APC-dependent changes in expression of genes influencing polyamine metabolism, and consequences for gastrointestinal carcinogenesis, in the Min mouse

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The colorectal mucosa of pre-symptomatic individuals with familial adenomatous polyposis (FAP) contains elevated levels of the proliferation-associated polyamines. The Min mouse, like humans with FAP, expresses an abnormal genotype for the APC tumor suppressor gene. In order to determine how APC mutation influences intestinal tissue polyamine content, we measured steady-state RNA levels of ornithine decarboxylase (ODC), the first enzyme in polyamine synthesis, antizyme (AZ), a protein which negatively regulates ODC, and the spermidine/spermine N1-acetyltransferase (SSAT), the first enzyme in polyamine catabolism. RNA content was increased 6- to 8-fold in both the small intestine and colon for ODC, decreased significantly in the small intestine but not the colon for AZ and was not statistically different in either intestinal tissue for SSAT in Min mice compared with normal littermates. Consistent with the changes in ODC and AZ gene expression, small intestinal, but not colonic, polyamine content was elevated in Min mice compared with normal littermates. Treatment of Min mice with the specific ODC inhibitor difluoromethylornithine (DFMO) suppressed small intestinal, but not colonic, polyamine content and tumor number. These data indicate that small intestinal tissue polyamine content is elevated in Min mice by a mechanism involving APC-dependent changes in ODC and AZ RNA. Further, ODC enzyme activity, which is influenced by both ODC and AZ RNA levels and inhibited by DFMO, is consequential for small intestinal tumorigenesis in this model. In the FAP population, DFMO may be of value in the chemoprevention of small intestinal adenocarcinoma that remains a risk following colectomy.

Introduction

Familial adenomatous polyposis (FAP), an inherited polyposis syndrome, is the result of germline mutation of the adenomatous polyposis coli (APC) tumor suppressor gene (1). This autosomal-dominant condition with variable expression is associated with the development of hundreds of colonic adenomas, which uniformly progress to adenocarcinoma by 40 years of age, two decades earlier than the mean age of diagnosis for sporadic colon cancer (2). In prior studies of pre-symptomatic individuals with FAP, increased levels of the polyamines spermidine and spermine and their diamine precursor putrescine have been detected in normal-appearing colorectal biopsies when compared with normal family member controls (3). The activity of ornithine decarboxylase (ODC), the first and rate-limiting enzyme in mammalian polyamine synthesis, is also elevated in apparently normal colon mucosal biopsies from FAP patients (3,4). These findings are of interest as elevated polyamine synthesis is associated with increased cell proliferation (5). Further, suppression of ODC activity, using the enzyme-activated irreversible inhibitor α-difluoro-methylornithine (DFMO), inhibits colon carcinogenesis in carcinogen-treated rodents (6,7). No information, however, is available regarding polyamine metabolism in the Min mouse model of gastrointestinal carcinogenesis nor have effects of DFMO on tumorigenesis in this model been reported to date.

This information is of interest, as recent studies have shown that one mechanism of tumor suppression by the APC gene involves its association with the β-catenin oncogene (8). This association prevents activation of the Tcf/Lef transcription factor by β-catenin (9), which in turn prevents expression of the c-myc oncogene by the Tcf/β-catenin transcription activator complex (10). Since the ODC gene has been established as a transcriptional target for c-myc (11,12), it follows that expression of ODC, and possibly other genes involved in polyamine metabolism, might be altered in the Min mouse as a result of APC mutation. If that scenario were true, then it would be of interest to determine if any alterations were consequential for tumorigenesis.

In order to test this hypothesis, we measured expression patterns of genes related to polyamine metabolism in the small intestinal and colonic tissues of Min mice, heterozygous for the APC allele, and littermates with two normal APC alleles. We chose to measure ODC, antizyme (AZ) and spermidine/spermine N1-acetyltransferase (SSAT). ODC acts to increase intracellular polyamine pools by increasing putrescine synthesis (5). AZ causes polyamine pools to decrease by targeting ODC for degradation and by suppressing polyamine uptake mechanisms (13). SSAT acts to suppress polyamine pools by acetylating spermidine and/or spermine, which facilitates further catabolism and/or export (14). We measured tissue polyamine contents, as this end-point reflects all aspects of polyamine metabolism and transport activities, and tumorigenesis in both the small intestines and colons of these mice.

Materials and methods

Rodent model

C57BL/6J-ApcMin+/Apc−/ mouse were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in The University of Arizona’s Animal Care Facility in accordance with The University of Arizona Institutional Animal Care Utilization Committee guidelines. These mice have a nonsense mutation in codon 850 of the murine Apc gene. Affected Min animals were obtained
by crossing Apc<sup>Min</sup>/Apc<sup>−</sup> males with B6 females. Offspring were screened as described elsewhere (15). Homozygous Apc<sup>1</sup>/Apc<sup>−</sup> littermates of both sexes were used as controls. DFMO was administered as a 2% solution in drinking water beginning on day 45 of life.

**Tissue collection**
Mice were killed at 65 and 114 days of age by CO₂ asphyxiation to evaluate intestinal tissues prior to (65 days) and after extensive tumorigenesis (114 days). After removal, the small intestine and colon were flushed with buffered saline and processed for RNA and polyamine analysis as described below. In the older mice, additional whole small intestine and colon segments were opened lengthwise, mounted and fixed in 70% ethanol for tumor scoring. Tumors from our Min colony were evaluated histologically. Lesions were primarily adenomas, with some displaying features of carcinoma in situ.

**RNA isolation and analysis**
Intestinal and colonic tissue samples from the mature mice were homogenized in TRIzol<sup>®</sup> reagent (Gibco BRL, Grand Island, NY) for the isolation of total cellular RNA as described (16). Purified total RNA from each sample was loaded onto a 1.0% agarose–formaldehyde gel and transferred to a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL). Membranes were subsequently hybridized with a [³²P]-labeled cDNA encoding mouse ODC (1.38 kb EcoRI–EcoRI fragment) and human SSAT (0.67 kb EcoRI–EcoRI fragment) utilizing a random priming technique (Boehringer Mannheim, Indianapolis, IN). Data are expressed (relative gene expression) as the ratio of the integrated [³²P]-labeled hybridization bands for the gene of interest and the integrated density of the ethidium bromide stained 18S ribosomal band to control for variation in loading. For analysis of ODC antizyme expression, mRNA was purified using an Oligotex™ mRNA isolation kit (Qiagen, Santa Carisa, CA). mRNA was separated and analyzed by northern blotting, as described elsewhere (17). As hybridization probes, whole cDNA inserts for AZ (0.95 kb EcoRI–XhoI fragment) and glyceraldehyde 3-phosphate dehydrogenase (GAPD) (0.75 kb PstI–XhoI fragment) were [³²P]-labeled. Autoradiograms were quantitated by densitometric analysis (Imagequant densitometer; Molecular Dynamics, Sunnyvale, CA). Amounts of AZ mRNA were expressed as the ratio of integrated densities of the AZ and GAPD bands.

**Polyamine analysis**
After collection tissue samples were stored frozen at −80°C. Samples were processed and assayed for polyamine (putrescine, spermidine and spermine) content by reverse phase high performance liquid chromatography as previously described (17). The residual acid-insoluble pellet from the initial centrifugation was assayed for protein content with the BCA protein assay kit (Pierce, Rockford, IL). Data are expressed as nmol polyamine/mg protein.

**Statistics**
Assessment of statistical differences between Min and littermate control mice were determined by ANOVA using a minimum of six mice for each group or data point. A P value < 0.05 was considered statistically significant.

**Results**

**Expression of genes affecting polyamine metabolism**
The relative expression of RNAs encoding ODC, AZ and SSAT were evaluated in intestinal tissues from 114-day-old Min mice and normal littermates. As shown in Figure 1A, relative steady-state levels of ODC RNA were similar in colon and small intestine when comparing similar genotypes, but were increased 6- to 8-fold in both the small intestine and colon of Min mice, compared with values measured in similar tissues of normal littermates.

Steady-state mRNA levels of AZ RNA were higher in small intestinal, compared with colonic, tissues of normal mice. The high level of AZ RNA in small intestinal tissue was reduced by ~75% in Min mice, compared with normal littermates. However, expression of antizyme was not affected by the Min phenotype in colonic tissue (Figure 1B). Steady-state levels of SSAT RNA were somewhat higher in colonic, compared with small intestinal, tissues of normal littermate controls (Figure 1C). This difference was not observed in Min mice. Differences in steady-state SSAT RNAs were not statistically significant, either comparing small intestine with colon values or when comparing values from Min mice with normal littermates.

**Evaluation of intestinal tissue polyamine contents**
Polyamine contents from small intestinal and colonic tissues of 114-day-old Min mice and normal littermates are shown in Figure 2. At this age, the Min small intestine displayed extensive adenoma development, such that assessment of polyamine content in apparently normal tissue distinct from adenomas was not possible. As shown in Figure 2A, putrescine, spermidine and spermine contents were elevated in a statistically significant manner in the small intestine from Min mice, compared with normal littermates. Similar differences in small intestinal polyamine content were seen in 65-day-old Min mice when compared with littermate controls (data not shown). At this younger age, the Min mice had not yet developed the dramatic small intestinal tumor load that characterizes the animal model. No differences in polyamine content were observed in colon tissues from either age group of mice (Figure 2B).

**Effect of DFMO on intestinal polyamine content and tumorigenesis in Min mice**
In order to determine if the elevation in polyamine content, which is associated with an increase in ODC expression and...
Polyamine metabolism in the Min mouse

Fig. 2. Polyamine content (means ± SEM) for 114 day old mice in (A) proximal and distal small intestine and (B) proximal and distal colon in control and Min mice (N = 6/group). Significant differences were seen in the proximal small intestine *P < 0.05 between Min and control for all three polyamines.

Fig. 3. Putrescine content in small intestine and colon for control and Min mice (n = 6/group) receiving 2% DFMO in the drinking water. A decrease in AZ expression, was influencing small intestinal tumorigenesis in this model, we treated Min and control mice with the ODC inhibitor DFMO. Administration of DFMO in the drinking water suppressed the Min-specific increase in putrescine content in small intestinal tissue (Figure 3). This effect was statistically significant in the proximal small intestine (P < 0.05). No differences were seen in small intestinal spermidine or spermine content with DFMO administration. Colonic polyamine content was unaffected by DFMO treatment in this rodent model of intestinal carcinogenesis.

DFMO suppressed small intestinal tumorigenesis in Min mice in a statistically significant manner, as shown in Figure 4. Untreated Min animals developed 41.0 ± 4.6 (mean ± SEM) intestinal tumors/mouse, while DFMO treatment reduced this number to 19.3 ± 5.5 tumors/mouse (P = 0.013). In contrast to the small intestine, where DFMO suppressed tissue polyamine content and tumor number, DFMO did not suppress colonic polyamine content and had little or no discernible effect on colonic tumorigenesis in this model. DFMO administration did not alter the tumor size distribution in either the small intestine or colon. No DFMO-related toxicity, as measured by body weight changes, was apparent in treated mice.

Discussion

Mutation of the murine homolog of the human APC gene in the Min mouse results in extensive gastrointestinal tumorigenesis, especially in the small intestine. We found that gastrointestinal
Polyamine metabolism is altered in these animals. Previous investigations have reported that polyamine metabolism is altered in humans with FAP (3,4). In the Min mouse model, tissue polyamine content was elevated in the small intestine, but not the colon. The increased polyamine content in the small intestine was associated with an increase in ODC and a decrease in AZ steady-state RNA levels. These two changes would predict an increased ODC-dependent polyamine content in the small intestine of these mice. In support of this hypothesis, we found that treatment of Min mice with the specific ODC inhibitor DFMO suppressed the Min-dependent increase in small intestinal polyamine content and small intestinal carcinogenesis in a statistically significant manner.

It is well known that ODC enzyme activity is labile and its measurement in gastrointestinal tissue is influenced by a number of technical factors (18). As a result of this consideration, and our own studies indicating that polyamine content is a more reliable measure of polyamine metabolism and DFMO effect in gastrointestinal tissues (19–21), we assessed the consequence of changes in ODC and AZ RNA levels in Min mice by measuring tissue polyamine contents directly.

Polyamine content was not elevated in colonic tissue of Min mice, although steady-state levels of ODC RNA were increased in a statistically significant manner in this tissue. One explanation for this observation might be that the not quite statistically significant decrease in SSAT RNA in colonic tissue might counteract the effects of elevated ODC RNA, since ODC and SSAT would have opposite effects on net polyamine pool sizes. DFMO did not suppress colonic polyamine content, suggesting that colonic polyamine pools are not strictly dependent on ODC. Others have documented that diet and intestinal bacteria, present in the gastrointestinal lumen, can serve as a significant source of polyamines in support of tumor growth (22,23). We measured polyamine content in our chow, which contained 0.87 (putrescine), 0.44 (spermidine) and 0.13 (spermine) mmol/mg dry wt polyamines. These mice ate ~5 g chow/day, indicating a dietary intake of polyamines of 0.5–4+ µmol polyamine/day. Thus, dietary polyamines could limit the effectiveness of DFMO in these studies.

Our data suggest that the Min-dependent increase in tissue polyamine content, as mediated by changes in expression of ODC and AZ, is one component of small intestinal carcinogenesis in this model. The lack of an effect of DFMO on colon carcinogenesis in this model appears to be due to the inability of this drug to suppress colonic mucosal polyamine content. This result may be unique to rodents, as we have previously shown that DFMO can suppress colorectal mucosal polyamine content in humans (20,21). The regulation of polyamine synthesis, catabolism, uptake and efflux in rodents and humans and the relative importance of each of these processes on determining tissue polyamine content is currently under active investigation in our laboratory.

It is unknown how the APC mutation influences ODC or AZ gene expression or how polyamines influence APC-dependent tumorigenesis in this model. The pathways leading from the APC mutation to ODC expression may involve the c-myc oncogene. ODC is a target gene for transcriptional transactivation by c-myc (11,12) and APC has recently been shown to suppress c-myc expression mediated by the Tcf/Lef transcriptional activation mechanism (10). Our results, reported here, showing that ODC RNA is increased in intestinal mucosa from Min mice are similar to those of Williams et al. (24) for cyclooxygenase-2 (COX-2), an enzyme involved in prostaglandin metabolism. COX-2, like ODC, has been linked to intestinal tumorigenesis. COX-2 expression is up-regulated in neoplastic colonic tissues (25) and COX-2-specific inhibitors suppress gastrointestinal tumorigenesis in APC mutant (26) and chemical carcinogen-treated (27) rodents. Thus, both ODC and COX-2 appear to be downstream effectors of the APC signaling pathway. The APC mutation is associated with an increase in ODC and a decrease in AZ RNA in the small intestine of Min mice. Future studies will be necessary to define the specific mechanisms by which APC modulates the expression of ODC, AZ and other genes, such as COX-2.

The younger mice in this study did not have obvious small intestinal tumors and were therefore lacking the adenoma burden that characterizes the older Min mice. We did not document the presence of microadenomas in our 65-day-old mice. However, others have identified small intestinal microadenomas and have speculated that these may develop from a single mutated multipotent crypt stem cell in animals <30 days of age (28). Adenoma mass, which can have an increased polyamine content, could not account for the increase in polyamine tissue content seen in the 65-day-old mice. Giardiello et al. (3) also found elevated putrescine content in the flat rectal mucosa of pre-symptomatic FAP carriers prior to the development of polyposis. These observations suggest that increased ODC expression is also phenotypic of either the heterozygous APC state, suggesting a possible dominant-negative effect resulting from expression of the mutated Apc allele, or that inactivation of the normal Apc allele occurs in apparently normal intestinal mucosa of 65-day-old mice. Multiple mechanisms for silencing the normal Apc allele have been documented (29). ODC may be either a direct or indirect downstream target of the β-catenin/Tcf-4 complex (10), which could account for the increased putrescine content seen in pre-symptomatic FAP patients and the Min mouse and could be postulated to accelerate the carcinogenesis process.

To date, the majority of cancer chemoprevention strategies for FAP have focused on the colon. However, up to 90% of FAP patients will also develop pre-neoplastic proximal small intestinal adenomas that remain a significant risk factor following colectomy (30). Future therapeutic options aimed at preventing or slowing adenoma progression in the colon and small intestine will, hopefully, influence the morbidity and mortality of FAP. Pharmacological interventions aimed at gene products activated as a consequence of APC mutation/deletion, such as ODC or COX-2, may help to correct the normal regulatory mechanisms that control cell turnover in the gastrointestinal tract.

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