Effect of Chemopreventive Agents on Intermediate Biomarkers during Different Stages of Azoxyomethane-induced Colon Carcinogenesis

Jagveer Singh, Gary Kelloff, and Bandaru S. Reddy

Abstract
Chemoprevention of colon cancer is emerging as an alternative to therapy with a broad potential for reducing cancer incidence in defined high-risk groups and the general population. Besides several chemopreventive agents in use and under investigation, D,L-α-difluoromethylornithine (DFMO) and piroxicam have been shown to effectively inhibit colon carcinogenesis in rodents. A variety of proliferation-related parameters have been suggested as potential intermediate markers of cancer risk that could be used to monitor the progress of chemoprevention in clinical trials. We have investigated the effect of chemopreventive agents, DFMO, and piroxicam on mucosal ornithine decarboxylase (ODC) and tyrosine-specific protein kinase (TPK) activities during different stages of azoxymethane (AOM)-induced colon carcinogenesis in male F344 rats in order to examine the plausibility of using these enzymes as intermediate biochemical markers of colon cancer. Groups of male F344 rats were fed modified AIN-76A diets containing 0 or 150 ppm piroxicam or 4000 ppm DFMO and given s.c. injections of AOM dissolved in normal saline at a dose of 15 mg/kg body weight/week, once weekly, for 4 weeks. Vehicle control groups received s.c. equal volumes of normal saline. Groups of animals were then sacrificed at 0, 4, 16, 24, and 32 weeks after AOM or saline treatment, and their colonic mucosa was analyzed for ODC and TPK activities. AOM treatment significantly increased mucosal ODC as well as TPK activities. AOM-induced ODC and TPK activities were significantly suppressed by dietary DFMO progressively at all stages of colon carcinogenesis. Dietary piroxicam increased AOM-induced colonic mucosal ODC and TPK activities but significantly reduced tumor incidence as well as tumor multiplicity. DFMO exerted a more pronounced inhibitory effect on AOM-induced colon tumor development. These results emphasize the importance of development of agent-specific intermediate biomarkers to be used as effective predictors of colon carcinogenesis.

Introduction
Colorectal cancer is a major cause of morbidity and mortality among men and women in the United States, afflicting 167,000 people and causing about 65,000 deaths in 1991 (1). Despite several advances made in the treatment of colon cancer, the cure rate has remained largely unchanged in the past two decades (2). Chemoprevention, which is emerging as an alternative to therapy, relies on the concept of inhibition or reversal of cancer formation through chemical intervention (3, 4). Several chemopreventive agents in use and under investigation include retinoids, purified dietary constituents, vitamins, micronutrients, antimutagens, dithiolthiones, metabolic products, protease inhibitors, and polyamines and prostaglandin synthesis inhibitors. DFMO, a specific, enzyme-activated, irreversible inhibitor of ODC activity, and piroxicam, a nonsteroidal, antiinflammatory drug have been shown to effectively inhibit carcinogen-induced colon tumor development in rodents (5, 6). Recent preclinical studies in laboratory animals and clinical investigations in high-risk patients of colon cancer have identified DFMO and piroxicam as ideal candidates for chemoprevention trials. However, the success of such clinical trials has been hampered by the lack of appropriate intermediate biomarkers or end points which could indicate the progress of chemoprevention and predict the subsequent reduction of cancer occurrence.

Intermediate end points may be defined as measurable markers of cellular or molecular events associated with specific stages of the multistep evolution and progression of carcinogenesis. This indicates that the risk of carcinogenic transformation must correlate with quantitative degree and pattern of biomarker expression. ODC, which catalyzes the conversion of ornithine to putrescine in a rate-limiting step of crucial polyamine biosynthesis, plays an important role in normal and neoplastic cell proliferation (7) and is associated with the tumor-promoting ability of a variety of agents (7–8). Increased ODC activity has been demonstrated in benign colonic adenomas (9–12) as well as normal-appearing mucosa adjacent to adenomas (13–15), reflecting the underlying hyperproliferative state of colonic mucosa. Significantly

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1 The abbreviations used are: DFMO, D,L-α-difluoromethylornithine; ODC, ornithine decarboxylase; TPK, tyrosine-specific protein kinase; AOM, azoxymethane; DTT, dithiothreitol.

2 To whom requests for reprints should be addressed, at American Health Foundation, Division of Nutritional Carcinogenesis, One Dana Road, Valhalla, NY 10595.
high levels of ODC activity have been reported for several rodent carcinogenesis models (16-17). TPKs which catalyze phosphorylation of tyrosine residues of protein substrates are important growth-promoting signal-transducing enzymes involved in cellular growth and differentiation (18-19). Tyrosine phosphorylation is now recognized as an important regulatory mechanism in response to a number of processes, including the action of several growth factors and oncogenes. Enhanced TPK activity has been demonstrated in neuroblastoma (20), rhabdomyosarcoma (21), and mammary (22) and colon (23) carcinomas. A significant number of TPKs that are products of protooncogenes have been implicated in the etiology of a number of human cancers (24).

Do these intermediate biomarkers, ODC and TPK, predict the known chemopreventive influence of DFMO and piroxicam on experimental colon tumor development? This study was designed to investigate the effect of chemopreventive agents, DFMO and piroxicam, on mucosal ODC and TPK activities during different stages of AOM-induced colon carcinogenesis in male F344 rats in order to examine the plausibility of using these enzymes as intermediate biochemical markers for colon cancer. The major goal of this study was to determine which intermediate biomarkers, if altered by these chemopreventive agents, could be used as effective predictors of colon cancer.

Materials and Methods

Animals, Diets, and Carcinogen. Weanling male F344 rats were obtained from Charles River Breeding Laboratories (Kingston, NY). Piroxicam and DFMO were generous gifts from Pfizer Central Research (Groton, CT) and Merrill-Dow Research Institute (Cincinnati, OH), respectively. AOM (COS: 25843-45-2) was purchased from Ash-Stevens (Detroit, MI). All ingredients of semipurified diet were obtained from Dyets, Inc. (Bethlehem, PA) and stored at 4°C prior to preparation of the diets. Piroxicam and DFMO were incorporated into the control diet at doses levels of 150 ppm and 4000 ppm, respectively. All control and experimental diets were prepared weekly in our laboratory and stored in a cold room.

Male F344 rats received at weaning were quarantined for 10 days. All animals were housed in plastic cages with filter tops under controlled environmental conditions of 21°C temperature, 50% humidity, and a 12-h light/dark cycle. At 5 weeks of age, all animals were assigned to AOM-treated and vehicle-treated groups and fed the control diet (Ref. 25; Table 1).

Experimental Procedure. Beginning at 6 weeks of age, animals from AOM- and vehicle-treated groups were reallocated into different dietary subgroups and fed one of the experimental diets containing 150 ppm piroxicam or 4000 ppm DFMO or control diet (Table 1). The rationale for selecting 150 ppm piroxicam and 4000 ppm DFMO has been based on our previous results, which indicated a colon tumor-inhibitory effect of these agents at these levels (5). One week later, animals intended for carcinogen treatment were administered s.c. AOM dissolved in normal saline at a dose rate of 15 mg/kg body weight/week, once weekly, for 4 weeks. Animals intended for vehicle treatment received s.c. an equal volume of normal saline. Animals were maintained on their respective dietary regimens until termination of the experiment. Body weights were recorded once weekly during carcinogen treatment and then every 4 weeks. Ten animals treated with AOM or vehicle from each dietary subgroup were sacrificed by decapitation at 0, 4, 16, 24, and 32 weeks after the last AOM or saline injection. Their colons were resected and opened longitudinally, and the contents were flushed with saline. Colon tumors, if any, were removed before scraping the colon mucosa with a microscope slide. Mucosal scrapings and tumors were quick-frozen in liquid nitrogen and stored at -80°C until use. No colon tumors were observed until 16 weeks after the AOM or vehicle treatment.

Determination of ODC Activity. Mucosal scrapings and tumor tissue were homogenized separately with polytron homogenizer in 10 volumes of 50 mM sodium phosphate buffer, pH 7.2 (containing 5 mM DTT, 0.2 mM pyridoxal phosphate, 0.1 mM EDTA), and centrifuged at 40,000 x g for 30 min at 4°C. Clear supernatant fraction was used for ODC assay. Enzyme activity was determined by measuring 14CO2 liberated from L-[1-14C]ornithine as described elsewhere (26). The standard assay mixture contained, in a total volume of 0.25 ml, an aliquot of supernatant, 50 mM sodium phosphate buffer (pH 7.2), 0.2 mM pyridoxal phosphate, 5 mM DTT, 0.1 mM EDTA, 0.4 mM L-ornithine, and 0.50 μCi of 14CO2. L-ornithine hydrochloride (56.6 mCi/mmol; Amersham Co., Arlington Heights, IL). The reaction mixture was incubated at 37°C for 1 h in a 16 x 100 mm glass tube sealed with rubber stopper supporting a center well (Kontes, Morton Grove, IL). The released 14CO2 was trapped on a microglassfiber filter disc (934-AH, GFA; Whatman) soaked in saturated solution of barium hydroxide. The reaction was stopped by injecting 0.1 ml of 2 M sulfuric acid through a rubber septum directly into the reaction mixture. The incubation was continued for an additional 1 h to completely trap the released 14CO2. Filter paper along with the center well was then transferred to a scintillation vial, and radioactivity was counted in 10 ml of scintillation cocktail (Scintisol; ISOLAB, Inc.) ODC activity is expressed as pmol 14CO2 released/mg protein/h.

Determination of Protein Tyrosine Kinase Activity. TPK activity was measured in both cytosolic and membrane

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<tr>
<th>Table 1</th>
<th>Percentage composition of experimental semipurified diets</th>
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<td><strong>Diet ingredients</strong></td>
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<td><strong>Piroxicam</strong></td>
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<td><strong>DFMO</strong></td>
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a Adapted from American Institute of Nutrition Reference Diet (AIN-76A), with the modification of source of carbohydrate.

b Piroxicam and DFMO were added to the diets at the expense of cornstarch.
fractions. Colonic mucosal or tumor samples were homogenized in 5 volumes of 10 mM Tris-HCl, pH 7.5, containing 0.25 mM sucrose, 1 mM MgCl₂, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mg/ml aprotinin. All debris and nuclei were removed by centrifuging at 800 x g for 10 min at 4°C. The supernatant (membrane fraction) was resuspended in 50 mM Tris-HCl, pH 7.5, containing 20 mM magnesium acetate, 5 mM NaF, 1 mM EDTA/ethyleneglycol-bis(-β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM DTT, 30 μM sodium-o-vanadate, and 0.5% Nonidet P-40; sonicated three times for 10 s each time; and centrifuged at 40,000 x g for 30 min at 4°C. The supernatant (solubilized membrane) was used to assay TPK activity. TPK activity was measured using a tyrosine kinase-specific synthetic polymer as substrate. The enzyme activity is expressed as pmol [³²P]ATP incorporated/mg protein/min.

**Protein Determination.** Protein content in cytosol and membrane fractions was determined by the method of Bradford (29), using bovine serum albumin as the standard.

**Statistical Analysis.** Wherever applicable the data were analyzed using an unpaired t test and one-way analysis of variance.

### Results

**General Observations**

The body weights were comparable in all the animals fed control and experimental diets (Table 2). However, the body weights of AOM-treated animals were slightly lower than those of vehicle-treated animals due to AOM carcinogenicity and consequent tumor burden.

### Tumor Incidence

Table 3 presents gross observations of the effect of piroxicam and DFMO on incidence and multiplicity of colon tumors observed at different stages. AOM induced multiple tumors toward the end of experiment in about 100% of animals fed the control diet. Animals fed the piroxicam diet developed relatively small colon tumors. As reported earlier using different protocol (3), the colon (1:1) and 100% ether. Radioactivity was counted in 10-ml scintillation cocktails (Scintiverse; Fisher Scientific, Fair Lawn, NJ). TPK activity was corrected for endogenous phosphorylation by assaying in the absence of polymer substrate. The enzyme activity is expressed as pmol [³²P]ATP incorporated/mg protein/min.

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tumor multiplicity was significantly reduced in animals fed the piroxicam diet ($P < 0.01$). The incidence and multiplicity of AOM-induced colon tumors were significantly decreased in animals fed the experimental diet containing 4000 ppm DFMO ($P < 0.001$). The latency period was greatly extended by dietary DFMO and piroxicam.

**ODC Activity**

**Induction of Colonic Mucosal ODC Activity.** Table 4 summarizes the colonic mucosal ODC activity at different time periods after AOM or saline treatment. AOM administration induced persistently elevated levels of ODC activity throughout the duration of this experiment. Although AOM-stimulated ODC activity exhibited a distinctly biphasic elevation pattern as demonstrated earlier (16), the differences in levels of ODC activity between AOM-treated and vehicle-treated animals remained highly significantly irrespective of dietary intake of chemopreventive agent ($P < 0.001$).

Dietary intake of DFMO resulted in a significant suppression of colonic mucosal ODC activity at all time points (Table 4). AOM-treated animals fed the DFMO diet demonstrated up to 90% inhibition of ODC activity. In saline-treated animals, DFMO suppressed the ODC activity by more than 90% at the end of the experiment. Interestingly, animals from both AOM-treated as well as saline-treated groups fed the piroxicam diet exhibited significantly increased levels of ODC activity as compared to their corresponding control dietary groups ($P < 0.001$).

**Colonic Tumor ODC Activity.** As summarized in Table 5, the ODC activity in colon tumors of animals fed the control diet was about 6 times higher than that of their uninjured colon mucosae. Interestingly, both cytosolic and membrane-bound ODC activities were significantly suppressed in colon tumors of animals fed piroxicam as compared to those fed the control diet ($P < 0.01$).

**TKP Activity**

**Mucosal Protein Tyrosine Kinase Activity.** As presented in Table 5, the modulation of colonic mucosal TKP activity by piroxicam and DFMO followed somewhat similar patterns of ODC activity, except that the differences in levels of TKP activity were not significant in the beginning of the experiment. AOM treatment significantly increased membrane-bound as well as cytosolic TKP activity in animals on the control diet as compared to their vehicle-treated counterparts throughout the duration of the experiment ($P < 0.05-0.001$). In addition, AOM administration induced progressively increasing levels of membrane-bound as well as cytosolic TKP activity. Dietary piroxicam significantly raised the TKP activity in AOM-treated as well as saline-treated animals as compared to their corresponding AOM- and saline-treated animals fed the control diet ($P < 0.05-0.001$). In contrast, animals fed the DFMO diet exhibited progressively decreasing levels of TKP activity in AOM-treated as well as saline-treated animals. In these animals, the levels of TKP activity were significantly reduced as compared to their corresponding control dietary groups ($P < 0.05-0.001$).

**Discussion**

The results of this experiment demonstrate that dietary DFMO and piroxicam significantly reduce colon tumor occurrence and multiplicity and greatly increase tumor latency in AOM-treated animals, corroborating earlier findings (5-6, 16-17, 30-32). Our data clearly demonstrate distinct peaks of ODC activity manifesting the multistep characteristics of AOM-induced colon carcinogenesis. The phasic increase in the colonic mucosal ODC activity of AOM-treated rats was 10-11 times higher than that of saline-treated rats. The levels of ODC activity in colon tumors of rats fed the control diet were 140-fold higher than the colonic mucosal ODC activity of vehicle-treated rats on the control diet. The results of present investigation and earlier studies (7-17) suggest that mucosal ODC activity can be used as a reliable and sensitive marker for colon carcinogenesis.

We expected that DFMO would inhibit the intermediate biomarkers, since it had inhibited colon tumor...
development; this was the case. DFMO has been shown to strongly inhibit cell proliferation and tumor development in rodents presumably by blocking ODC-catalyzed decarboxylation of ornithine into putrescine, thereby depleting intracellular polyamines (5–6). These polyamines are known to play an important regulatory role in the control of normal growth and neoplastic transformation (33–34). In most of the animal studies on chemoprevention reported to date, ODC inhibitors are shown to be very effective antitumor agents when treatment was begun prior to, concomitant with, or shortly after tumor initiation (31–32). In this study, where DFMO treatment began 1 week before AOM administration, we observed more than 90% inhibition of AOM-induced increase in ODC activity in a time-dependent manner, as well as suppression of colon tumor development. Tatsula et al. (35) found that prolonged administration of putrescine inhibited AOM-induced colon tumor incidence, the mucosal labeling index, and ODC activity and suggested that putrescine-augmented down-regulation of ODC is the basis of chemoprevention of AOM-induced carcinogenesis. It thus appears that inhibition of ODC activity interferes with the sequence of events in tumor development. Significant differences in the levels of colonic mucosal ODC activity between saline-treated and AOM-treated rats on the control diet as well as on the DFMO diet, as observed in this study, further attest to the fact that ODC may be used as a marker of DFMO-mediated suppression of colon tumor progression.

We expected that piroxicam would also exert its inhibitory effect on the biomarkers just as DFMO had. However, this was not the case. The results of the present study demonstrate that, in contrast to suppressive effect of DFMO on ODC activity, piroxicam significantly increased colonic mucosal ODC activity. Direct evidence establishing any mechanism by which piroxicam may exert its antitumor activity is lacking. However, the chemopreventive influence of piroxicam and other nonsteroidal antiinflammatory drugs like indomethacin and aspirin might be mediated through the inhibition of prostaglandin synthesis at the cyclooxygenase level or possibly by interfering with cellular mitosis at the G1 stage (31, 36–37). To our knowledge, there are no data pertaining to the modulation of ODC activity by piroxicam. Indomethacin is the only compound of this category so widely studied in the context of its chemopreventive action. DeRubertis et al. (38), in a related study, demonstrated that treatment of rats with indomethacin rapidly increased colonic mucosal ODC activity, [3H]thymidine incorporation into mucosal DNA, and enhancement of proliferative activity of colonic epithelium. These authors also showed that concurrent administration of prostaglandin analogue in rats suppressed an indomethacin-induced increase in colonic mucosal ODC activity, DNA synthesis, and epithelial proliferative activity. Recently, Noguchi et al. (39) demonstrated that indomethacin decreased mammary tumor incidence and tumor multiplicity and increased tumor latency but significantly promoted proliferation of DMBA-induced tumors in both high- and low-fat diet groups as indicated by increased tumor size, increased bromodeoxyuridine labeling index, and decreased tumor doubling time. Furthermore, since prostaglandins have a major role in the maintenance of normal gastrointestinal physiology, it is not surprising that drugs which inhibit the formation of prostaglandins interfere with normal gastrointestinal function. The nonsteroidal antiinflammatory drugs have been shown to cause gastric mucosal cell loss due to gastric erosion and peptic ulcer formation and perforation (40), which may initiate several rounds of compensatory hyperproliferation in gastrointestinal mucosa. In several other studies, aspirin and indomethacin have been shown to increase gastric and duodenal epithelial proliferation (41–42).

TPK activity has only recently been implicated in cellular proliferation, differentiation, and neoplastic transformation. The biochemical actions of most of the oncogenes and certain growth factors are thought to be mediated through the effects of specific protein kinases that are involved in selective phosphorylation of cellular proteins (18–20). Hennipman et al. (22) observed progressively increasing levels of TPK activity in benign to malignant breast tumors as compared to normal breast tissue. Elevated levels of TPK activity have been demonstrated in human colon cancer and colon cancer cell

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<th>16</th>
<th>24</th>
<th>32</th>
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<sup>a</sup> TPK activity is expressed as [32P]ATP phosphorylated/mg protein/min; values are mean ± SEM (n = 10).

<sup>b</sup> Significantly different from their respective control groups: 1, P < 0.001; 2, P < 0.01; 3, P < 0.05.

<sup>c</sup> Significantly different from their respective control groups: 1, P < 0.001; 2, P < 0.01; 3, P < 0.05.
lines. Arlow et al. (43) recently reported that an OAM-induced increase in colonic ODC activity in rats was associated with increased TPK activity. In our study, colonic mucosal TPK activity has exhibited a pattern similar to that of ODC activity. There was a consistent and significant increase in AOM-induced TPK activity in both cytosolic and membrane fractions of rats fed the control diet as compared with their saline-treated counterparts. A similar pattern of difference in AOM-induced TPK activity was observed in animals fed the piroxicam or DFMO diet. Although piroxicam appears to moderately enhance TPK activity both in AOM-treated as well as saline-treated animals, the dietary DFMO significantly suppressed the TPK activity in a systematic time-dependent manner (Table 5). Furthermore, we observed drastically reduced levels of membrane-bound TPK activity in relation to cytosolic TPK activity in AOM-treated animals fed the piroxicam diet both in colonic mucosa and tumors as compared to those in AOM-treated animals fed the control diet. Sakanoue et al. (44) have reported reduced levels of cytosolic tyrosine kinase activity and increased levels of membrane TPK activity in human colon carcinomas as compared with their adjacent normal mucosa. It is possible that the progressively increasing translocation of TPK from cytosol to membrane may be occurring with the advancing sequence of events in AOM-induced tumor development as in the case of phorbol ester-induced translocation of protein kinase C (45). It may also be possible that the cytosolic (nonreceptor) tyrosine kinase could be an antioncogene product itself. Recently, multiple genetic alterations have been detected that appear to contribute to the malignant transformation of colonic mucosa. Allergic deletions of chromosomes 5, 17, and 18 sequences have been identified in human colonic carcinoma and in adenomas, suggesting that tumor suppressor genes may be important regulators of cell growth (46).

In conclusion, the results of this study demonstrate the usefulness of mucosal ODC and TPK in analyzing the state of proliferating mucosa and events leading to tumor development. But these results also point to the fact that some chemopreventive agents, such as piroxicam, can inhibit cancer development without reducing proliferation. This of course raises interesting questions about the diverse mechanisms by which different agents exert their chemopreventive effects and the general usefulness of proliferating markers such as ODC as an intermediate end point. We suggest that it may be necessary to use “agent-specific intermediate biochemical markers” for evaluating the effectiveness of specific agent(s) in chemopreventive studies.

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