-well plates and cultured in the absence of DFMO. When the cells became confluent, one-half of each cell layer was peeled off with a rubber policeman along a line marked with black ink. The remaining halves of the cell layers were then cultured further in the presence of DFMO (5 mM) and/or putrescine (10 $\mu$M) or spermidine (10 $\mu$M). The medium was renewed 48 h later with addition of DFMO and polyamines at the same time. Phase-contrast photomicrographs of cells from which the monolayers had been removed were taken 48 h after the second change of medium (E–H) ($\times$ 67) and then the cultures were fixed with methanol and stained with Coomassie blue (A–D). Addition to culture medium: (A and E) PBS; (B and F) 5 mM DFMO; (C and G) 5 mM DFMO and 10 $\mu$M putrescine; (D and H) 5 mM DFMO and 10 $\mu$M spermidine.

apparently due to its cytotoxic effect (data not shown). Inhibition by DFMO of Proliferation of Bovine Carotid Artery Endothelial Cells in Culture and Its Reversal by Exogenous Polyamines. In addition to BPAE cells, DFMO also strongly inhibited proliferation of bovine carotid artery endothelial cells in culture (Table 5). DFMO at 2 mM almost completely inhibited their proliferation and its inhibition was reversed by the addition of 10 $\mu$M putrescine or 1 $\mu$M spermidine.
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Table 6 Effects of DFMO on neovascularization in yolk sac membrane of chick embryos

Twenty µl of DFMO and/or polyamines in PBS were mixed with the same volume of 1% methylcellulose and introduced into silicon rings placed on yolk sac membrane of 4-day-old chick embryos. Forty-eight h after the application of agents, angiogenesis was assessed as described in the text. Other experimental procedures were as described in the text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of eggs used</th>
<th>No. of eggs with antiangiogenesis activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>DFMO (2 mg/egg)</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>+ putrescine (1.2 µmol/egg)</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>+ spermidine (0.6 µmol/egg)</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>DFMO (1 mg/egg)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>DFMO (0.5 mg/egg)</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>DFMO (0.2 mg/egg)</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

* The scoring of angiogenesis is described in the text.

DFMO Acts on Only Endothelial Cells Induced to Proliferate.
For determination of whether DFMO acts only on rapidly proliferating endothelial cells, BPAE cells were first grown in confluence without DFMO and then one-half of the BPAE cell layer was peeled off with a rubber policeman along a line marked with black ink (Fig. 4). Then, 5 mM DFMO was added and the cultures were incubated for 4 days. BPAE cells in control cultures proliferated and extended into the vacant area (Fig. 4A), whereas BPAE cells cultured in the presence of DFMO did not (Fig. 4B). However, on addition of 10 µM putrescine with DFMO or 10 µM spermidine with DFMO BPAE cells proliferated and extended into the vacant area (Fig. 4, C and D). DFMO had no effect on the morphology of BPAE cells in the remaining half of the monolayer (Fig. 4E and F), and putrescine and spermidine also did not affect the morphology of the cells (Fig. 4, G and H).

Inhibition by DFMO of Neovascularization in Yolk Sac and Its Reversal by Exogenous Polyamines. When DFMO (2 mg/ml) was administered to the yolk sac membrane of 4-day-old chick embryo which showed rapid neovascularization, marked inhibition of angiogenesis was observed on day 6 (Table 6; Fig. 5, A and B), indicating that DFMO also inhibits normal neovascularization. The inhibitory effect of DFMO on angiogenesis was dose dependent and observable at a dose of 0.2 mg/egg (Table 6). The inhibitory activity was also time dependent and observable as early as 12 h after administration (data not shown).

To determine if the effect of DFMO resulted from depletion of polyamines, the effects of putrescine and spermidine on DFMO-inhibited angiogenesis were investigated (Table 6; Fig. 5). Administration of 1.2 µmol/egg of putrescine on yolk sac with DFMO resulted in reappearance of normal neovascularization in yolk sac. Spermidine also reversed the effect of DFMO on the neovascularization.

DISCUSSION

In the present study we showed that DFMO inhibited B16 melanoma-induced angiogenesis in chick embryo CAM and the
growth of the tumor on CAM (Fig. 1; Tables 1 and 2) and that DFMO inhibited the proliferations of both BPAE cells and B16 melanoma cells in culture (Fig. 3). Moreover, DFMO inhibited normal, rapid neovascularization in chick yolk sac membrane (Fig. 5; Table 6). During tumor development there is a kind of symbiosis of tumor cells and endothelial cells, and the tumor tissue must be vascularized to grow, so that DFMO may exert its antitumor activity by two mechanisms: (a) the generally accepted mechanism of direct inhibition of tumor cell proliferation by polyamine depletion; (b) through inhibition of rapid tumor-induced proliferation of endothelial cells. Our findings that the antiangiogenic activity preceded the antitumor action, demonstrated by CAM assay, and that DFMO caused greater inhibitions of DNA synthesis and proliferation of BPAE cells than of B16 melanoma cells (Figs. 2 and 3) suggest that DFMO exerts its antitumor activity by inhibiting the proliferation of endothelial cells induced by tumors rather than by a direct inhibitory effect on the proliferation of tumor cells. The finding that DFMO inhibited not only tumor-induced angiogenesis but also normal, rapid neovascularization, demonstrated by yolk sac assay (Fig. 5; Table 6), also supports this proposal.

In contrast to its effect on rapidly proliferating cells, DFMO had no cytotoxic effect on endothelial cells in confluent cultures (Fig. 4). This finding is important because it suggests that DFMO would not have side effects on preexisting blood vessels. In this regard, DFMO has been reported to be cytostatic to various cells (6).

DFMO inhibited the ODC activity in rapidly proliferating BPAE cells in culture and decreased their intracellular putrescine and spermidine concentrations (Table 3). Addition of putrescine increased the intracellular putrescine and spermidine concentrations in DFMO-treated BPAE cells while addition of spermidine increased the intracellular spermidine concentration only (Table 3). Addition of putrescine and spermidine both reversed the inhibition by DFMO of proliferation of endothelial cells (Tables 3 and 5) and exogenous putrescine and spermidine also both restored DFMO-inhibited tumor angiogenesis in ovo (Tables 1 and 2; Fig. 1). These findings suggest that depletion of polyamines, especially spermidine, in endothelial cells induced by DFMO caused growth arrest of endothelial cells, thereby inhibiting angiogenesis.

Some angiogenesis factors are known to be proteins or peptides (27, 28). Because polyamines have been shown to play important roles not only in cell proliferation but also in protein synthesis (5–9), DFMO might inhibit the production of angiogenesis factors by B16 melanoma, although its inhibitory effect on the proliferation of B16 melanoma cells was weak (Figs. 2 and 3). However, in many in vivo experiments which have shown the antitumor activity of DFMO, DFMO has been administered systemically (13–18), and as systemically administered drugs reach tumors through blood vessels, the primary target of DFMO is probably endothelial cells which are induced to proliferate by a tumor angiogenesis factor(s).

In addition to inhibiting tumors that have already developed and become vascularized, DFMO has been reported to inhibit tumor promotion in two stage carcinogenesis in mouse skin (15, 16). Angiogenesis is required for development of visible papillomas and a potent tumor promoter, 12-O-tetradecanoyl-13-acetate, has been reported to induce angiogenesis in CAM and rabbit cornea (29) and formation by endothelial cells of vessel-like structures in a collagen matrix in vitro (30, 31). Thus the inhibitory effect of DFMO on tumor promotion in mouse skin may be due not only to its direct effect on promoted epidermal cells but also to its antiangiogenic effect. If this is the case, tumor promotion in mouse skin should be divided into at least two stages, a true, avascular stage of epidermal cell promotion and a stage of angiogenesis.

DFMO has been reported to inhibit spontaneous metastasis of Lewis lung carcinoma (32–34) and spontaneous pulmonary metastasis (34) as well as pulmonary metastasis induced by i.v. injection of B16 melanoma cells (35). However, the mechanism by which DFMO inhibits tumor metastasis has not been clarified. Neovascularization should be required in formation of new metastatic foci. Therefore, the inhibitory effect of DFMO on metastasis may be due to its antiangiogenic activity.

From the above findings and considerations, it is clear that in development of antitumor agents, not only direct effects on tumor cells but also effects on endothelial cells must be investigated, because angiogenesis is important in various steps of development of tumors, such as tumorigenesis, the growth of tumor tissues and metastasis.

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EFFECT OF ODC INHIBITOR ON ANGIOGENESIS


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