Inhibition of Ornithine Decarboxylase with 2-Difluoromethylornithine: Reduced Incidence of Dimethylhydrazine-induced Colon Tumors in Mice

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ABSTRACT

2-Difluoromethylornithine (DFMO) was administered to 1,2-dimethylhydrazine (DMH)-treated mice to reduce colonic polyamine levels and mucosal hyperplasia. Mice received 1% DFMO in drinking water throughout the experiment and were given injections of DMH (20 mg/kg) weekly for 28 weeks. DFMO inactivated 93% of colonic ornithine decarboxylase activity. Although DMH treatment did not induce colonic ornithine decarboxylase activity by Week 28, the putrescine content was increased 31% in DMH-treated mice (p < 0.01). Concurrent treatment with DFMO depressed putrescine content (42 to 63%) and spermidine content (27 to 38%), but it increased spermine content (18 to 22%). At Week 28 of treatment with DMH alone, RNA content was increased 8.6% (p < 0.01), DNA content 10% (p < 0.01), DNA specific activity 24% (p < 0.01), and crypt depth 20% (p < 0.01), but not in mice receiving DMH and DFMO. At 28 weeks, 13 of 17 mice (76%) treated with DMH alone had histologically confirmed colon cancers; of mice treated with DMH and DFMO, two of 18 (11%) had colon tumors. Throughout the experiment, 50 colon cancers developed in 16 DMH-treated mice (mean, 3.12 tumors/mouse); three mice treated with DMH and DFMO developed three colon cancers total (p < 0.001). Reduction of colonic polyamine levels after DFMO treatment prevents proliferative changes induced by DMH and reduces the incidence of tumors.

INTRODUCTION

Intracellular polyamine levels (putrescine, spermidine, and spermine) and polyamine-synthetic enzyme activities are high in proliferating normal cells (4, 13, 17), neoplastic cells (30, 33), and cells undergoing neoplastic transformation by viruses (1, 11, 16, 37). They may be necessary for the increase in cellularity of the colonic mucosa that precedes the development of cancers in experimental animals and in human beings (10, 18, 21, 22, 24).

MATERIALS AND METHODS

Male CD-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.) were received 2 weeks before the start of the experiment at the age of 40 days (26 to 28 g). They were housed in plastic containers 4 to 6 per cage, under 12-hr light-dark cycles, beginning at 6 a.m. Mice received Purina chow and were allowed free access to water or a fresh solution of 1% DFMO (RMI 71, 782A; Merrell Dow, Cincinnati, Ohio), which is stable in these circumstances (9, 12). The average daily intake of DFMO was estimated from the fluid intake per cage.

Mice were randomly allocated to one of 4 groups. The 4 groups (42 in each) were: (a) mice receiving s.c. DMH (20 mg/kg) weekly for 28 weeks; (b) control mice (no treatment); (c) DMH-treated mice, also given 1% DFMO in drinking water throughout; and (d) mice receiving 1% DFMO in drinking water throughout the experiment.

DMH (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was dissolved in 0.001 M EDTA immediately before use, and the solution was brought to pH 6.5 with 1 N sodium bicarbonate. Weekly injections of DMH were given to animals in Groups 1 and 3 throughout the experiment.

Two mice from each group were killed at 2-week intervals from Weeks 2 to 28; the remainder were sacrificed at Week 28.

After receiving an injection of 25 μCi [14C]thymidine 30 min before death, mice were killed by cervical dislocation. The whole colon was removed, slit lengthwise, washed in ice-cold 0.9% NaCl solution, blotted, and weighed before being placed in ice-cold buffer for immediate assay of ODC or coded and being stored at –30° for subsequent nucleic acid and polyamine analysis. Colon segments were sampled for the different assays from exactly the same part of the bowel in each animal. At Week 28, the whole colon was analyzed.

ODC activity assayed by a modification of the method of Lapointe and Cohen (19) was done immediately after harvesting tissue. Specimens were homogenized in ice-cold 50 mM sodium phosphate buffer at pH 7.2, containing 1 mM EDTA and 2 mM dithiothreitol, and then centrifuged at 30,000 × g for 20 min at 4°. The assay was carried out by collecting 14CO2 released from 20 μCi of L-[14C]ornithine (carboxyl labeled, 47.2 mCi/mmol; New England Nuclear, Boston, Mass.). A Pyrex spot plate was used to contain the reaction, with a glass fiber disc wet with saturated barium hydroxide (7%, w/v) placed over the well to absorb the radiolabeled carbon dioxide released by the reaction. In the incubation wells, 160 μl of supernatant were added to 1.1 mm pyridoxal phosphate, 0.1 mM L-ornithine, and 0.25 μCi of L-[14C]ornithine in a final volume of 0.25 ml. Blanks were heated-inactivated supernatant. After 60 min at 37°, the reaction was stopped by adding 0.1 ml of 2 N sulfuric acid; the disc was replaced for a further 30 min to ensure complete CO2 collection. Radioactivity of the discs was counted for 5 min in 3 ml of toluene-based scintillation medium at 25% efficiency; quench correction was by internal
standard. Counts were converted to equivalent amounts of CO₂, and ODC activity was expressed as pmol CO₂ liberated per mg wet tissue per hr incubation. Assays were performed in duplicate.

DNA content was determined by the method of Burton (3). Radioactivity in aliquots from the acid-insoluble fraction was counted in 3 ml PCS scintillation fluid (Amersham Corp., Arlington Heights, Ill.) for 5 min at 20% efficiency; quench correction was by internal standard. RNA was assayed by the method of Scott et al. (35) modified by the method of Hinrichs et al. (14).

Colon segments without any neoplastic component (except for Week 28 mice treated with DMH alone) were analyzed for polyamine content (putrescine, spermidine, and spermine) by a modification of the method of Marton and Lee (25). Samples were homogenized in 4 volumes of 0.4 M perchloric acid, left overnight on ice, and centrifuged at 15,000 × g for 15 min. The supernatant was filtered through a 0.45-μm Microfilter (type MF-1; Bioanalytic Systems, West Lafayette, Ill.). Aliquots (20 μl) of the filtered extracts were put into a Dionex D-300 amino acid analyzer with a Dionex P/N 30831 cation separator column. Polyamines were eluted using an eluent flow rate of 36 ml/hr, reagent (O-phthalaldehyde) flow rate of 18 ml/hr, and column temperature of 45°C. The eluent buffer for equilibration and for putrescine was 0.3 M sodium buffer, pH 5.8 [trisodium citrate (0.98 g/liter):NaCl (16.94 g/liter); phenol (1 ml/liter)]. For spermidine, it was 0.7 M sodium buffer, pH 5.55 [trisodium citrate (1.97 g/liter):NaCl (42.63 g/liter); phenol (1 ml/liter); NaCl (83.0 g/liter); phenol (1 ml/liter)]. For spermine, it was 1.5 M sodium buffer, pH 5.55 [trisodium citrate (7.84 g/liter); NaCl (83.0 g/liter); phenol (1 ml/liter)].

Crypt depths in coded slides were measured by ocular micrometry in crypts sectioned from top to bottom without interruption. Each value reported represents the means of measurements of 100 crypts.

Student’s t test for unpaired data was used for statistical analysis.

RESULTS

Weight Gain (Chart 1). DMFMO did not affect weight gain until Weeks 26 to 28, when DFMO-treated animals were 8.5% (Group 4) to 11.4% (Group 3) lighter than controls.

Mortality. One animal died in the DMH-alone group shortly after receiving an injection of DMH at Week 27. No colonic tumor was present.

ODC Activity (Chart 2). Administration of DMH caused a fall in ODC activity for 24 hr, 55% of ODC activity being lost at 6 hr and a 36% loss still being present at 24 hr. Loss of ODC activity paralleled the DMH-induced inhibition of [³H]dThd intake (36% fall in DNA specific activity at 6 hr). Colonic ODC activity did not change during the 28-week period up to cancer development (Chart 3). Ingestion of DFMO (11.5 ± 0.4 ml 1% DFMO per day; 2.78 ± 0.05 g DFMO per kg body weight per day) caused 92 to 94% inactivation of ODC activity throughout the 28 weeks.

Polyamine Content (Chart 4). After DMH, there was a 31% rise in colonic putrescine content at Week 28 compared with control animals. DFMO reduced putrescine levels by 63% in DMH-treated mice and by 46% in control mice. Spermidine levels fell in DFMO-fed, DMH-treated mice (27%) and in DFMO-fed mice (38%). Spermine levels rose 18% in DFMO-fed, DMH-treated mice and 27% in control DMFMO-fed mice.

Nucleic Acid Content. DNA content and DNA specific activity of the colon did not change during the first 24 weeks of tumor induction. At Week 28, RNA content was increased 9%, DNA content 10%, and DNA specific activity 24% in DMH-treated mice (Chart 5). The RNA:DNA ratio in DMH-treated mice was the same as in controls. DFMO prevented the rise in RNA, DNA, and DNA specific activity caused by DMH treatment.

Crypt Depth (Chart 6). Crypt depth was the same in all 4 groups of mice during the first 24 weeks. At the time of the sacrifice, crypt depth was increased 20% by DMH. There was no increase in crypt depth in DFMO-fed, DMH-treated mice.

Tumors (Chart 7). There were no extracolonic tumors except for one invasive squamous cell carcinoma of the anorectal junction in a DMH-treated mouse. All tumors were malignant and...
DFMO Effect on DMH Colon Cancer

were confirmed histologically. Some were well-differentiated tubular or papillary adenocarcinomas; the others were poorly differentiated carcinomas with deep invasion into the muscularis propria. In total, there were 50 tumors present in DMH-treated mice (16 mice; mean, 3.12 tumors/mouse) and 3 in DMH-treated, DFMO-fed mice (3 mice; one tumor in each). Of DMH-treated mice, 76% had tumors at Week 28 (13 of 17 mice). Only 11% of DMH-treated and DFMO-fed mice had tumors at Week 28 (2 of 18 mice).

DISCUSSION

In contrast to the rise in ODC activity occurring soon after administration of organ-specific carcinogens to experimental animals (7, 27-29, 34, 38), administration of DMH produces a fall in colonic mucosal ODC activity that lasts for 24 hr after administration but causes no long-term changes in activity during 28 weeks of DMH treatment. However, rises in tissue ODC activity and polyamine content occurring before the appearance of carcinogen-induced tumors are not universal. For instance, a rise in hepatic polyamine content does not always occur before the appearance of malignant hepatic tumors induced by feeding diethylnitrosamine or 4-dimethylaminoazobenzene. Any elevation of cellular ODC activity during the formation of preneoplastic foci produced by diethylnitrosamine and 4-dimethylaminoazobenzene could rather be related to cellular proliferation occurring as a result of liver necrosis than to a specific action of the carcinogen on the target cell DNA (6). Similarly, the transient rise in ODC activity in the rat colon occurring after intrarectal instillation of N-methyl-N-nitro-N-nitrosoguanidine (38) may be related to mucosal regeneration (23) resulting from damage caused by the
instillation. Moreover, colonic [H]dThd uptake is reduced for 48 hr after a single injection of DMH (40); therefore, reduction in ODC activity seen in our study is likely to be an aspect of reduced mucosal cell proliferation.

Because overall ODC activity at the times measured was not increased in tumor-bearing colonic mucosa in this study, the increase in putrescine content might have arisen from interconversion of putrescine from spermidine and spermine (15), which have long half-lives (36). Certainly, a 31% rise in the colonic putrescine levels indicates that polyamine biosynthesis or degradation had been altered by the carcinogen.

The use of DFMO prevents small increases in crypt depth, RNA content, DNA content, and DNA specific activity occurring in the colon of DMH-treated mice. Interference with production of the carcinogenic metabolite of DMH, methylazoxyster (8), can be discounted, however, because DFMO does not alter the action of carcinogen-metabolizing enzymes (32). Although there is no direct evidence that DFMO does not otherwise alter the metabolism of DMH, this possibility is unlikely, inasmuch as DFMO binds to the protein of ODC only (31), is specific in its effects, and is not metabolized (5, 9). Furthermore, DFMO decreases the number of rat mammary tumors induced by 7,12-dimethylbenz[a]anthracene even when given 30 days after 7,12-dimethylbenz[a]anthracene (9); 7,12-dimethylbenz[a]anthracene is not a strict alkylating agent like DMH, however. Finally, although polyamine depletion resulting from DFMO treatment could, in principle, affect DMH metabolism or alkylating properties, the DNA-alkylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea has its cytotoxic effects potentiated by DFMO, rather than being inhibited (28). DFMO also inhibits formation of cutaneous tumors induced in mice by croton oils (39). In this instance, however, there is an enormous induction of cutaneous ODC by the croton oil derivative (12-O-tetradecanoylphorbol-13-acetate), which in turn is blocked by the surface application of DFMO.

Whatever effect DMH has on inducing colonic cancer in mice, there is not a generalized rise in colonic ODC activity, unlike the situation in the colonic mucosa of rats (23), even though there is a small, but significant, rise in the putrescine level of DMH-treated mouse colonic mucosa. Nevertheless, localized changes in polyamine biosynthesis occurring during DMH-induced colon tumor development are obligatory, inasmuch as inhibition of putrescine biosynthesis by administration of DFMO prevents the biochemical and proliferative changes that precede cancer development, reducing the incidence of tumors.

REFERENCES

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