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Effects of Difluoromethylornithine on Growth Inhibition and Apoptosis in Human Cervical Epithelial and Cancerous Cell Lines[1]

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Objective. **Difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase and an angiogenesis inhibitor, has been used in phase I cervical intraepithelial neoplasia (CIN) trials, producing a 50% regression of CIN 3 lesions. DFMO is currently in phase II trials. In the experiments reported here, DFMO's growth inhibition and apoptosis induction were explored in an** *in vitro* **model to elucidate mechanisms of action.**

Methods. **Four immortalized cervical epithelial cell lines, serving as** *in vitro* **models of precancerous CIN lesions, and nine cervical carcinoma cell lines were studied. DFMO's growth inhibitory effect was tested in monolayer culture and in semisolid medium, and concentrations required for a 50% growth inhibition (IC_{50}) with a 5-day treatment were determined. Apoptosis induction was analyzed using the terminal deoxynucleotidyl transferase assay of DNA fragmentation.**

Results. **DFMO inhibited growth of immortalized cervical epithelial cell lines and cervical cancer cell lines in monolayer culture and in semisolid medium. The immortalized cervical epithelial cell lines were more sensitive than the cervical cancer cell lines to DFMO's growth inhibitory effect. Concentrations required for 50% growth inhibition after a 5-day treatment ranged from 100 μM to >5 mM for cervical carcinoma cell lines and from 100 μM to 1 mM for immortalized cervical epithelial cell lines. DFMO induced apoptosis in precancerous and cancerous cell lines at a concentration of 5 mM, regardless of the cells' human papillomavirus status.**

Conclusion. **DFMO inhibits the growth of cervical precancerous and cancerous cells** *in vitro* **in a dose-dependent and time-dependent manner, partially through inducing apoptosis.**

INTRODUCTION

Chemopreventive agents that block cervical carcinogenesis yet are well tolerated are needed [1–4]. One attractive strategy involves blocking the synthesis of polyamines (e.g., putrescine, spermidine, spermine) with ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis. The polyamines are organic polycations known to play important roles in biological functions [5–7], especially in cell maintenance, proliferation, differentiation, neoplastic transformation [8–13], apoptosis, and antiangiogeneis [7, 14]. Although the mechanisms of action of natural, ubiquitous polyamines are not entirely clear, it is known that they interact with nucleic acids, proteins, membranes, and several intracellular organelles. Polyamines can interact with DNA by inducing conformational changes in its structure [15]. The gene encoding ODC is a putative protooncogene considered crucial for the regulation of cellular growth and transformation [5, 9]. The most effective way to inhibit polyamine biosynthesis is to inhibit ODC selectively. Difluoromethylornithine (DFMO) covalently binds to ODC, thus inhibiting proliferation and inducing apoptosis by acting as a specific suicide inhibitor. DFMO has shown antitumor and antimetastatic activity in several animal models of carcinogen-induced cancers, including cancers of the colon, bladder, trachea, breast, stomach, liver, intestine, oral cavity, and skin [5, 8, 9, 14, 16].

A clinical trial of DFMO in patients with cervical intraepithelial neoplasia (CIN) 2 and 3 (high-grade dysplasia) is now being carried out by our group. A phase I study of DFMO in the cervix was completed previously in which 50% of patients with CIN 3 had partial or complete responses [17]. Quantitative analysis of histopathological biomarkers showed statisti-

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cally significant decreases in DNA content in all specimens at all dosages (0.06 to 1.0 g/m^2). Decreases in DNA content were seen in both types of patients (those whose histology indicated a response and those whose histology did not), though decreases were most significant among those whose histology indicated a response [18]. In the present study, we are using a number of immortalized human cervical epithelial cell lines as precancerous cell lines that mimic CIN *in vivo* and carcinoma cell lines as models of cervical malignancy to study the mechanism of DFMO's effects on cervical carcinogenesis. Here we report the effects of DFMO on growth inhibition and apoptosis induction in cell lines and discuss how the results will help design new cervical cancer chemoprevention trials using DFMO.

MATERIALS AND METHODS

Cell Cultures

Nine human cervical carcinoma cell lines (C33A, C4I, C4II, Caski, HeLa, HT3, ME180, MS751, and SiHa) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Four human cervical epithelial cell lines (Z132, Z173, Z183, and TCL1) were kindly provided by Dr. Vittorio Defendi, Kaplan Cancer Center, New York University Medical Center, New York). All four of the epithelial cell lines were transfected with cloned viral DNAs from HPV types 16 and 18 by electroporation [19].

Cervical cancer cells were cultured in monolayers in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's minimal essential medium and Ham's F12 medium containing 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2:95% air.

DFMO

DFMO was obtained from Dr. Kenji Nishioka (M. D. Anderson Cancer Center, Houston, TX) and Dr. Ajit Verma (University of Miami, Miami, FL). DFMO was dissolved in distilled water at a concentration of 1 M, sterilized by filtration through a 0.22-μm-pore membrane, and stored as a stock solution at −20°C. Before each experiment, the stock solution was diluted to the appropriate final concentrations (0.1, 0.5, 1, and 5 mM) with growth medium.

Growth Inhibition Assays

The exponentially growing cells were seeded at densities ranging from 1000 to 3000 cells per well in 96-well culture plates overnight. The cells were treated with DFMO at concentrations of 0.1, 0.5, 1, and 5 mM for 5 days. Cell cultures grown in medium alone served as controls. In all cultures, the medium was changed at the third day of treatment.

Growth inhibition was determined by the sulforhodamine B (SRB) assay. In brief, the medium on each plate was discarded, and the adherent cells were fixed with 100 μl of 10% (w/v) cold trichloroacetic acid and incubated for 60 min at 4°C. The plates were then rinsed five times with deionized water, air-dried, replenished with 50 μl of 0.4% (w/v in 1% acetic acid) SRB solution (Sigma Chemical Co., St. Louis, MO), and incubated for 10 min at room temperature. Then the staining SRB was solubilized with 100 μl of 10 mM unbuffered Tris base (pH 10.5), and the optical density was read by using a microtiter plate reader at 492 nm. The percentage of growth inhibition (% GI) was calculated by using the formula

$$\% \text{ GI} = (1 - N_t/N_c) \times 100,$$

where N_t is the optical density of stained cells in treated cultures and N_c is the optical density of stained cells in control cultures. All experiments were performed in triplicate, and the means ± SD were calculated. The drug concentration required to inhibit cell growth by 50% (IC_{50}) was determined by interpolation from dose–response curves.

Colony Formation Assays

The cervical cancer cell line HeLa and the immortalized human cervical epithelial cell lines Z173, Z183, and TCL1were grown in matrigel basement membrane matrix (MBMM) (Collaborative Biomedical Products, Bedford, MA). The MBMM was prepared as described by Oridate *et al.* [20]. In brief, the MBMM was mixed to homogeneity and added to Costar six-well culture plates at 200 μl/well. The plates were then stored on ice until used (cooled pipes were used to mix and suspend the MBMM). When needed, the plates were taken off the ice, allowed to stand at 37°C for 30 min, and then covered with a 1:1 (v/v) mixture of DMEM and Ham's F12 medium containing 10% FBS. Cervical cancer cells and precancerous cells were then placed on top of the gel and supplemented with 0.1, 0.5, or 1 mM DFMO or distilled water (control). Cells were then grown in the MBMM for 2 to 3 weeks until colonies grew. The colonies were counted under a microscope, and growth inhibition was calculated.

TUNEL Assay of Apoptosis

Cells were analyzed for apoptosis by using a modification of the method described by Gavrieli *et al.* [21]. In brief, after incubation with 1 or 5 mM DFMO, cells were fixed in 1% formaldehyde in PBS (pH 7.4) for 15 min at 4°C. The cells were then washed twice with PBS, resuspended in 70% cold ethanol, and transferred to a freezer, where they were kept at −20°C until use. When needed, the cells were resuspended in 1 ml of a wash buffer containing cacodylic acid, Tris–HCl-buffered solution, and sodium azide (Phoenix Flow Cytometry Kit, Phoenix Flow Systems, San Diego, CA). Then approximately 5×10^6 cells were resuspended in 50 μl of a staining buffer containing Tris–HCl buffer, TdT enzyme, and fluorescein-12-dUTP (Phoenix Flow Cytometry Kit); incubated at 37°C for 60 min; and then rinsed twice with PBS. The cells were stained with 500 μl of propiodium iodide/RNase A so-

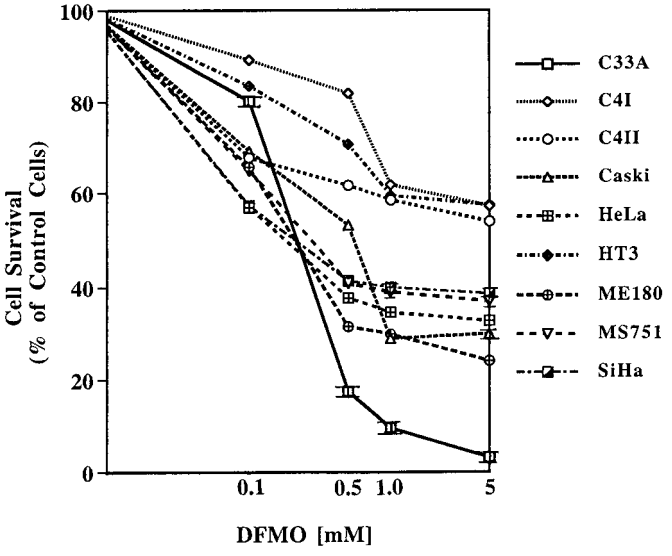

FIG. 1. Effect of DFMO on growth of human cervical carcinoma cell lines. Cells were grown in the absence (control) or presence of 0.1, 0.5, 1, and 5 mM DFMO. Data presented are means ± SD of triplicate determinations.

lution in the dark for 30 min at room temperature and analyzed by flow cytometry with a FACScan flow cytometer (Epics Profile, Coulter Corp., Hialeah, FL) equipped with a 15-mW argon laser for excitation at 488 nm. Fluorescence was measured at 570 nm. The flow cytometry kit included cell suspensions that served as negative and positive controls for apoptosis. Computer analysis of the data provided information on the percentage of apoptotic cells as well as the proportion of cells in the hypodiploid (HD), G_1, S, and G_2 phases of the cell cycle.

RESULTS

Growth Inhibition

DFMO inhibited the growth of both the immortalized cells and cervical cancer cells at concentrations of 0.1, 0.5, 1, and 5 mM. Growth of most of the cancer cell lines, including ME180, HeLa, Caski, SiHa, and MS751, was inhibited about 60–70% at 1 mM (Fig. 1). The C33A cell line was the most sensitive of all the cell lines to the effects of DFMO and showed 80% inhibition at 0.5 mM. At that same level, DFMO inhibited growth by at least 50% in all immortalized cervical epithelial cell lines (Fig. 2).

The time course of DFMO effect on cervical cancer cell lines was determined by analyzing the 5-day growth curve. Most of the cervical cancer cell lines started to respond to DFMO treatment at Day 3 (Fig. 3). Three of these lines, C33A, HeLa, and ME180, were especially sensitive to DFMO's growth inhibitory effect.

The morphology of growth inhibition in cervical cancer cells ME180 and immortalized cervical epithelial cells TCL1 are shown in Fig. 4. The IC_{50} of DFMO for each cell line was determined, and the response of these cell lines to DFMO appeared to be independent of the HPV status of the cell line (Table 1) [22].

Inhibition of Colony Formation

The ability of DFMO to inhibit colony formation was also tested in MBMM. Selected lines—the cervical cancer HeLa cells and the immortalized human cervical epithelial cell lines Z173, Z183, and TCL1—were grown in MBMM. Cervical cancer cells and immortalized cells were placed on the top of the matrigel and were incubated with 0.1, 0.5, 1, and 5 mM DFMO for 2 to 3 weeks. The results of their growth inhibition assay were similar to those from the assay of cells (TCL1, Z183, and Z173) grown in monolayer cultures (Fig. 5). However, there was a difference in response to DFMO between monolayer culture and MBMM culture in cervical cancer HeLa cells. The growth inhibitory effect of DFMO was approximately 80% at 0.5 mM in MBMM, but there was a 50% growth inhibitory effect in monolayer culture. At 1 mM DFMO inhibited HeLa colony formation by 90% (Fig. 5) compared with 60% inhibition in monolayer cultures.

Induction of Apoptosis by DFMO

After treatment with 1 or 5 mM DFMO for 5 days, cells were analyzed for apoptosis by the TUNEL assay. Apoptosis induction was found to be dose dependent in both immortalized cervical epithelial cell lines and cervical carcinoma cell lines, with apoptotic cells increasing, for example, in the C33 cell line from 12.2% in the control to 18% in cells treated with 1 mM DFMO to 35.3% in cells treated with 5 mM DFMO (Fig. 6).

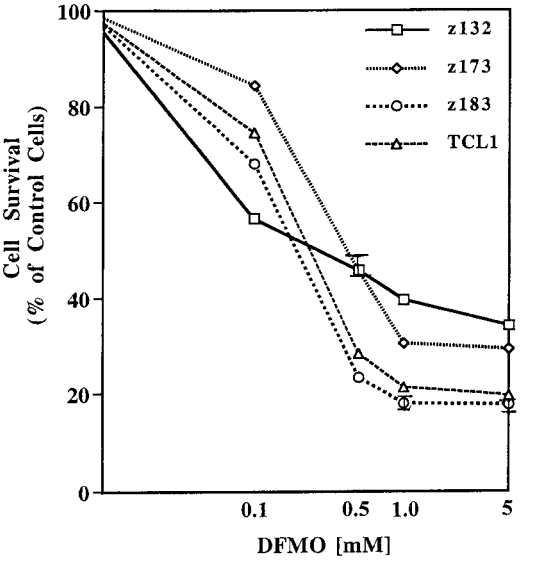

FIG. 2. Effect of DFMO on growth of immortalized cervical epithelial cell lines. Cells were grown in the absence (control) or presence of 0.1, 0.5, 1, and 5 mM DFMO. Data presented are means ± SD of triplicate determinations.

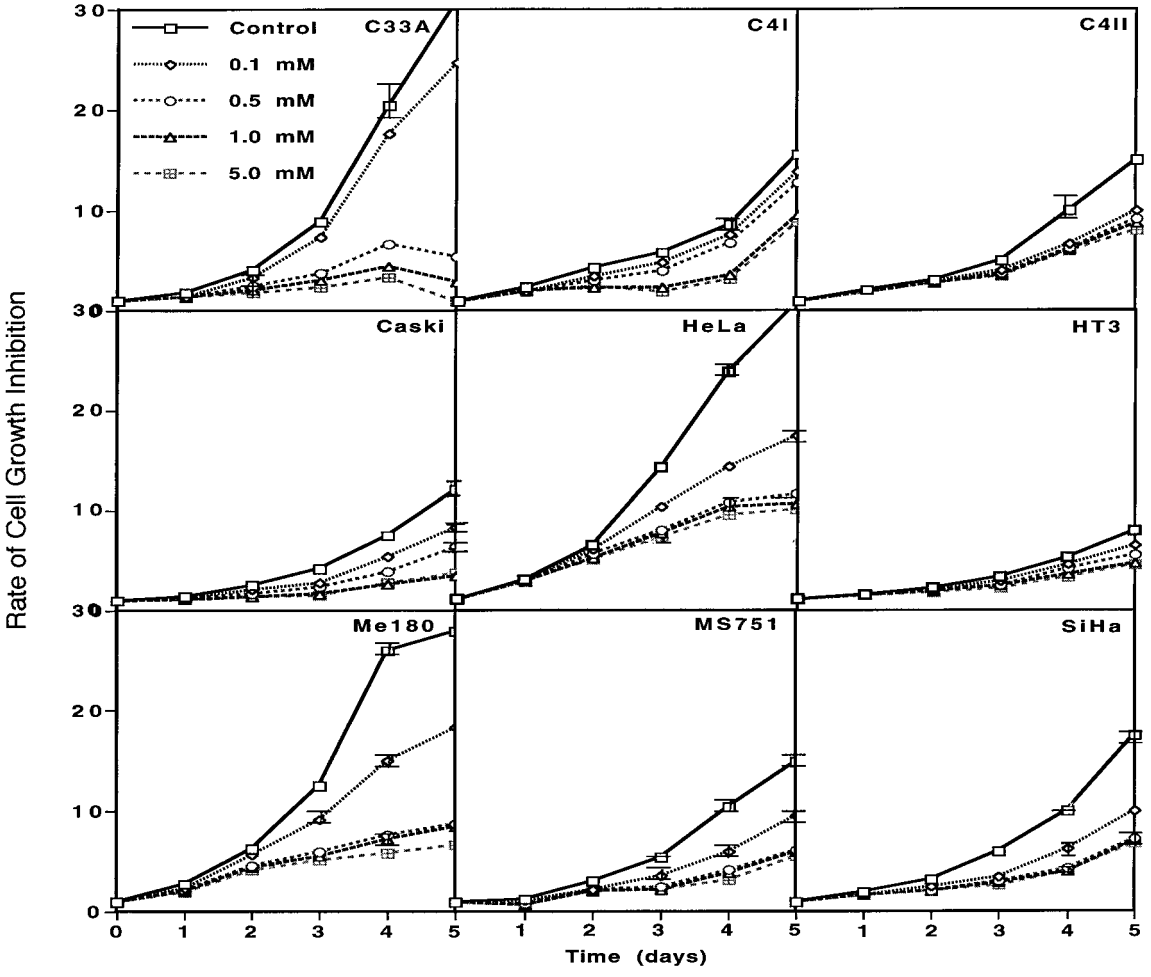

FIG. 3. Time course of effect of DFMO on growth of human cervical carcinoma cell lines. Cells were treated with 0.1, 0.5, 1, and 5 mM DFMO. Cells were harvested daily from Day 1 to Day 5, and their optical density was determined.

DISCUSSION

As a chemopreventive agent, DFMO has been found to be effective in experimental animals [12, 16, 23] and also effective in a phase I dose deescalation trial among women with a histological diagnosis of CIN 3 [6, 7, 17, 18]. In the human trial, a dose of 1.0 g/m^2 DFMO produced no major toxicity in patients with reduced tissue spermidine/spermine ratio and increased the plasma arginine level [17, 18]. In the present study, we investigated the ability of DFMO to inhibit growth and induce apoptosis in two *in vitro* models: one for CIN (immortalized cervical epithelial cell lines) and the other for cervical cancer (cervical carcinoma cell lines). To our knowledge, this is the first report of the effect of DFMO on both cervical precancerous and cancerous cell lines.

Apoptosis can be induced by a variety of intracellular signals and external agents, including those that induce terminal differentiation, DNA damage, or both. Apoptosis induction may protect the organism by eliminating genetically damaged cells from replication, thus decreasing the risk of malignant transformation of damaged cells [24, 25]. Carcinogenesis is often associated with a decreasing tendency of cells to undergo apoptosis in response to certain physiological stimuli and cytotoxic agents. The result is the initiation, promotion, and, ultimately, growth of a tumor. Therefore, agents that can induce apoptosis, growth arrest, or both in premalignant cells have potential as agents that prevent future cancers and treat existing ones [6, 26–28]. Recently, several reports have demonstrated that DFMO, in addition to regulating cell proliferation and differentiation in tumor cells *in vivo* and *in vitro*, can also induce apoptosis [11, 27–34]. We plan to examine p53 and p21 regulation in our next series of experiments.

The doses of DFMO were chosen based on the literature. A comparative report is that by Seidenfeld *et al.* [35]. Using a molecular weight for DFMO of 236.65 g/mol and using the following formula to convert the drug concentration used in the laboratory,

$$\text{drug concentration (mM/liter)} = \text{drug concentration } (\mu\text{g/ml}) \times 1000/\text{molecular weight of drug (g/mol)},$$

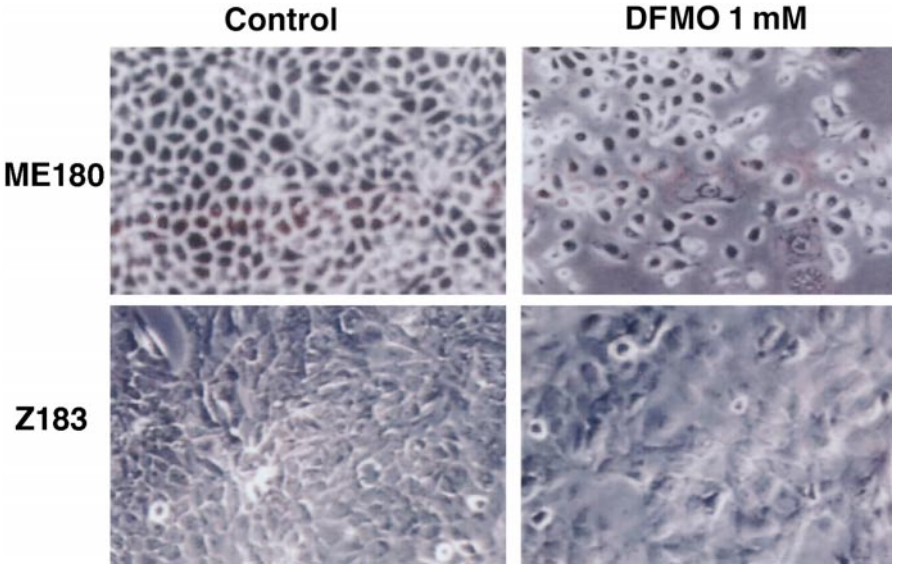

FIG. 4. Effect of DFMO on morphology of cervical cancer cells. Here ME180 cells (cervical cancer cells) and Z183 (cervical epithelial cells) are compared before treatment (control) and after treatment with 1 mM DFMO for 5 days.

we determined the drug concentrations in micrograms per milliliter (Table 2). Table 3 indicates the drug concentrations based on the five dose levels used in our phase I trial, calculated by multiplying the total drug dose by an average body surface area of 1.4, converting to micrograms, and dividing the result by the average blood volume of 5000 ml. From this table, we see the concentrations chosen for the laboratory study,

TABLE 1
DFMO Concentrations Required for 50% Growth Inhibition *in Vitro*

Cervical cell line[a]	Type of differentiation[b]	Human papillomavirus status[c]	IC_{50} for DFMO (mM)
Cancer cells			
ME180(met)	Epidermoid	HPV 39+ HPV 18+	0.6
MS751	Epidermoid	HPV 18+ HPV 45+	0.8
C4I	Epidermoid	HPV 18+	>5.0
C4II	Epidermoid	HPV 18+	>5.0
HeLa	Epithelioid	HPV 18+	0.8
C33A	Epithelioid	ND	0.3
Caski(met)	Epidermoid	HPV 16+ HPV 18+	0.5
SiHa	Epidermoid	HPV 16+	0.6
HT3	Epithelioid	ND	>5.0
Epithelial cells			
Z132	Epithelioid	HPV 16+	0.8
Z173	Epithelioid	HPV 18+	0.6
Z183	Epithelioid	HPV 18+	0.3
TCL1	Epithelioid	HPV 18+	0.4

[a] "Met" indicates that the cell line was derived from metastasis.
[b] Differentiation based on the American Type Culture Collection catalog.
[c] HPV status as reported by Hamada *et al.* [22] or the American Type Culture Collection catalog; ND, HPV not detected.

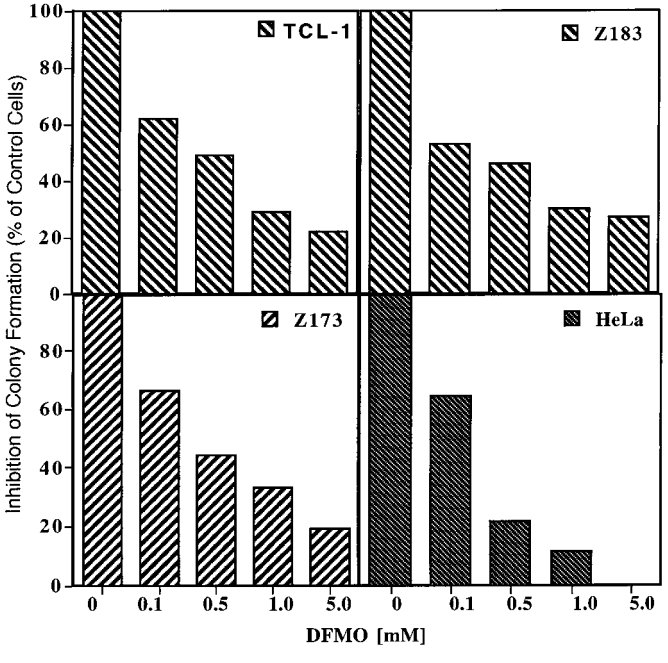

FIG. 5. DFMO-induced inhibition of colony formation by immortalized cervical epithelial TCL1, Z183, and Z173 cells and human cervical carcinoma HeLa cells in MBMM. In each experiment, 105 cells/well were placed on top of the MBMM and treated with DFMO at concentrations ranging from 0.1 to 5 mM in TCL1, Z183, and Z173 cells and from 0.1 to 1 mM in HeLa cells. Untreated cells were used as controls. The colonies were counted under a microscope, and the growth inhibition was calculated as described under Materials and Methods.

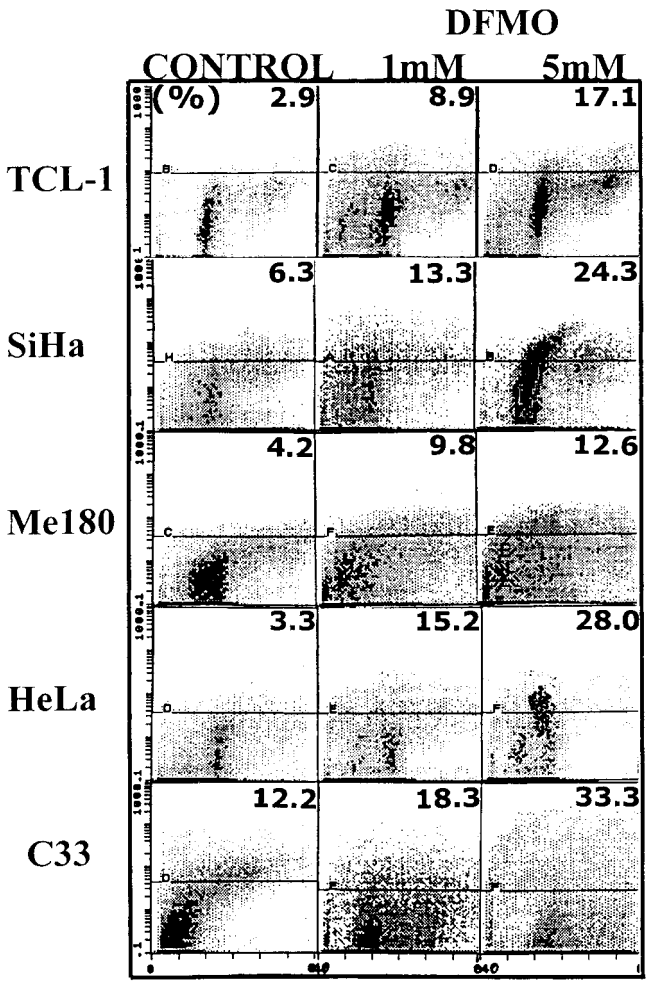

FIG. 6. Effects of DFMO on apoptosis in cervical cancer cell lines (SiHa, ME180, HeLa, and C33) and an immortalized cervical epithelial cell line (TCL1). As described under Materials and Methods, cells were treated with 1 and 5 mM DFMO for 5 days and then harvested, stained with fluorescein-labeled dUTP to label DNA fragments, and then stained with propidium iodide for TUNEL analysis of DNA content. The fluorescence of viable and apoptotic cells is represented by the dark dots below (viable) and above (apoptotic) the demarcation line, which was determined by a standard cell provided with the labeling kit. The percentage of apoptotic cells as determined by flow cytometry is shown in each panel.

ranging from 0.1 to 1.0 mM, correspond to doses ranging from 0.125 to 1 g/m^2/day. The 5 mM laboratory dose would correspond to 3 g/m^2/day, a dose that has been used in some trials.

We evaluated DFMO's effect on both precancerous and cancer cell lines and found that it effectively inhibited growth and induced apoptosis in both types. However, this growth inhibition was stronger in cancer cells grown on MBMM than in cancer cells grown in monolayers, suggesting the assay of colony formation on MBMM was more sensitive than the assay of growth inhibition in monolayers. The MBMM is solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm mouse sarcoma, a tumor rich in matrix proteins containing tumor growth factor β, fibroblast growth factor, tissue plasminogen activator, and other growth factors. MBMM is effective for the attachment and differentiation of both normal and transformed anchorage-dependent epithelium and other cells because there is less death from causes other than the effect of the drug under study. We also found that DFMO's growth inhibitory effect on cervical cancer cells was especially strong (about 80% at 0.5 mM). DFMO induced apoptosis in both precancerous and cancer cells in a dose-dependent manner.

A subject of controversy is DFMO's ability to cause apoptosis. A rat ulcer model showed that polyamines repair gastric and duodenal erosion [36, 37]. Here DFMO depletes the polyamine supply, thus inhibiting cell proliferation and preventing healing [36, 37]. There have been several reports of the inhibition of polyamine synthesis causing G_1 arrest and a concomitant increase in p53 and p21 expression without inducing apoptosis [33, 34]. In fact, polyamine depletion by DFMO or other agents can increase p53, p21, and p27 expression in IEC-6 epithelial cells of the small intestine and MALME-3M melanoma cells and induce wild-type p53 function in the regulation of cell cycle arrest [31–34]. DFMO has also been found to completely block ODC activity and inhibit the growth of Ramos cells and human pre–T cells without inducing apoptosis [38, 39]. Together these results suggest that polyamine depletion by DFMO affects cell growth through cell cycle arrest but not through apoptosis. Nonetheless, Seidenfeld *et al.*

TABLE 2
Laboratory Drug Concentration Conversion to Drug Concentration

Drug concentration in lab cell culture			
mM	μmol/liter	Conversion formula	Drug concentration (μg/ml)
0.1	100	$n \times 1000/237$	23.7
0.5	500	$n \times 1000/237$	118.5
1.0	1000	$n \times 1000/237$	237
5.0	5000	$n \times 1000/237$	1185

TABLE 3
Drug Concentrations in Phase I Study

Drug dose (g/m^2/day)	Total drug $\times$ 1.4 (average BSA)[a]		Average blood volume (ml)	Drug concentration (μg/ml/day)
	g	μg		
0.06	0.08	80,000	5000	16
0.125	0.18	180,000	5000	36
0.25	0.35	350,000	5000	70
0.5	0.7	700,000	5000	140
1.0	1.4	1,400,000	5000	280

[a] Average body surface area.

and others [35, 40, 41] have shown that DFMO could markedly change the cell cycle fraction in four different noncervical carcinoma cell lines (HuTu-80, HT-29, MCV-7, and A-427), but not in a cervical cancer line (ME180). In our study, apoptosis was identified in ME180 cells. Thus the growth inhibitory effect may involve two different regulatory pathways: G_1 arrest and apoptosis induction. In our study, the growth inhibitory effect of DFMO in most of the cervical precancerous and cancerous cell lines we tested was mediated by apoptosis induction.

The growth of cancer or precancer cells can be inhibited by causing cell cycle arrest, inducing apoptosis, or both. In this study, we found that the chemopreventive agent DFMO affects cervical precancerous and cancer cells in a dose- and time-dependent manner by induction of apoptosis. We plan more studies in both precancerous and cervical cancer cells of DFMO's regulation of apoptosis-related and cell cycle-regulated genes. By combining clinical data on the chemoprevention of cervical dysplasia with data from the laboratory, it should be possible to develop new surrogate biomarkers and design a rationale for future chemoprevention studies to reduce both the progression of severe cervical dysplasia to cervical cancer and the recurrence of invasive cervical cancer.

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