Identifying molecular changes that predict the risk for developing colon cancer is critical for designing effective prevention strategies. In the present study, we determined early-stage molecular alterations within the colonic epithelium of A/J and AKR/J mice that are sensitive and resistant to Azoxymethane (AOM)-initiated tumor development, respectively. Six week-old male mice were injected intraperitoneally with AOM (10 mg/kg body weight) once a week for six weeks. One week after the last injection, distal colons from both strains were analyzed for cell proliferation using a proliferating cell nuclear antigen (PCNA) assay. Unlike AKR/J, a significant increase (2.5-fold, p<0.05) in the number of PCNA-positive cells within the upper third of the crypt compartment was observed in the A/J colons. This proliferative response was associated with a sizeable increase in the levels of c-myc mRNA, quantified by RNase protection assay. cDNA sequencing, protein expression and localization of \( \beta \)-catenin, an upstream activator of c-myc, however, showed no aberrant changes within AOM-exposed A/J colons. Interestingly, TdT-mediated dUTP nick-end labeling assay revealed a significant increase (4-fold) in the number of apoptotic colonocytes in A/J mice following AOM treatment. Consistent with this finding, a modest increase in the expression of pro-apoptotic Bak was limited to the sensitive A/J colons. In summary, the current study suggests that a significant alteration in the rate of cell turnover in the normal appearing colonic mucosa, as observed in susceptible A/J mice, may be one of the earliest events predisposing the colon to neoplastic growth.

Introduction

Repetitive treatment with the methylating carcinogen, Azoxymethane (AOM), produces tumors in the distal colon of susceptible rodent strains (1). This model has been used extensively to study underlying mechanisms involved in the pathogenesis of human sporadic colorectal cancer. In addition, AOM-induced tumors exhibit pathological and genetic changes similar to those seen in sporadic forms of the human disease (1-7). As in human populations, the genetic background of laboratory animals is a significant component of organ-specific carcinogenesis. For example, A/J mice injected with AOM once a week for six weeks develop multiple in situ adenocarcinomas 24 weeks after carcinogen treatment. Interestingly, AKR/J mice, maintained under the same dosing regimen are highly resistant. Cancer, in general, evolves from an imbalance in the mechanisms that control proliferation and apoptosis, processes that may become dysregulated during the initial stages of tumorigenesis. We hypothesized that alterations within the normal appearing colonic epithelium, even before the manifestation of preneoplastic lesions such as aberrant crypt foci (ACF), may prognosticate cancer risk. Using the well-characterized AOM murine model, we demonstrate an enhanced rate of cell turnover that is limited to the colons of A/J mice which, in part, may underlie their susceptibility to chemical carcinogenesis.

Materials and methods

Treatment of animals. Five-week old A/J and AKR/J male mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed in a ventilated, temperature controlled (23±1°C) facility with a 12-h light/dark cycle. Mice were allowed access to laboratory rodent chow and water ad libitum up to the time of sacrifice. Animals were randomly assigned to vehicle control and treatment groups and were treated with 10 mg/kg of AOM (Sigma Chemical Co., St. Louis, MO) by intraperitoneal injection at weekly intervals for a total of six weeks. Animals were sacrificed one week after the last injection and distal colons were excised and processed for subsequent analyses.
Analysis of colon cell proliferation. Five-μm formalin-fixed, paraffin-embedded colon sections were incubated overnight at 4°C with anti-PCNA mouse monoclonal antibody (Signet laboratories Inc., Dedham, MA) at a dilution of 1:800 in 5% goat-serum. The sections were washed with PBS-Tween and incubated at a 1:400 dilution with biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA) at room temperature for 30 min. After washing, sections were incubated with avidin-biotin peroxidase complex at room temperature for 30 min using the Vectastain Elite ABC kit (Vector Laboratories). Color was developed using 3,3-diaminobenzidine as the substrate and sections were counterstained with hematoxylin. To demonstrate the specificity of the immunostaining, primary antibody was replaced with an equivalent concentration of normal mouse IgG. PCNA-positive cells were counted in a minimum of ten different fields per section and from three different cellular compartments (base, middle and upper third) of the colonic crypts, and resultant data was statistically analyzed for differences in proliferation rates between A/J and AKR/J mice.

TUNEL assay. Five-μm frozen sections from distal colons were fixed in 1% paraformaldehyde in PBS for 10 min, then post-fixed in ethanol:acetic acid (2:1) for 5 min prior to quenching of the endogenous peroxidase activity with 3% H₂O₂ in PBS for 5 min. The protocol for the ApopTag
mean mRNA levels for the respective treatment groups. The mean ± SE of each treatment group. *Significant difference (p<0.05) in the followed by PDIFF analysis for comparing the means. Each column represents
Statistical analysis was performed using the general linear model procedure

peroxidase In situ apoptosis detection kit (Intergen Co., Purchase, NY) was followed per the manufacturer's instructions. Briefly, digoxigenin-labeled deoxyribonucleotides (dUTPs) were incorporated into the free 3'-OH terminus of fragmented DNA within apoptotic cells by the TdT enzyme during a 1-h incubation at 37°C. The integrated dUTPs were then conjugated to anti-digoxigenin peroxidase and the color was developed with the peroxidase substrate, 3,3'-diaminobenzidine (DAB). Tissue sections similarly processed in the absence of the terminal deoxynucleotidyl transferase (TdT) enzyme were used as a negative control. Sections that were treated with nuclease enzyme prior to quenching to generate double-stranded DNA breaks served as positive controls. A counterstain of the surrounding tissue using methyl-green allowed visualization of the brown apoptotic controls. A counterstain of the surrounding tissue using Harris acid hematoxylin. was developed using 3,3',-diaminobenzidine substrate and washings, the sections were incubated with avidin-biotin peroxidase complex at room temperature for 30 min using Immunoglobulin G at 1:100 dilution (Vector Laboratories, Burlingame, CA) at room temperature for 1 h with mouse anti-β-catenin monoclonal antibody (Sigma-Aldrich, St. Louis, MO). The blot was then incubated with horseradish peroxidase-conjugated secondary antibody and visualized using the ECL system (Santa Cruz Biotechnology, Santa Cruz, CA).

For immunohistochemical analysis, 5-μm formalin-fixed, paraffin-embedded colon tissue sections from A/J and AKR/J mice were incubated at room temperature for 1 h with mouse monoclonal anti-β-catenin antibody (Sigma-Aldrich) at a dilution of 1:50 in 10% normal goat-serum. The sections were then washed and incubated with biotinylated goat anti-mouse immunoglobulin G at 1:100 dilution (Vector Laboratories, Burlingame, CA) at room temperature for 30 min. After washing, the sections were incubated with avidin-biotin peroxidase complex at room temperature for 30 min using the Vectastain Elite ABC kit (Vector Laboratories). Color was developed using 3,3'-diaminobenzidine substrate and sections were counterstained with Harris acid hematoxylin. As a negative control, duplicate sections were immunostained with mouse IgG in place of the primary β-catenin antibody.

Statistical analysis. The General Linear model procedure using SAS software was employed to determine the overall treatment (AOM) effect across the mouse strains. Significant differences in the expression levels of the genes within each strain were determined by the probability of difference (PDIFF) between the means. A p<0.05 was considered statistically significant.

Results
Enhanced colonic epithelial cell proliferation in AOM-sensitive A/J mice. Hyperproliferation of epithelial cells is a critical event underlying tumorigenesis in many organ systems

Western blotting and immunostaining of β-catenin. Thirty μg of protein from vehicle control and AOM-treated colon tissues was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was probed with either mouse anti-β-catenin monoclonal antibody at a dilution of 1:1,000, or rabbit anti-β-actin monoclonal antibody at a dilution of 1:2,000 (Sigma-Aldrich, St. Louis, MO). The blot was then incubated with horseradish peroxidase-conjugated secondary antibody and visualized using the ECL system (Santa Cruz Biotechnology, Santa Cruz, CA).

Figure 2. Quantitative analysis of c-myc expression. (I) Radiograph from an RPA showing c-myc mRNA levels in the distal colons of A/J and AKR/J. C, distal colon tissues from saline controls; and A, tissues from AOM-treated mice. As indicated, L-32 and GAPDH were used as loading controls for normalization. (II) Quantitative analysis of mRNA levels using NIH imaging

RNase protection assay. mRNA levels for a panel of proliferation and apoptosis-related genes were evaluated using the RiboQuant Multiprobe RNase protection assay system from Pharmingen (San Diego, CA). Customized template sets from Pharmingen containing c-myc, Bak and bcl-XL were used to probe the RNA samples. L-32 and GAPDH were used as internal controls. The probes were synthesized according to the manufacturer's instructions (Pharmingen). Five-μg RNA samples were incubated with the labeled probes overnight at 56°C. The samples were then digested with RNase and resolved on 7% denaturing gels. Gels were dried and exposed to X-ray film at -70°C. Image densitometry was performed using NIH image software.

Semi-quantitative RT-PCR and sequencing analysis of β-catenin cDNA. Two μg of total RNA extracted using TRIzol reagent (Gibco/BRL, Gaithersburg, MD) was reverse transcribed and PCR amplified using the forward 5'-GGCGTGA CAATGGCTACTCAAG-3', and reverse 5'-TATTAATAC CACCTGGTCTTC-3' primers, which includes the GSK-3β phosphorylation sites of β-catenin. The PCR products from control and treated samples were directly sequenced and compared with the reference sequence from GenBank (accession no. M90364) using Sequencher software (Gene Codes Corp., Ann Arbor, MI).
and typically occurs as an early and necessary step in the development of cancer (8). In the present study, epithelial cell proliferation was measured in sections of distal colon of A/J and AKR/J mice one week after the last dose of AOM. No significant changes were observed in the levels of β-catenin protein in both strains after AOM treatment. (II) Immunohistochemical analysis showing β-catenin localization in the distal colon epithelial cells. Panels a and b, distal colon sections from control and AOM-treated A/J mice respectively (x400). Panels c and d, sections from control and AOM-treated AKR/J mice respectively (x400). A bilateral membrane localization pattern (brown staining) was observed for β-catenin in all the sections. Furthermore, staining was restricted to the colonocytes in the upper compartment of the crypts and in the superficial layer. Hematoxylin was used as a counter stain for visualizing the nuclei. As shown in the figure, no nuclear accumulation of β-catenin was observed in any of the sections.

Increased c-myc expression in A/J colons. The c-myc proto-oncogene plays a pivotal role in tumor initiation/promotion, mainly by promoting cell proliferation and blocking terminal differentiation (9). To determine whether enhanced epithelial cell proliferation in A/J colons is associated with c-myc gene regulation, mRNA levels were quantified using an RNase protection assay (RPA). While AKR/J colons revealed no AOM-induced changes, a statistically significant increase in c-myc expression was observed one week after AOM treatment in A/J colons (Fig. 2). These data suggest that the enhanced expression of c-myc, limited to the A/J colon, may play a role in the expansion of the proliferative crypt compartment in direct response to carcinogen treatment.

Absence of AOM-induced changes in β-catenin. β-catenin is a key component of the Wnt signaling pathway and directly controls the transcriptional activation of a panel of genes involved in cell growth and proliferation (10). Cellular
alterations that may trigger activation of the Wnt pathway have been shown to cause transcriptional activation of c-myc. In particular, mutations within the GSK-3β phosphorylation motif of the β-catenin gene have been shown to occur within a subset of human colorectal cancers (10). These mutations render the protein resistant to proteosomal degradation, resulting in the accumulation and nuclear translocation of β-catenin (10). This in turn induces transcriptional activation of c-myc and other downstream targets (10). Previous studies have also reported mutations within this motif in colon tumors induced by AOM in ICR (11) and C57BL/6J (12) mice. To determine whether alterations in β-catenin may account, in part, for the increased expression of c-myc observed in A/J colons, we analyzed cDNA for AOM-induced mutations within the GSK-3β phosphorylation motif. Direct PCR-sequencing of ten AOM-exposed distal colon tissues, however, did not reveal sequence alterations within this key regulatory domain. In addition, Western blot and immunohistochemical analyses of β-catenin revealed neither an increase in expression nor the appearance of nuclear localization of this protein within AOM-exposed A/J colons (Fig. 3). These results suggest that c-myc may be transcriptionally induced in A/J colons via β-catenin-independent mechanisms.

Induction of apoptosis in AOM-exposed A/J colons. Resistance to apoptosis is an additional mechanism whereby cells attain a selective growth advantage that may enhance tumorigenesis (13). In the present study, we performed a TUNEL assay on distal colon sections from A/J and AKR/J mice to determine the apoptotic response to AOM. As shown in Fig. 4, no change in the number of apoptotic nuclei within the colonic epithelium was observed in AOM-exposed AKR/J colons. In striking contrast, however, a significant increase (4-fold, p<0.05) in the number of apoptotic cells was found within the colonic epithelium of AOM-treated A/J mice (Fig. 4). To gain further insight into potential mechanisms that may account for this strain-specific enhancement in the rate of apoptosis, the expression levels of a panel of genes, including pro-apoptotic Bak and caspase 3, and anti-apoptotic bel-XL, were determined by quantitative RPA. Although AKR/J colons revealed no significant changes in the expression of these genes in response to AOM treatment, carcinogen exposure resulted in a modest increase in the levels of Bak within the A/J colons (Fig. 5). These results suggest that the pro-apoptotic machinery may be activated in A/J colons to counteract the hyperproliferation seen during early stages of tumor development.

Discussion

The main goal of this study was to determine strain-specific, AOM-initiated alterations that are associated with tumor risk. AOM, an indirect-acting genotoxic carcinogen, forms DNA methyl adducts within target tissues (14). As mentioned earlier, repeated exposures to AOM produces distal colon tumors only in sensitive mouse strains (1). However, the underlying mechanism for this differential sensitivity to AOM has not been fully defined. One possible explanation for this differential response may be a strain-specific activation of the carcinogen. However, previous studies from our laboratory have shown that the levels of DNA methyl adducts formed following an acute dose of the carcinogen are, in fact, similar between sensitive and resistant mice, arguing against metabolism as a critical component of strain sensitivity (14). This earlier study, however, tested the effects of a single, acute dose of carcinogen. Thus, it is possible that repetitive exposures to AOM may yield differential levels of DNA adducts when compared between the two strains. In the present study, however, we found no detectable levels of the adduct (data not shown) in either strain at one week after the last dose of AOM that the levels of DNA methyl adducts within target tissues (14). As mentioned earlier, repeated exposures to AOM produces distal colon tumors only in sensitive mouse strains (1). However, the underlying mechanism for this differential sensitivity to AOM has not been fully defined. One possible explanation for this differential response may be a strain-specific activation of the carcinogen. However, previous studies from our laboratory have shown that the levels of DNA methyl adducts formed following an acute dose of the carcinogen are, in fact, similar between sensitive and resistant mice, arguing against metabolism as a critical component of strain sensitivity (14). This earlier study, however, tested the effects of a single, acute dose of carcinogen. Thus, it is possible that repetitive exposures to AOM may yield differential levels of DNA adducts when compared between the two strains. In the present study, however, we found no detectable levels of the adduct (data not shown) in either strain at one week after the last dose of AOM suggesting a similar metabolic efficiency between the strains.

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of signaling pathway alterations in response to genotoxic carcinogens.

We therefore focused on identifying potential mechanisms that may promote hyperproliferation during early stages of tumorigenesis in the sensitive mice. c-myc is an important protooncogene and plays a key role in promoting tumorigenesis by driving cell cycle progression (9). Furthermore, colonic epithelial hyperproliferation has been shown previously to be associated with enhanced c-myc expression in a transmissible murine colonic hyperplasia model (15). In addition, significantly elevated levels of c-myc mRNA within normal-appearing colonic mucosa were observed in rats after a single exposure to AOM (16). Our current findings of enhanced c-myc expression in A/J mice (Fig. 2), taken together with earlier reports (15,16), suggest that c-myc induction at an early stage may play a key role in promoting the formation of colon tumors in response to carcinogen. In addition, the absence of AOM-induced mutations or nuclear accumulation of the β-catenin protein suggests that c-myc may be transcriptionally induced within the colonic epithelium via a β-catenin-independent mechanism during early stages of AOM-induced tumorigenesis.

Finally, we determined whether there exists a difference in apoptotic rates within the normal-appearing colonic cells before and after AOM treatment. Using TUNEL assay, we found a marked increase in the number of apoptotic cells within the A/J colons, while no significant changes were observed in AKR/J following carcinogen exposure (Fig. 4). Furthermore, this enhanced apoptosis in A/J mice was associated with a modest increase in the expression of the proapoptotic Bak (Fig. 5), a principal endogenous promoter of apoptosis within the intestinal epithelium (17). Our results indicate that intrinsic apoptotic mechanisms may be activated to counteract the hyperproliferation induced by AOM in sensitive mice.

In conclusion, this study shows that altered cell turnover is one of the earliest detectable changes within the colons of susceptible strains which, in turn, may be associated with tumor risk. More importantly, the fact that these perturbations were observed, albeit in the absence of detectable levels of DNA adducts, suggests that a tumor initiating event may have occurred early on in response to repeated carcinogen exposures within sensitive colons. Such an alteration in crypt dynamics to DNA alkylating agents may predispose the colon for acquiring additional genetic aberrations that promote tumor progression.

Acknowledgements

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References