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Aging

Increased Responsiveness of Colorectal Mucosa to Carcinogen Stimulation and Protective Role of Folic Acid

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Recent investigations have demonstrated a protective role for folic acid in dysplasia and neoplasia, through an unknown mechanism. The current study was designed to evaluate whether the protective role of folic acid is due, in part, to its antiproliferative properties. In addition, because colorectal neoplasia is more common with increasing age, we have compared results in both old (22 months) and young (3 months) rats. Colorectal mucosal explants of rats treated with the known carcinogen methylazoxymethanol, were supplemented with folic acid. Ornithine decarboxylase was then measured as an index of cellular proliferative activity. We observed that supplemental folic acid suppressed carcinogen-induced ornithine decarboxylase activity by 64% in the old and 74% in the young rats. Furthermore, a similar phenomenon was observed for tyrosine kinase, which was measured for comparison. The suppression of hyperproliferative activity by supplemental folic acid may contribute to the protective effect of folic acid in colorectal neoplasia.

KEY WORDS: folic acid; antiproliferative effect; ornithine decarboxylase activity; tyrosine kinase activity; dysplasia.

Recent studies have demonstrated that supplemental folic acid (FA) can suppress dysplasia arising in a variety of tissues including the colon (in ulcerative colitis), the cervix, the bronchi, and breast (1–5). Although the mechanism is unknown, it is suggested that supplemental FA may, in part, be beneficial by suppressing abnormal cell proliferation through its DNA-stabilizing effect.

Ornithine decarboxylase (ODC) and tyrosine kinase (Tyr-k) are two enzyme markers of proliferative activity that are increased in conditions predisposed to dysplasia and neoplasia (6). Since increased cellular proliferative activity is present in both dysplasia and neoplasia, the current study was designed to evaluate whether supplemental FA can suppress proliferative activity induced by a carcinogen. Furthermore, since the development of colorectal neoplasia is commonly an age-related phenomenon, we have compared results in both young and old animals. Aging itself has been associated with the predisposition to neoplasia and a hyperproliferative status (7). We have previously shown that aging rats have increased basal proliferative activity in colorectal mucosa and increased ODC activity. A similar increase in colorectal mucosal ODC activity has been reported in patients with ulcerative colitis (8) and in patients with adenomatous colorectal polyps (9). In the present study, we investigate the effect of folic acid on carcinogen-induced ODC activity in colorectal mucosal explants of young and old rats. As a pilot study, these experiments were conducted *in vitro* to eliminate systemic effects associated with the metabolism of folic acid.

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MATERIALS AND METHODS

Animals and Diets. Pathogen-free male Fischer 344 rats were obtained at eight weeks of age (young group) from . Harlan Breeding Labs, Walkersville, Maryland. Old rats

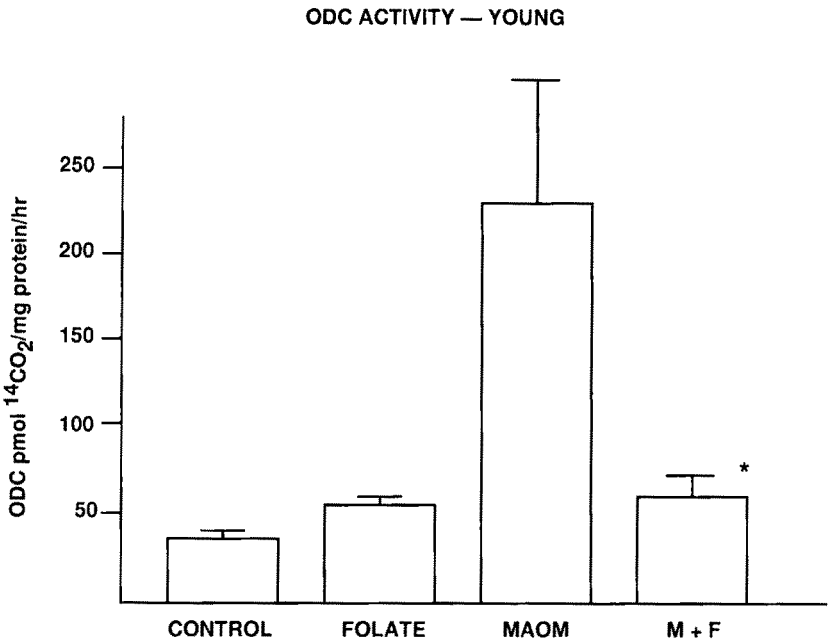

Fig 1. Inhibition of ODC activity (pmol $^{14}CO_2$ released/mg protein/hr) in MAOM (100 μg/ml)-treated colonic mucosal explants of young rats (3 months) by folic acid; 74% inhibition of ODC ($P < 0.05$) was seen with folic acid (500 ng/ml) when compared with MAOM alone. Columns are means of four observations; bars, SEM. * P value < 0.05.

were obtained from the National Institute of Aging, Bethesda, Maryland, at 20 weeks of age.

Animals were quarantined in our facilities for two weeks prior to experimentation, at which time their weight was 150–250 g. Animals were housed in metal cages in a light- (12 hr/day) and temperature- (20° C) controlled rodent colony with daily care. They were given water and Ralston Purina rat chow diet *ad libitum* and fasted over night prior to experiments. All experiments were conducted *in vitro*, in mucosa prepared for organ culture (see below). Folate was added directly to the nutrient media after a stabilization period and incubated for 4 hr prior to removal for tissue homogenization and enzymatic assays.

Carcinogen Procedures. All procedures involving carcinogen were reviewed and approved by the Animal Use Committee. Methylazoxymethanol (MAOM) acetate was purchased from Ash Stevens Co., Detroit, Michigan. MAOM was diluted in sterile water to a concentration of 50 mg/ml for use in organ culture studies and stored at 4° C in the dark until used.

Organ Culture. *In vitro* experiments were performed to assess whether folic acid exerts a direct suppressant effect on MAOM-induced ODC and Tyr-k activity. For this purpose an organ culture system was used, similar to that used by us previously (6). MAOM was used instead of azoxymethane (AOM) because, unlike AOM, MAOM does not require *in vivo* liver metabolism to be an active carcinogen (10).

The procedure to prepare explants was the same as described previously (6) in which the intestinal explants remained viable and metabolically active for up to 36 hr (11). Briefly, male Fischer 344 untreated rats were sacrificed by CO_2 narcotization and decapitation, and colonic tissue was obtained from the abdominal cavity. After careful cleansing in ice cold saline multiple 2 × 2-mm mucosal biopsies (10–15 mg each) were obtained and four were floated on 1 × 1-cm sterilized paper rafts in 2 ml Eagle's minimum essential media (1 μg/ml folate) containing 10% dialyzed fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 mg/ml) in 60-mm Petri dishes. Following a 4-hr equilibrium period at 37° C CO_2/oxygen (5/95%) incubation, explants were incubated for 4 hr in the presence of MAOM (10 and 100 μg/ml) and added folic acid (250 and 500 ng/ml). A dose–response curve was previously done to determine optimum concentrations of MAOM and folic acid required (data not shown). Following the 4-hr test period, four mucosal explants were homogenized in 0.25 ml of homogenizing buffer and processed for ODC or Tyr-k.

There were four groups, with each condition completed in quadruplicate: (1) saline (control), (2) MAOM, (3) folic acid, and (4) MAOM + folic acid. Specimens were homogenized, processed, and assayed for ODC and Tyr-k activity.

Biochemical Assays. ODC buffer contains 55 mM HEPES (pH 7.4), 1.05 mM EDTA, 0.025 mM pyridoxol-5-phosphate, and 1.0 mM dithiothreitol. The ODC activity in colonic mucosa was determined by a modification of the micromethod of Beaven et al (12), similar to that previously described from our laboratory (6). The assay reagent contained 50 mM HEPES (pH 7.4), 1.05 EDTA, 0.25 mM pyridoxol-5-phosphate, 1.0 mM dithiothreitol, and 0.144 μCi of L-[1-^{14}C]ornithine (40–60 mCi mmol; NEN, Boston, Massachusetts) in 0.01 N HCl. A 10-μl sample of the supernatant extract of the tissue homogenate was mixed with 10 μl of assay reagent in a 1.5-ml Eppendorf vial. This

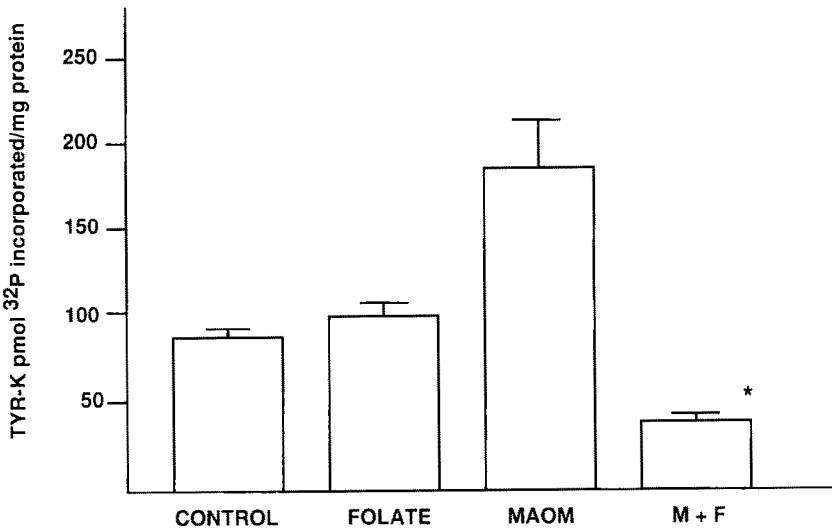

Fig 2. Inhibition Tyr-k activity (expressed as pmol ^{32}P incorporated/mg protein) in MAOM (100 μg/ml)-treated colonic mucosal explants of young rats (3 months) by folic acid; 80% inhibition of Tyr-k (* $P < 0.05$) was seen with folic acid (500 ng/ml) when compared with MAOM alone. Columns are means of four observations; bars, SEM.

was placed inside a 20-ml screw cap scintillation vial, and 20 μl of 1 M methylbenzethonium hydroxide in methanol was placed on the bottom of the scintillation vial. The scintillation vials were tightly capped and incubated at 37° C for 60 min; then they were placed on ice, uncapped one at a time, and 10 μl of 2 M citric acid was added to the Eppendorf vial. The scintillation vials were quickly recapped and reincubated for 60 min. At the end of the second incubation, the Eppendorf vials were removed, rinsed with 2 ml of 95% ethanol and discarded, and 10 ml of liquid scintillation cocktail were added for assay of $^{14}CO_2$. Protein content was determined by the method of Bradford (13), utilizing bovine serum albumin as the standard.

To determine tyrosine kinase activity, membrane (30,000*g* pellet) fractions were prepared from colonic mucosa homogenized in Tyr-k buffer containing 10 mM HEPES (pH 7.2), 150 mM NaCl, and 1 mM $MgCl_2$ according to the method of Dangott et al (14) as described by Majumdar et al (7). Tyr-k activity was measured using poly(L-Glu-Tyr) 4:1; (Sigma Chemical Co., St. Louis, Missouri) as substrate (7, 14), which has been shown to be highly specific for Tyr-k (15). The reaction mixture contained 2.5 μCi ATP, 11.7 Ci/mmol (New England Nuclear, Boston, Massachusetts) and 50 μg of Glu-Tyr polymer, in a final volume of 50 μl. The reaction was initiated with membrane preparations (10–20 μg protein). Orthovanadate was added to inhibit degradation of ATP and dephosphorylation of the phosphopeptide. The reaction was terminated by applying 20 μl of the reaction mixture onto 3-cm^2 Whatman No. 2 MM filter paper. The filters were washed three times in 10 ml of 10% trichloroacetic acid containing 10 mM sodium pyrophosphate, rinsed with ethanol, dried, and radioactivity quantitated in 5 ml scintillation cocktail. Results are expressed as picomoles ^{32}P incorporated per milligram protein.

Statistical Analysis. Where applicable, data were analyzed using the two-tailed, unpaired Student's *t* test, and Kruskal-Wallis one-way analysis of variance taking $P < 0.05$ as the level of significance. For purposes of data analysis, this was a completely randomized design with four treatment groups.

RESULTS

Four hours of exposure of explants to MAOM produced increased colonic mucosal ODC activity: by 544% and 274% in young and old rats, respectively. Aging was associated with an increased responsiveness of the colorectal mucosa to a lower dose of MAOM compared to younger rats (10 vs 100 μg/ml). ODC activity was suppressed by folate in both age groups: 74% for young ($p < 0.05$) and 64% for old ($P < 0.04$). However, a higher dose P < was required for suppression in the younger rats (500 vs 5) 250 ng/ml). A similar phenomenon was observed for Tyr-k activity with 80% inhibition using a dose of 500 ng/ml for young ($P < 0.05$) and 57% inhibition with 250 ng/ml in the old rats (see Figures 1–4).

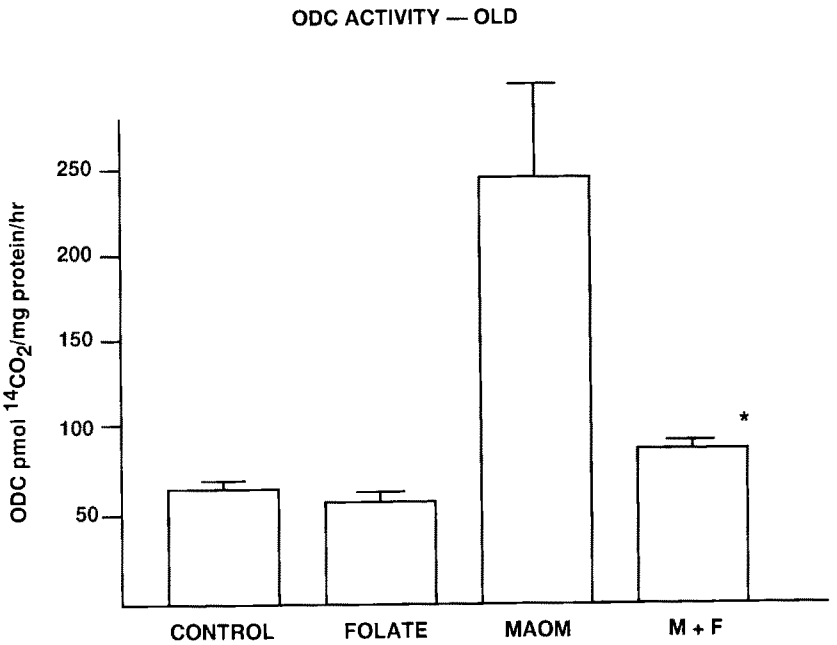

Fig 3. Inhibition of ODC activity (pmol $^{14}CO_2$ released/mg protein/hr) in MAOM (10 μg/ml)-treated colonic mucosal explants of old rats (22 months) by folic acid; 64% inhibition of ODC ($P < 0.042$) was seen with folic acid (250 ng/ml) when compared with MAOM alone. A lower dose of both MAOM and folic acid were required for stimulation and suppression in the old compared to the young rats. Columns are means of four observations; bars, SEM.

DISCUSSION

ODC and Tyr-k are two enzyme markers of proliferative activity that are increased in conditions predisposed to dysplasia and neoplasia (6). We used these markers to assess whether supplemental FA can suppress carcinogen-induced ODC and Tyr-k and whether there is a different response in young and old animals. Folic acid supplementation has recently been shown to suppress dysplasia arising in a variety of tissues, through an unknown mechanism (1–5). Utilizing an *in vitro* model of colorectal mucosal hyperproliferative activity, we assessed the effect of supplemental FA on rodent tissue stimulated by the carcinogen methylazoxymethanol (MAOM). This model was used because it mimics the hyperproliferative and dysplastic changes that occur in the colon during the process of carcinogenesis. MAOM, the active metabolite of the rodent colorectal carcinogen azoxymethane, induces a stepwise increase in colorectal ODC activity prior to the development of macroscopic tumors (16). ODC is the rate-limiting enzyme in polyamine biosynthesis, whose activity increases in conditions of cell turnover such as injury, regeneration, or neoplasia (17). Accumulated data suggest that polyamines regulate cellular functions via their effects on enzyme activities, nucleic acid and protein synthesis, and stabilization of membrane structures, functions also associated with folate activity.

ODC is a useful enzyme for measuring proliferative activity *in vitro*, because it responds rapidly to proliferative stimuli and may be detected within 2–4 hr after induction (18). An *in vitro* rather than *in vivo* system was studied so that tissue levels of FA could be readily manipulated and systemic or metabolic interactions such as that between FA and vitamin B_{12}, could be eliminated. Lashner et al postulated that in conditions of inflammation such as ulcerative colitis, colonic cells entered a rapidly proliferating state, developing a "relative" folate deficiency and a tendency for dysplasia (1). This, in part, may explain why the colorectal mucosa in ulcerative colitis is predisposed to neoplasia and is associated with increased ODC activity (8).

Other investigators have also observed a beneficial effect of FA in suppressing neoplasia. Cravo et recently demonstrated that folate-deficient rats exposed to the colorectal carcinogen dimethylhydrazine al developed significantly more colorectal carcinoma and dysplasia (19) than folate replete controls. An inverse

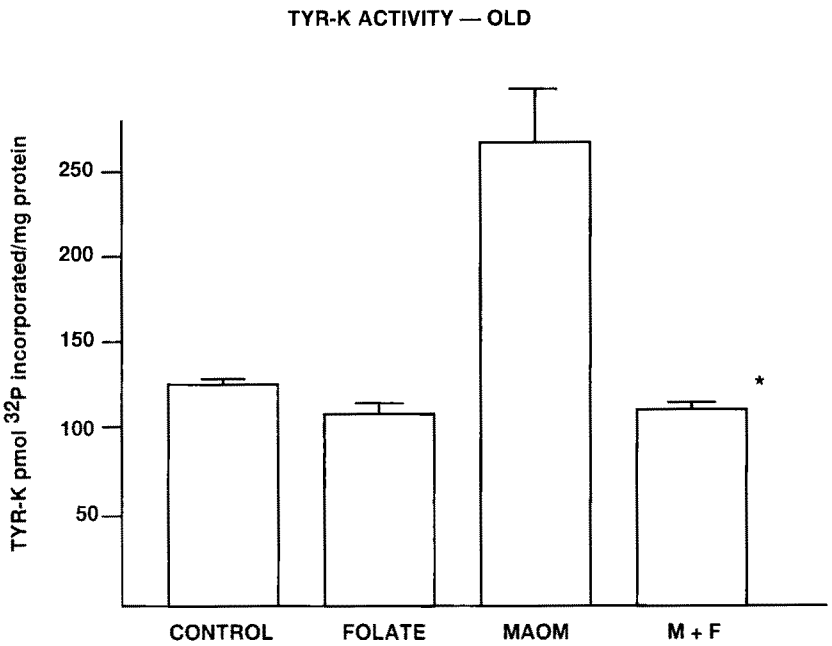

Fig 4. Inhibition of Tyr-k activity (pmol ^{32}P incorporated/mg protein) in MAOM (10 μg/ml)-treated colonic mucosal explants of old rats (22 months) by folic acid; 57% inhibition of Tyr-k ($P < 0.001$) was seen with folic acid (250 ng/ml) when compared with MAOM alone. A lower dose of both MAOM and folic acid were required for stimulation and suppression in the old compared to the young rats. Columns are means of four observations; bars, SEM.

correlation was found between the severity of histologic lesions and tissue folate levels.

In the current study, Tyr-k, an enzyme that also responds rapidly to a variety of proliferative stimuli, was measured for comparison (7). In human studies, increased ODC and Tyr-k activity have been measured in adenomatous colorectal polyps as they become progressively more dysplastic (9).

Colorectal cancer and polyps are known to be associated with aging. We demonstrated a differential response between young and old animals such that the mucosa of young rats was resistant to induction of ODC activity, requiring a higher dose of carcinogen than that of the old. In addition, a higher dose of FA was required for suppression of ODC activity in the young compared to the old. The reason for this different response is unknown but may possibly involve an age-related increased susceptibility to carcinogenesis. We have recently shown that the gastric and colorectal mucosa of aging rats have increased levels of basal ODC activity and altered responsiveness to trophic polypeptides (20). Lipkin et al demonstrated that many of the premalignant conditions of the gastrointestinal tract associated with aging, such as Barrett's metaplasia, atrophic gastritis, and colorectal polyps are characterized by hyperproliferative mucosa (21).

We have postulated that FA supplementation may affect the proliferative status of the colorectal mucosa through its ability to stabilize DNA. Although we did not directly measure DNA synthesis, FA is an important coenzyme in purine and pyrimidine synthesis as well as in amino acid metabolism, methylation of biogenic amines, and the initiation of protein synthesis (22, 23), all directly related to DNA synthesis. In a relatively folate-deficient state, cells that are rapidly dividing have been shown to develop defective DNA synthesis and chromosome breaks, which may partially explain the tendency to develop neoplasia and dysplasia.

Taken together, these observations suggest that folic acid may in part, suppress dysplasia through an antiproliferative effect, with greater benefit in older animals. Whether this would result in less progression to carcinoma is unknown, although the opposite has recently been demonstrated (19). Other possible mechanisms for the beneficial effect of supplemental FA include combining directly with a carcinogen or a tumor promoter to render them inactive. Further

studies are necessary to explore these possibilities and elucidate any potential clinical relevance.

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