Ape Mutation Is Associated with a Dominant-Negative Effect upon Intestinal Cell Migration

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ABSTRACT

Apc-associated intestinal tumor formation appears to require functional loss of both Apc alleles. Apc has, therefore, been classified as a tumor suppressor gene. Loss of Apc protein function results in increased intracellular β-catenin, a molecule important to both cell-cell adhesion and regulation of cellular growth. In mice bearing a germ-line Apc mutation, we found that enterocyte β-catenin expression was also increased in histologically normal intestinal mucosa. Enterocyte crypt-villus migration was decreased by 25%, and treatment of Min/+ animals with sulindac sulfide normalized both β-catenin expression and enterocyte migration. Our data suggest that alterations in enterocyte migration occur in cells bearing a single mutant Apc allele, and that sulindac sulfide may normalize enterocyte growth in these cells.

INTRODUCTION

The Apc3 gene mutation was first identified in patients with FAP, a disorder characterized by the presence of multiple colorectal polyps and early onset of colorectal cancer. Studies of the adenoma-carcinoma sequence in both sporadic cancers and tumors from FAP patients suggest that Apc mutation is an early event in the progression of colorectal cancer, occurring before ras or p53 mutation (1–3). These observations led to designation of Apc as the "gatekeeper" for colorectal carcinogenesis. The Apc gene encodes a Mr 310,000 protein that, in association with GSK-3β, a serine threonine glycogen synthase kinase, regulates intracellular levels of β-catenin. The role of β-catenin in colorectal carcinogenesis is unclear. β-catenin is a component of the adherens junction of intestinal epithelial cells and may, therefore, be a modulator of cell-cell adhesion or contact-associated growth regulation. β-catenin also participates in Wg-Wnt signal transduction.

Recent work shows that β-catenin binds the DNA-binding proteins, Tcf and Lef. In association with β-catenin, Tcf and Lef alter expression of genes that may govern cell proliferation and apoptosis (4). One function of Apc protein in human cells may be down-regulation of β-catenin/Tcf-4-mediated transcription. Recently, colorectal cancer cell lines mutant for Apc were found to contain a stable nuclear Tcf-4/β-catenin complex that constitutively transactivates transcription. When wild-type Apc was re-introduced in these cell lines, β-catenin released its association with Tcf-4, and transcription was inhibited (5). These data suggest that overexpression of β-catenin, through lack of Apc gene function, results in abnormal gene transcription that promotes tumor development.

Tumors arising in individuals with germ-line Apc mutations demonstrate loss of the normal allele (6). This loss occurs at the earliest visible stage of tumor formation, the aberrant crypt foci or microadenoma (7). The histologically normal intestinal mucosa retains the normal allele; thus, Apc is designated a tumor suppressor gene. In this study of the C57Bl/6J-Min/+ (Min/+) mouse, an animal model of FAP, we show that alterations in the intestinal epithelium occur before tumor development. We found that histologically normal Min/+ intestinal epithelium exhibits elevated β-catenin expression, which is associated with decreased proliferation, decreased apoptosis, and a decreased rate of enterocyte crypt-villus migration. We conclude from these observations that mutation of Apc exerts a dominant-negative effect resulting in decreased intestinal epithelial cell migration. In addition, we found that tumor-preventing doses of the NSAID, sulindac sulfide, normalized enterocyte proliferation and apoptosis and restored a normal enterocyte migration rate. These results suggest that NSAIDs act at a stage prior to tumor formation to reverse the effect of Apc mutation.

MATERIALS AND METHODS

Determination of Small Intestine Morphology. To compare the morphology of the small intestinal mucosa from Min/+ and +/+ animals, we measured the crypt-villus length, crypt-villus area, nuclear density, and total number of cells per crypt-villus unit using a Feulgen stain. Serial sections of formalin-fixed mid-small intestine were deparaffinized in xylene for 10 min. Following alcohol dehydration, the slides were rinsed in PBS and hydrolyzed in 5 N HCl for 1 h. The slides were then placed in Feulgen stain, covered with paraffin, and allowed to stain for 1 h before rinsing in Feulgen rinse solution. The slides were then washed in tap water for 5 min, followed by 1% acid alcohol for 5 min. The slides were then placed in 100% methanol for 6 min and cleared in xylene before coverslipping. Analysis of crypt-villus length and area was accomplished using the micrometer function of the Cell Analysis System 200 (CAS) software. Nuclear density was analyzed using the quantitative DNA program of the CAS 200. Five animals in each group were examined. For each animal, 10 sections of mid-small intestinal mucosa and 10 intact crypt-villus units from each section were measured.

Identification of Intact APC Protein in Intestinal Mucosa. Serial sections of mid-small intestinal mucosa from Min/+ and +/+ animals were deparaffinized and rehydrated as described above. Endogenous peroxidases were quenched in 3% H2O2, and the slides were rinsed in PBS. The sections were then incubated with goat anti-rabbit IgG for 30 min at 25°C, followed by 30 min treatment using the Vector Elite Avidin Biotin Complex (ABC) kit (Vector Laboratories, Inc., Burlingame, CA). The slides were then stained with DAB chromogen solution and counterstained with methyl green. As a control, the anti-APC antibody was absorbed using 10 times the amount of intact APC protein. This mixture was then used as a primary antibody in the method described above. As expected, this resulted in no staining of the Min/+ and +/+ tissue sections, thereby demonstrating specificity of the antibody staining. As an additional control, a polyclonal rabbit anti-human APC antibody that identifies the NH2 terminus of APC protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used. The slides were then stained with DAB chromogen solution and counterstained with methyl green. As a control, the anti-APC antibody was absorbed using 10 times the amount of intact APC protein. This mixture was then used as a primary antibody in the method described above. As expected, this resulted in no staining of the Min/+ and +/+ tissue sections, thereby demonstrating specificity of the antibody staining. As an additional control, a polyclonal rabbit anti-human APC antibody that identifies the NH2 terminus of APC protein (Santa Cruz Biotechnology,
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Inc., Santa Cruz, CA) was also used as the primary antibody in the method outlined above. This resulted in ubiquitous staining of both the tumor and the adjacent normal mucosa in Min/+ tissue. Absorption of this antibody with 10 times the amount of APC protein revealed no staining and was thus used as a negative control.

Determination of β-Catenin Expression. Slides were deparaffinized in xylene for 10 min followed by alcohol rehydration. After quenching endogenous peroxidases with 0.45% H₂O₂ in methanol, the slides were rinsed in PBS, and an antigen retrieval step was carried out in a 700-W microwave oven for 10 min in preheated pH 6.0 citrate buffer. The slides were then incubated with a monoclonal antibody to β-catenin (Transduction Laboratories, Lexington, KY) at 25°C for 1 h. Horse anti-mouse IgG secondary antibody was added for 30 min at 25°C followed by the Vector Elite ABC kit according to the manufacturer’s instructions. The slides were stained with DAB for 5 min, followed by counterstaining with methyl green. Analysis was carried out using the Cell Analysis System 200 (CAS 200) and CAS 200 Quantitative Nuclear Analysis Software.

Treatment of Min/+ Mice with Sulindac Sulfide. Female C57BL/J-Min/+ (Min+/+) mice were obtained at 5 weeks of age from The Jackson Laboratory (Bar Harbor, ME). Beginning at 5–6 weeks of age, Min/+ mice were fed AIN-76A chow diet (Research Diets, New Brunswick, NJ) and given solutions of sulindac sulfide (Merck, Rahway, NJ) to drink. Sulindac sulfide was solubilized in water by stirring 180 mg of the compound with 360 mg of sodium bicarbonate until dissolved, followed by dilution with water to a final concentration of 0.167 mg/ml of sulindac sulfide. The dose administered is estimated at 0.5 ± 0.1 mg/day (0.05 mg/kg/day or 200 μg/mg feed). As controls, Min/+ mice and C57BL/J-Min/+ nonaffect ed mice (+++/+) were fed AIN-76A diet and given tap water to drink. The animals were fed daily for signs of distress or anemia, and animals and their food were weighed weekly. During the course of the experiment, there was no difference in body weight or food consumption among the various study groups, thus documenting the approximate intake of 2.5 g/day of food and 3.0 ml/day of water. Mice were euthanized at 110 days of age for measurement of proliferation and apoptosis and 65 days of age for determination of enteroctye migration, and their intestinal tracts were examined by an observer blinded to the animal’s genetic status and treatment group. Samples of grossly normal mucosa were harvested from each group of mice and processed for studies of apoptosis, proliferation, and enteroctye migration as described.

Measurement of Enteroctye Proliferation. Small bowel sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked by incubating the slides with 0.45% methanol/H₂O₂. Antigen retrieval was achieved by microwaving (700-W microwave oven for 10 min in preheated pH 6.0 citrate buffer). PCNA (Dako, Carpinteria, CA) antibody was applied and incubated for 1 h at room temperature. Indirect detection was performed by incubating with secondary biotinylated horse-anti-mouse IgG followed by Vector Elite ABC for 30 min at room temperature. Incubation for 5 min in DAB was used for color development. The specimens were counterstained with methyl green. For each animal, eight crypt-villus units were chosen randomly from serial sections of small bowel mucosa by an individual blinded to the animal’s genetic status. The percentage of staining of enterocytes in these crypt-villus units was measured using the Cell Analysis System 200 (CAS 200) and CAS 200 Quantitative Nuclear Analysis Software.

Measurement of Enteroctye Apoptosis. Apoptosis was identified using the ApopTag kit (Oncor, Gaithersburg, MD). Small bowel sections were processed according to the manufacturer’s directions. For each animal, eight crypt-villus units were chosen randomly from serial sections of small bowel mucosa by an individual blinded to the animal’s treatment group and genetic status. The percentage of staining of enterocytes in these crypt-villus units was measured using the Cell Analysis System 200 (CAS 200) and CAS 200 Quantitative Nuclear Analysis Software. To confirm that uniform sampling was achieved, nuclear density was measured and confirmed to be equal throughout the three study groups. All of the positively stained cells were located in the upper one-third of the crypt-villus.

Measurement of Enteroctye Migration. After 1 month of AIN-76A diet, with or without sulindac sulfide as described above, 10 mice from each experimental group (Min, ++/+, and Min/sulindac sulfide) were injected with 0.3 μg/kg of BrdUrd (Sigma Chemical Co., St. Louis, MO). One animal from each of the three groups was euthanized by CO₂, injection at 1 h after injection and then at eight other time points at 6–10 h intervals. At harvest, the intestinal tracts were immediately removed from duodenum to distal rectum, opened, flushed with PBS, and examined under ×3 magnification. Multiple samples of grossly normal, full-thickness bowel were obtained from the mid small intestine. Samples were fixed in 10% formalin for histological examination and immunohistochemistry. The animals were harvested, and the specimens were assayed by an individual blinded to the animal’s genetic status and treatment.

To detect BrdUrd incorporation into newly synthesized epithelial cells, specimens of mid small bowel of approximately 5 mm in length were formalin fixed, embedded in paraffin, and sectioned at 5 μm. Several serial sections were obtained. Prior to staining, the sections were deparaffinized in Hemo-De and rehydrated through an alcohol series. The first section was stained with H&E to document normal mucosal histology. The sections were then stained with anti-BrdUrd antibody as follows. Endogenous peroxidase activity was blocked by incubating the specimens in 3% H₂O₂ for 10 min at 37°C. DNA was then denatured by immersing the specimens in 2 N HCl for 30 min, followed by enzymatic pretreatment by immersing specimens in trypsin for 20 min. Five % goat serum was then applied to the slides for 15 min, followed by a 2-h incubation with anti-BrdUrd (Dako, Carpinteria, CA) at 37°C. Specimens were then incubated with biotinylated horse-anti-mouse IgG and avidin-biotin complex for 30 min at room temperature, followed by color development by incubation in DAB for 5 min. The specimens were counterstained with Vector Alkaline Phosphatase substrate kit (Vector Laboratories, Inc., Burlingame, CA) and coverslipped. Eight intact histologically normal crypt-villus units from each animal were examined.

RESULTS

Enterocyte β-Catenin Expression, Proliferation, and Apoptosis. As a result of Apc mutation, Min/+ mice develop multiple tumors of the small intestine, with few tumors of the large intestine or duodenum (6, 8). We, therefore, focused our study on the small intestinal mucosa from Min/+ mice. To ensure uniformity of sampling, all specimens were taken from the mid portion of the small intestine. The histologically normal small intestinal epithelium from Min/+ animals was rigorously compared to that of wild-type littermates (+/+ ) by light microscopy using Feulgen stain. No differences in crypt length, crypt area, nuclear density, or number of cells per crypt-villus unit were observed (Table I). Immunohistochemical staining of small intestinal mucosa from Min/+ and ++/+ animals, using an antibody to the COOH terminus of APC protein, documented the presence of full-length APC protein in the histologically normal mucosa of Min/+ animals, as well as the absence of normal protein in an adjacent adenoma (Fig. 1). Staining with antibody to β-catenin demonstrated a 75% increase in staining intensity in the histologically normal Min/+ mucosa compared to that of +/+ animals (Fig. 2).

In spite of its normal histological appearance, further analysis of Min/+ small intestine revealed striking differences in cellular proliferation and apoptosis. By immunohistochemistry using an antibody to PCNA, proliferation in the +/+ animals was 44.4%. This overall high level of proliferation may be due to the PCNA technique, which is known to demonstrate higher levels than that reported using [3H]thymidine or bromodeoxyuridine incorporation (9). Nevertheless, we found that Min/+ animals exhibited a 45% decrease in proliferation compared to their fully wild-type littermates (Fig. 3A). This decrease

<table>
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<th>Table 1 Cellular morphology</th>
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<td>Specimens of grossly normal small intestine from animals at 110 days of age were formalin fixed, embedded in paraffin, and sectioned at 5 μm. Sections were stained with Feulgen for evaluation of mucosal histology and nuclear density. Five animals in each group were studied. For each animal, 10 sections of small intestinal mucosa and 10 crypt-villus units from each section were analyzed. Values are expressed as ±SE.</td>
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<th>+/+</th>
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<td>Crypt-villus length (μm × 10²)</td>
<td>3.01 ± 0.05</td>
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<td>Crypt-villus area (μm² × 10⁶)</td>
<td>77.4 ± 2.4</td>
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<td>Nuclear density (μm² × 10⁶)</td>
<td>52.6 ± 2.7</td>
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<td>Cell number per crypt-villus unit</td>
<td>78.9 ± 2.8</td>
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Fig. 1. Intact APC protein in Min/+ small intestinal mucosa. Specimens of small intestine from animals at 110 days of age were formalin fixed, embedded in paraffin, and sectioned at 5 μm. Sections were stained with antibody to the COOH terminus of APC protein. A, Min/+ (X40); B, Min/+ small intestine with adjacent tumor (+) showing loss of full-length APC protein (X10); C, Min/+ adenoma (X40).

was opposed by a concomitant decrease in enterocyte apoptosis in the Min/+ mucosa, as measured by TUNEL, an in situ measure of genomic DNA degradation induced by programmed cell death (Fig. 3B). As expected, PCNA-positive cells were located in the middle portion of the crypts, whereas cells labeling by TUNEL were predominantly found at the apex of the villi. Although the immunohistochemical methods available for detection of cell proliferation and apoptosis are not accurate enough for a quantitative comparison, the observed relative change suggests that the life span of enterocytes in Min/+ mucosa is increased with respect to that of normal animals.

These data suggest that the histologically normal mucosa of Min/+ animals retains a normal copy of Apc but exhibits an increase in β-catenin expression and a parallel decrease in proliferation and apoptosis, suggesting prolonged enterocyte life span.

Enterocyte Migration in Min/+ Animals. We hypothesized that an increased life span for enterocytes in Min/+ mucosa would result in alterations in enterocyte migration from the proliferative zone to the apex of the villus. To test this hypothesis, we injected Min/+ and +/+ animals with the thymidine analogue, BrdUrd, thus labeling all proliferating cells. Immunohistochemistry using anti-BrdUrd anti-
body was performed at 6–10-h intervals up to 72 h after injection (Fig. 4A). As expected, by 72 h, the tips of the villi were labeled in the +/+ animals, indicating that cells proliferating at t = 0 had progressed beyond the full length of the crypt-villus unit. For Min/+ animals, however, labeled cells had only traversed approximately 80% of the crypt-villus length by 72 h after BrdUrd injection. ANOVA with time as a covariate was used to analyze the differences between the groups over time. A significant group-time interaction ($P < 0.0001$) was observed. At all time points, migration of the enterocytes in the Min/+ mucosa lagged behind those in the wild-type animals by 20–25% (Fig. 4B).

One possible explanation for increased life span in Min/+ enterocytes is a “field effect” upon enterocyte proliferation and apoptosis produced by tumors in the Min/+ animals. To rule this out, we compared measurements of proliferation and apoptosis in the small intestinal mucosa of 5-week-old Min/+ and +/+ animals. At 5 weeks of age, the Min/+ mice had not yet developed histological evidence of intestinal tumors; however, as in the older, tumor-bearing animals, their small intestinal mucosa demonstrated decreased proliferation and apoptosis (Table 2). These data argue against field effect as a reason for altered enterocyte migration in histologically normal Min/+ mucosa.

### Effect of Sulindac Sulfide Administration

The NSAID sulindac induces intestinal tumor regression in humans with FAP (10–12) and prevents tumor formation in Min/+ mice (8). We, therefore, studied the effect of sulindac sulfide administration upon the crypt-villus migration rate in Min/+ animals. Min/+ mice were administered sulindac sulfide at doses of 0.05 mg/kg/day in drinking water. This dose has previously been associated with prevention of tumor formation in Min/+ animals (8). Control Min/+ mice and homozygous C57BL/6J+/+ littermates lacking the Min +/+ gene were fed AIN-76 diet without sulindac sulfide. Treatment began with mice at 5–6 weeks of age. Following 1 month of treatment, all mice were euthanized, and their intestinal tracts were examined. Samples of normal mid small intestine were harvested from each group of mice. Histological examination confirmed normal mucosal architecture in these tissues. These tissues were then analyzed by determining β-catenin expression, PCNA expression, and percentage of apoptosis.
(TUNEL method). Enterocyte migration was also determined by tracking enterocytes labeled with BrdUrd as described above.

In all cases, treatment with sulindac sulfide normalized the effect of the Apc mutation. Sulindac sulfide decreased cellular levels of β-catenin (Fig. 2), increased enterocyte apoptosis and proliferation (Fig. 3), and accelerated enterocyte mucosal transit time to that of normal littermates (Fig. 4).

**DISCUSSION**

The tumors that develop in Min/+ mice are adenomas containing multiple epithelial cell lineages, suggesting that the tumor-producing defect arises in a multipotent epithelial stem cell. In addition, these adenomas show loss of the wild-type Apc locus, consistent with the tumor suppressor gene designation of Apc (6). It is obvious that, by the time an intestinal adenoma has developed, homeostasis in the epithelium is deranged, with proliferating cells outnumbering those undergoing apoptosis and shedding into the gut lumen. Abnormalities in enterocyte growth have, however, been reported in nonadenomatous tissue from Min/+ mice and humans with FAP. In a study by Bedi et al. (13), apoptosis was decreased in the flat rectal mucosa from FAP patients, compared to rectal mucosa from normal individuals. Studies of proliferation in the rectal mucosa of FAP patients have yielded variable results (14, 15). Although alterations in the magnitude of proliferation in rectal mucosa from FAP patients were not consistently observed, detailed studies suggest that the proliferative compartment is shifted toward the lumen in these tissues (14, 16, 17).

In previous work by our laboratory, we found that apoptosis was decreased in the histologically normal mucosa of Min/+ mice (8). Maintenance of tissue architecture in histologically normal tissue requires balanced conditions of epithelial cell production and loss. For these conditions to be met in a tissue with decreased apoptosis, a compensatory decrease in proliferation must occur. Our data, therefore, led to the hypothesis that, in Min/+ animals, the basic architecture of the crypt-villus unit was maintained by a balance between proliferation and apoptosis, but the length of time a given enterocyte remained in the intestinal mucosa was increased. The migration of enterocytes in the intestinal mucosa is a dynamic equilibrium that results in continuous renewal of the epithelium. Stem cells are present in the middle of the mucosal crypt and undergo continuous division (18). After division, the daughter cells migrate in an orderly progression up to the tip of the villus, where they become apoptotic and are shed into the lumen of the intestine. In normal murine small intestine, cells produced in the proliferative zone have an average cell cycle time of 13 h and undergo vertical migration without cell division until they are lost at the apex of the villus in 48–72 h (19). The crypt-villus migration rate of an intestinal epithelial cell varies widely according to age, diet, or health of the animal, as well as location in the gastrointestinal tract (20, 21). Enterocyte migration, therefore, is a variable that can be influenced by both genetic and environmental factors.

Studies in Drosophila demonstrate that functional β-catenin and APC protein are required for embryogenesis (22), a time when properly timed cell migration is crucial. An additional suggestion that APC protein modulates cell migration came with the discovery that APC binds β-catenin, a component of the adherens junction. The adherens junction is a cadherin-associated transmembrane complex mediating Ca²⁺-dependent cell adhesion in epithelial cells. Human cancer cell lines expressing mutated β-catenin demonstrate defective cadherin-mediated adhesion (23). The intracellular distribution of β-catenin suggests that it regulates cell adhesion in association with APC protein. When β-catenin is stabilized by deletion of its NH₂ terminus, it co-localizes with APC protein to the ends of microtubule bundles at the tips of plasma membrane protrusions (24, 25). Madin-Darby canine kidney epithelial cells expressing the NH₂-deleted β-catenin exhibit inhibition of early cell-cell contact formation (26).

APC protein exists as a dimer in vivo (27) and associates with the cytoskeleton by binding microtubules (28). Truncated APC proteins are unable to bind to microtubules; however, they can associate with a wild-type APC protein that retains binding to the cytoskeleton (25). This association provides a mechanism by which a mutant APC protein could alter β-catenin-mediated cell-cell signaling in a dominant-negative fashion. It is likely that this dominant-negative effect alone is not sufficient to produce a tumor, and that additional mutations, such as ras-activating mutations, loss of p53 function, or loss of the wild-type Apc allele may be required for tumor formation. In a recent study by Shoemaker et al. (29), Min/+ mice treated with N-ethyl-N-nitrosourea developed tumors without loss of the wild-type allele, presumably as a result of additional transforming mutations not yet identified.

In humans with FAP, sulindac induces regression of rectal adenomas (10–12). Sulindac administration in FAP patients is associated with an increase in apoptosis in the rectal mucosa (30) and has variable effects upon enterocyte proliferation (15). We reported recently that sulindac and its active metabolite, sulindac sulfide, prevent tumor formation in the Min/+ mouse (8). The present study demonstrates that sulindac sulfide acts at the earliest onset of carcinogenesis in the Min/+ mucosa by decreasing enterocyte β-catenin levels and restoring a normal rate of enterocyte crypt-villus migration. Most data suggest that the antitumor efficacy of anti-inflammatory agents such as sulindac and sulindac sulfide resides, at least in part, in their ability to inhibit prostaglandin synthesis. The relationship between prostaglandin synthesis and epithelial cell migration is unclear.

In summary, a balance of proliferation and apoptosis, coordinated by cell-cell interactions, is required for maintaining the architecture of the intestinal epithelium. Breakdown of normal cell-cell interactions is associated with carcinogenesis. Demonstration of altered apoptosis, proliferation, and migration in enterocytes bearing a single mutated Apc allele suggests that truncated APC protein contributes to carcinogenesis in a dominant-negative fashion. Furthermore, sulindac sulfide, which is effective in mediating regression of tumors in animals with germ-line Apc defects, acts to normalize enterocyte crypt-villus migration that is altered by Apc mutation.

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