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Polyamine Contents in Rectal and Buccal Mucosae in Humans Treated with Oral Difluoromethylornithine¹

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Abstract

Difluoromethylornithine (DFMO) is an investigational chemopreventive agent that inhibits ornithine decarboxylase (ODC) activity, lowers cellular polyamine concentrations, and decreases cell proliferation *in vivo* and *in vitro*. In five subjects we have compared the polyamine concentrations in rectal mucosal biopsies and in exfoliated buccal mucosal cells (EBM) before and after DFMO treatment to assess the suitability of EBM as an easily accessible marker tissue for DFMO suppression of polyamine synthesis in the rectal mucosa. One month of 3 g/m²/day of DFMO treatment caused a statistically significant decrease in putrescine and spermidine concentrations in rectal mucosa biopsy specimens but not in EBM samples. ODC activity in EBM was high (~1 μmol/min/mg protein), resistant to DFMO inhibition ($K_i = 4200 \mu\text{M}$), dependent on GTP concentration (maximal at 0.1 mM), and was reduced concomitantly with bacterial concentration by antiseptic mouthwashing. Bacteria adherent to EBM were visible by electron microscopy. Forty bacterial colonies/ng protein were culturable from washed EBM samples. Oral bacteria preclude the use of EBM samples as a marker tissue of DFMO effect in the rectal mucosa, but oral DFMO therapy is effective in depleting polyamines in rectal mucosa.

Introduction

ODC¹ activity is elevated in many epithelial cancers and precancers, and polyamine synthesis has been implicated in carcinogenesis in the gastrointestinal tract (1-3). Polyamines are cytoplasmic and nuclear polycations that are required for cell proliferation and differentiation (4, 5). ODC is the rate-limiting enzyme in polyamine synthesis. The competitive suicide enzyme inhibitor DFMO decreases ODC activity, polyamine concentrations, and

growth in cell cultures (3, 6). The antiproliferative effect of DFMO results from the depletion of polyamine contents in the target tissue (6) and is reversed by the replacement of exogenous polyamines. In animal models, DFMO reduces the incidence and multiplicity of carcinogen-induced tumors and reduces the growth and metastasis of existing tumors (3, 7, 8). DFMO is under investigation as a chemopreventive agent, a radiosensitizer, and a potentiator of cytotoxic therapy in human cancers (3, 9, 10).

In this study, we have compared the polyamine concentrations in rectal mucosal biopsies and in EBM before and after oral DFMO treatment to assess the suitability of EBM as a marker tissue for DFMO suppression of polyamine synthesis in the rectal mucosa. We studied the impact of bacterial contamination on ODC activity and polyamine concentration measurements in EBM samples. We also report our preliminary data on the efficacy of long-term oral DFMO in decreasing polyamine levels in colonic mucosa.

Materials and Methods

Patients. As part of a larger study to determine the safety and efficacy of oral DFMO therapy in humans, five subjects consented to be treated with DFMO p.o. (1 g/m² body surface area every 8 h) and to undergo pre- and posttreatment sigmoidoscopy and biopsy. Three biopsies of rectal mucosa were taken before and after 1 month of 3 g/m² daily DFMO treatment, and the samples were snap frozen. EBM samples were also obtained before and after DFMO treatment by brushing the inner surfaces of the cheek with a soft toothbrush for 1 min, rinsing the mouth with 20 ml of 1 N phosphate-buffered saline, centrifuging this mouthwash, and freezing the cell pellet. Samples were thawed and washed twice with cold phosphate-buffered saline, and the polyamine concentrations were determined as described below.

To determine the relationship between the concentration of bacteria in the oral cavity and the ODC activity in EBM, an antiseptic mouthwash protocol was established for normal volunteers. Oral intake was standardized, and 7 times/day 30-s mouthwashes with Listerine antiseptic mouthwash were performed for 2 days. EBM samples were collected at 15, 22, 39, and 46 h after initiation of the mouthwash protocol. Each sample was divided, and determinations of bacterial concentration, ODC activity, and total protein were made.

Polyamine Determinations. Cells were lysed by ultrasound on ice in 0.05 M sodium-potassium buffer, pH 7.2, containing 20 μM pyridoxal phosphate, 0.1 mM EDTA, and 1.0 mM 1,4-dithiothreitol. This lysate was adjusted to 0.2 N HClO₄. The acid-soluble fractions were analyzed for polyamine content by high-performance liquid chro-

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³ The abbreviations used are: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; EBM, exfoliated buccal mucosal cells.

matography using the method of Seiler (11), while the pellet was assayed for protein content.

ODC Activity Assay. ODC activities were measured by the collection of liberated $^{14}\text{CO}_2$ from labeled ornithine as described by Fuller (12). Two hundred μl of the cell lysate were reacted with 20 μl of 5.4 mM ornithine for 30 min at 37°C in sealed reaction tubes. The $^{14}\text{CO}_2$ was collected on filter papers moistened with tissue solubilizer and suspended in the reaction vials. Radioactivity was measured by scintillation counting. One unit of enzyme is defined here as 1 μmol $^{14}\text{CO}_2$ collected/min of reaction in the lysate volume assayed.

ODC Kinetic Parameter Determinations. DFMO was used to inhibit ODC activity and was the gift of Marion Merrell Dow Pharmaceuticals, Inc. Cell lysates were incubated for 30 min in final DFMO concentrations of 0, 2, 4, and 8 mM, before the addition of labeled ornithine to 20 or 100 μM final concentrations, and incubated as described under "ODC Activity Assay." The K_i , or inhibition constant, for DFMO was calculated as the negative abscissa intercept of the plots of inverse activity versus DFMO concentration (13). To determine the K_m (concentration at half-maximal enzyme activity) for pyridoxal phosphate, ODC was measured in the presence of 0.9–14.4 μM concentrations of this cofactor. Similarly, to determine the K_m for ornithine substrate, ODC activities were measured in the presence of 0.001–5 mM concentrations of ornithine. The K_m s were calculated to be the x-axis intercepts of double-reciprocal plots of ODC activity versus substrate concentration (13).

GTP concentration dependence was determined by measuring ODC activity in the presence of GTP at 0, 0.01, 0.1, or 1 mM concentrations.

Techniques. Protein contents were determined in 0.5 N NaOH solutions of acid precipitates by the bicinchoninic acid (14) method or by the method of Bradford (15).

Bacterial concentrations in EBM samples were determined by dilutional plating. Suspensions of the washed EBM were diluted serially, plated on agar, and incubated at 37°C for 48 h, and the colonies were counted. Bacterial concentration was expressed as colonies cultured/ng protein in the original EBM sample.

Mixed oral flora were cultured from twice-washed EBM cells and grown in nutrient agar under reduced oxygen tension at both 25°C and 37°C. Gram-positive cocci predominated at both temperatures. Rare fungal hyphae and bacilli were also seen on Gram stains.

Statistical Analysis. The paired *t* test was used to establish the statistical significance of changes of polyamine concentrations in individual patients before and after oral DFMO treatment.

Results

Fig. 1 shows characteristic high-performance liquid chromatography tracings obtained from rectal biopsy samples (A) and buccal mucosa samples (B) before DFMO treatment. Spermine and spermidine are the dominant polyamine species in rectal mucosal cells. Putrescine and cadaverine are evident in these samples, but at lower levels than the longer chain amines. Conversely, putrescine and cadaverine are the dominant polyamine species in EBM samples, and the concentrations of the longer chain amines, especially spermine, are low (see also Table 1).

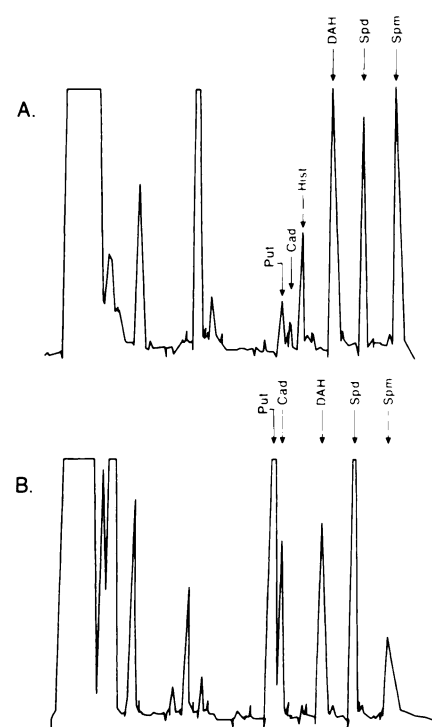


Fig. 1. High-performance liquid chromatography tracings from rectal (A) and EBM (B) samples. Put, putrescine; DAH, diaminoheptane; HIST, histamine; SPD, spermidine; SPM, spermine; CAD, cadaverine.

Polyamine concentrations, before and after treatment with DFMO, in rectal mucosa (average of three biopsies) and in EBM are shown in Table 1. DFMO causes a statistically significant ($P < 0.005$) decrease in putrescine and spermidine concentrations in the rectal mucosa. Putrescine levels are markedly decreased (below detectable limits in four of five patients) by ODC inhibition. Spermidine but not spermine levels are also decreased in a statistically significant manner.

Oral DFMO administration did not cause a statistically significant decrease in polyamine levels in EBM samples. Of the five subjects whose rectal polyamine concentrations decreased after DFMO treatment, two had an increase in EBM polyamine levels after treatment, and three had only a slight decrease in EBM polyamine levels after treatment. The dramatic reduction in putrescine levels that occurred in rectal mucosa after DFMO treatment did not occur in the EBM samples.

A determination of kinetic parameters of ODC enzyme from EBM samples was performed and compared with parameters determined in a human cell culture line (A549 cells, which are of lung cancer origin) and in mixed bacterial flora cultured from washed EBM (Table 2). ODC activity levels in these cultured bacteria and in lysates of EBM samples (1 unit/mg protein) was much higher (10^5 – 10^6 times) than that in other human tissues (e.g., normal human colon mucosa, ~ 0.00001 units/mg protein) and in A549 cells (~ 0.00003 units/mg protein as a maximal value during log phase growth). The inhibition constant K_i for DFMO in buccal mucosa cells was 20 times higher than that measured in A549 cells. Likewise, the K_i for

Table 1 Polyamine contents in gastrointestinal tissue samples from patients, either before or after treatment with DFMO

Patient no.	DFMO ^a	Polyamine contents (nmol/mg protein) ^b					
		Rectal mucosa			Buccal mucosa		
		Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
205	-	0.99 ± 0.12	3.47 ± 0.04	9.14 ± 0.23	2.46	1.18	0.59
	+	ND ^c	1.69 ± 0.21	6.67 ± 0.30	2.05	0.64	0.31
206	-	0.66 ± 0.05	3.35 ± 0.05	7.74 ± 0.30	0.29	0.08	0.83
	+	ND	1.65 ± 0.18	6.21 ± 0.26	0.61	0.47	0.98
207	-	0.89 ± 0.11	4.03 ± 0.49	9.18 ± 1.22	0.95	0.46	0.51
	+	0.30 ± 0.03	2.16 ± 0.11	7.50 ± 0.30	0.42	0.30	0.16
208	-	1.14 ± 0.14	3.55 ± 0.38	9.28 ± 1.02	2.20	1.05	0.40
	+	ND	1.81 ± 0.05	7.20 ± 0.63	1.57	0.66	0.33
209	-	0.74 ± 0.10	2.93 ± 0.51	7.06 ± 1.54	1.02	0.36	0.18
	+	ND	2.32 ± 0.03	9.80 ± 0.20	1.60	0.69	0.54
Mean ± SD	-	0.89 ± 0.20	3.46 ± 0.51	8.58 ± 1.28	1.39 ± 0.82	0.62 ± 0.42	0.50 ± 0.22
	+	<0.10 ± 0.10	1.93 ± 0.30	7.05 ± 1.05	1.25 ± 0.63	0.55 ± 0.15	0.46 ± 0.29
<i>p</i> ^d		<0.005	<0.005	0.35	0.61	0.72	0.79

^a -, samples collected prior to DFMO therapy; +, after DFMO therapy.

^b Rectal biopsy values are means ± SD of 3 independently assayed tissue samples taken from an individual patient at the same time. Buccal mucosa contents are values obtained from a single sample collected either before or after DFMO therapy. Mean values ± SD shown in the last line were computed using individual sample values.

^c ND, none detected; limit of detection is 0.05 nmol polyamine/mg protein.

^d Paired *t* test of statistical significance of the reduction of polyamine concentration after DFMO treatment.

Table 2 Ornithine decarboxylase enzyme activity and kinetic parameters in bacterial cultures and in human cells and tissue extracts

Cell type	Enzyme activity (units/mg protein) ^a	<i>K_i</i> , DFMO (μM)	<i>K_m</i> s (μM)	
			Ornithine	Pyridoxal phosphate
A549	0.00003	200	54	1.1
EBM	1	4200	2127	3.2
Mixed bacteria from oral cavity	10	>8000	ND	ND

^a One unit of enzyme activity is defined as 1 μmol ¹⁴C₂O₂ released from radiolabeled ornithine/minute at 37°C.

DFMO in mixed-flora bacteria from the oral cavity was much higher than that found in the human A549 cells. In addition, *K_m* for ornithine and *K_m* for pyridoxal phosphate values for ODC in EBM are inconsistent with those determined for A549 and other human tissues (data not shown). Washing the EBM twice with phosphate-buffered saline did not cleanse them of bacteria. Scanning electron micrographs of these washed cells revealed a large number of bacteria adherent to their surface (Fig. 2). In addition it was possible to culture approximately 40 colonies of mixed bacterial flora/ng of protein in the original EBM sample. Minimal bacteria could be cultured from washed rectal biopsy specimens.

ODC activity in EBM cells was dependent on GTP concentration. The profiles of GTP dependence of ODC activity in EBM and in oral mixed flora were similar (Fig. 3). The absolute value of activity in bacteria was approximately 10 times higher than that in EBM, when normalized to total sample protein. EBM sample protein included both bacterial and EBM cell protein. In both profiles, ODC activity was maximal at 0.1 mM GTP, which is consistent with the optimal GTP concentration re-

ported for bacterial species. Finally, Fig. 4 shows a concomitant decrease in the bacterial flora cultured from and the ODC activity measured in EBM samples during 2 days of frequent antiseptic mouthwashing.

Discussion

Our results show that oral DFMO can deplete polyamine content, particularly putrescine, in the rectal mucosa. This suggests that DFMO therapy p.o. might decrease cell proliferation of the rectal mucosa and inhibit adenoma or carcinoma formation in humans. A larger patient population is currently being studied to prove the safety and efficacy of long-term DFMO p.o. as a chemopreventive agent. In animal models, DFMO effects are maximized when DFMO treatment p.o. is combined with diets containing low polyamine concentrations and colonic decontamination (16, 17). It is proposed that colonic mucosal cells depleted of polyamines by DFMO may take up polyamines from the colonic bacterial flora, and this exogenous polyamine rescue might preclude any benefit of ODC inhibition by DFMO in the colon and throughout the body. However, the nondetectable levels of cellular putrescine and the lowering of putrescine and spermidine levels in the rectal mucosa of most of the DFMO-treated patients studied here suggest the potential for significant alterations in proliferative activity in colorectal mucosa by DFMO therapy p.o. alone.

The effect of DFMO therapy p.o. on the ODC activity in EBM is not accurately measurable. ODC activity and polyamines measured in this tissue are primarily due to contamination of the EBM samples by the normal flora of the mouth. The mouth contains a myriad of contaminating organisms including Gram-negative, Gram-positive, anaerobic, and aerobic bacteria as well as fungi and parasites. Specifically, 80% of the bacteria that colonize

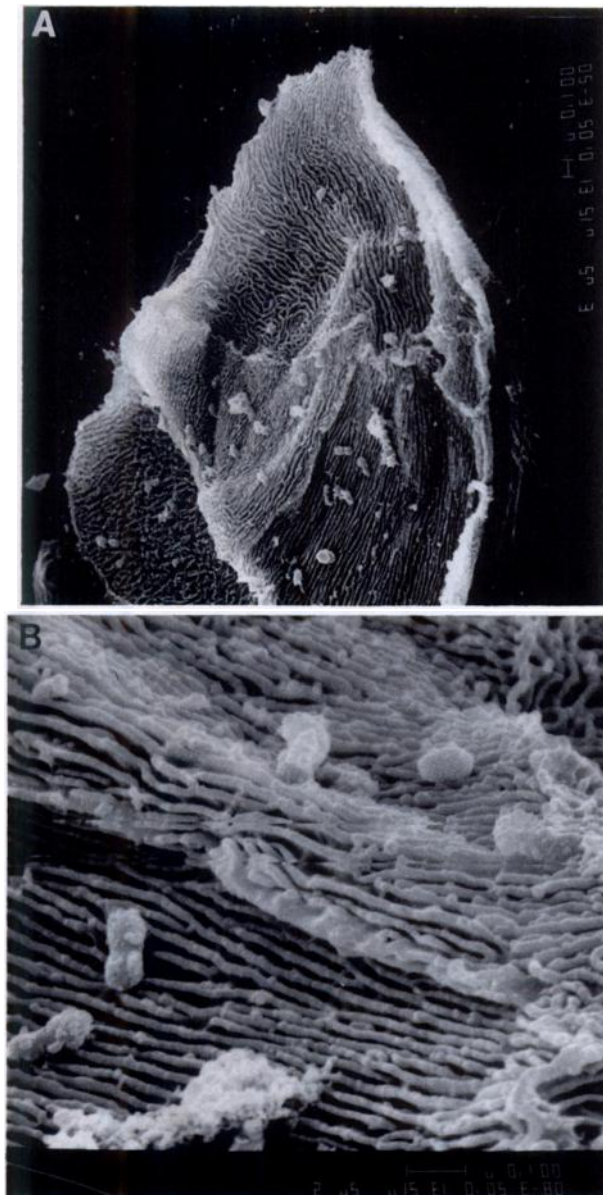


Fig. 2. A, electron micrograph of washed EBM cells with adherent bacteria; B, higher magnification. Bar, 1 μ m.

the mucosa are streptococcal species of three major strains: *S. mitis*, *S. salivarius*, and *S. sanguis* (18).

These bacteria are highly adherent to the EBM, and the ODC activity measured in EBM samples is characteristic of the prokaryotic enzyme, based on several findings. First, the ODC activity measured in EBM and the putrescine and cadaverine contents were unexpectedly high. The ODC activities are consistent with bacterial ODC activities (19). Likewise, EBM polyamine contents resemble those of bacteria, which contain high concentrations of putrescine and spermidine but almost no spermine (20). The human oral epithelium is a stratified squamous epithelium with basilar proliferating compartments and superficial differentiated compartments. The exfoliating cells collected in this study are completely

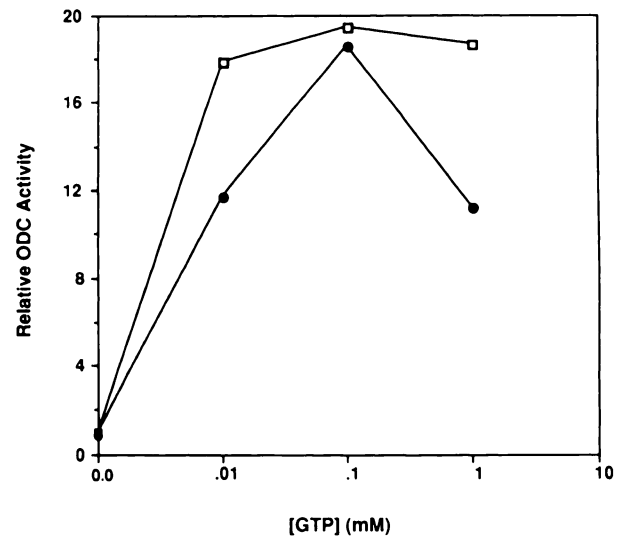


Fig. 3. Ornithine decarboxylase activity as a function of GTP concentration in EBM (□) and bacteria (●). Initial activities in the absence of GTP are as stated in Table 1.

differentiated and nonviable by dye exclusion or dye uptake and metabolism (data not shown) and serve a physical protective function (21). Because they are not proliferating, low ODC activity and polyamine levels would be predicted in the EBM cells.

Second, resistance of ODC to DFMO inhibition (20, 22) and GTP concentration dependence of ODC activity are characteristic of bacterial enzyme and suggest that bacterial ODC is present in EBM lysates. Mammalian ODC is generally not dependent on GTP, while prokaryotic ODC is known to be dependent on GTP concentration (23). Finally, as concentrations of bacteria in EBM samples were reduced by antiseptic mouthwash, the measured ODC activity was also reduced.

The results shown in Fig. 1 suggest that rectal mucosa samples are also contaminated with bacteria, as detectable levels of cadaverine, an amine not generally found in mammalian tissues but found in bacteria under certain conditions, are evident. However, either the type of bacteria or the degree of contamination is different in rectal mucosa compared to EBM, since DFMO does suppress putrescine and spermidine in rectal mucosa but not in EBM. Furthermore, the ODC activity found in rectal mucosa has characteristics of human ODC (activity levels/mg protein, K_m , K_i for DFMO and general lack of stimulation by GTP; data not shown), while EBM ODC activity is characteristic of bacterial ODC. These findings underscore the importance of identifying the source (bacterial versus human) of polyamines in specific tissues in the gastrointestinal tract.

We have not found a technique to eliminate bacteria from the collected EBM cells that does not affect ODC activity in control cells. Antibiotic mouthwashes are generally ineffective (24), and *in vitro* antiseptics like 70% ethanol are cytotoxic and denature proteins. It is unlikely, however, that the level of endogenous ODC activity of EBM is significant compared to that of the bacterial flora adherent to the EBM cells.

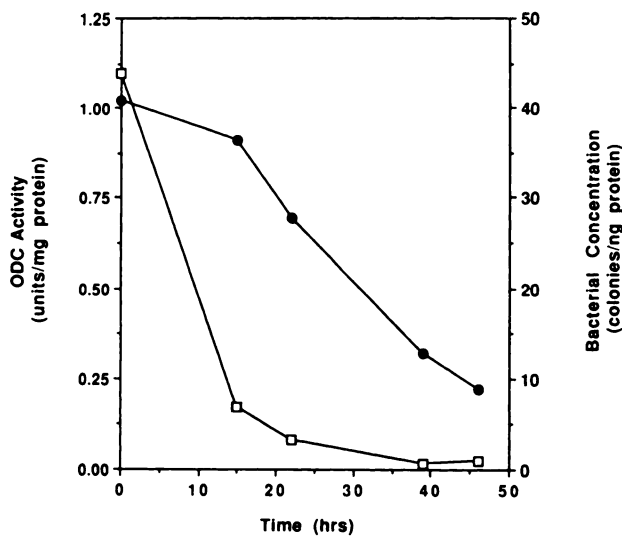


Fig. 4. Effect of frequent antiseptic mouthwashing on ornithine decarboxylase activity (□) and bacterial concentration (●) measured in EB samples.

Although EBM is attractive for its accessibility and noninvasive acquisition, it does not represent a good marker tissue for assessing DFMO effect on polyamine concentrations in the distal gastrointestinal tract. Perhaps biopsy of the buccal mucosa may be superior to exfoliated cell collection. The cornified and contaminated surface of oral biopsy specimens could be microdissected away before assaying the proliferating layers for polyamine concentrations of ODC activity. The skin is also a possible marker tissue of systemic polyamine depletion. Skin is continuously proliferating and can be nearly sterilized by topical antiseptics before collection, and microdissection of the cornified layer is easily performed. The skin is accessible and can be obtained under local anesthesia from nonsensitive and noncosmetic areas like the back. Subjects, however, may prefer sigmoidoscopic biopsy of the noninnervated mucosa of the rectal colon to the discomfort of biopsy of the oral mucosa or skin.

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