Strain-specific homeostatic responses during early stages of Azoxymethane-induced colon tumorigenesis in mice

KISHORE GUDA, JILLIAN N. MARINO, YONGHWON JUNG, KEN CRARY, MEI DONG and DANIEL W. ROSENBERG

Center for Molecular Medicine, University of Connecticut Health Center, Farmington, CT 06030, USA

Received January 29, 2007; Accepted March 9, 2007

Abstract. 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 *ß-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.

Correspondence to: Dr Daniel W. Rosenberg, Center for Molecular Medicine, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030-3101, USA
E-mail: rosenberg@nso2.uchc.edu

Abbreviations: AOM, Azoxymethane; RPA, RNase protection assay; TUNEL, TdT-mediated dUTP nick-end labeling; PCNA, proliferating cell nuclear antigen; IHC, immunohistochemistry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; PCR, polymerase chain reaction; SAS, statistical analysis system

Key words: Azoxymethane, mouse colon, tumorigenesis, proliferation, apoptosis, PCNA

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.

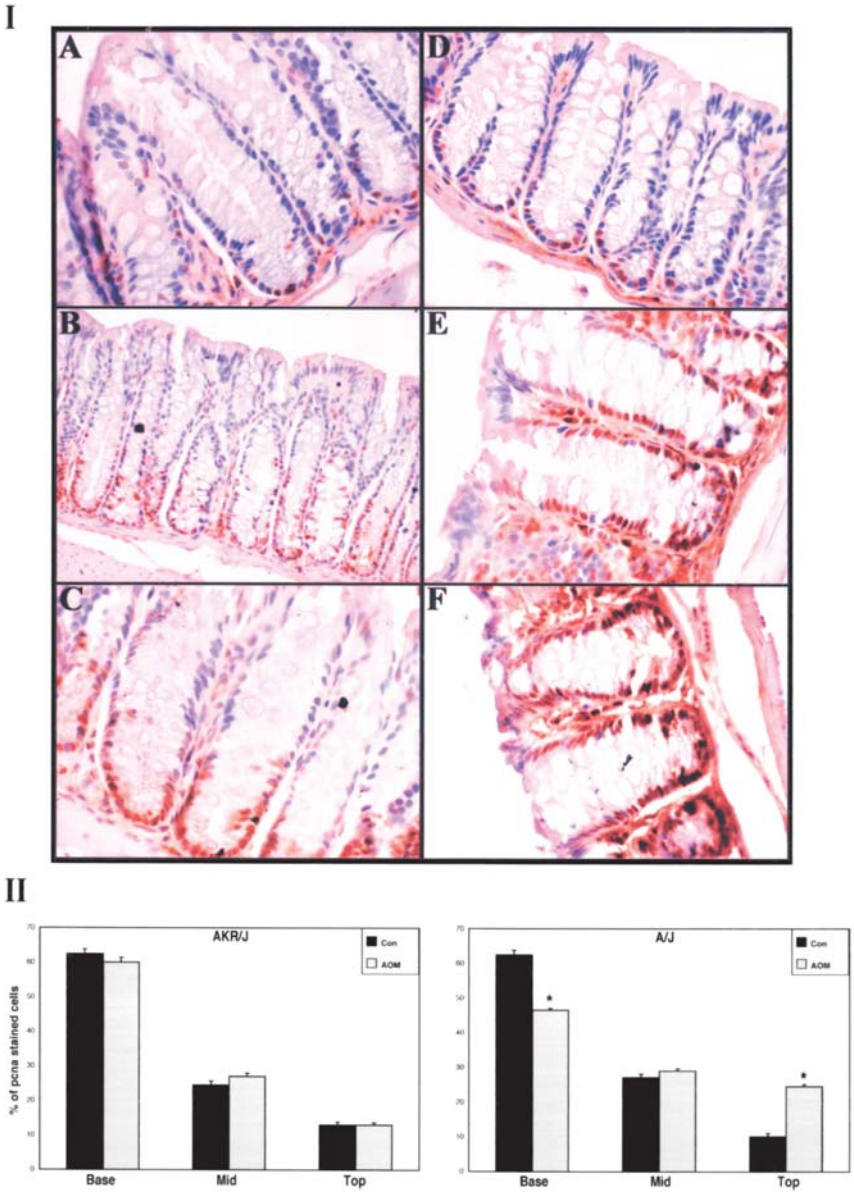

Figure 1. Proliferating cell nuclear antigen staining in mouse colons. (I) Representative distal colon sections from A/J and AKR/J mice stained for PCNA. Panel A represents colon section from untreated control AKR/J (x400), B and C from AOM-treated AKR/J at magnification x200 and x400. Panel D represents section from an A/J control (x400) whereas E and F represent sections from treated mice (x400). Nuclei with brown staining were considered positive for PCNA. Note the expansion of proliferative crypt compartment in AOM-treated A/J colon. (II) Enumeration of PCNA-positive cells in basal, middle and top compartments of colonic crypts. The bar graphs show the percentage of PCNA-stained cells in different compartments of the colon crypts in control and AOM-treated AKR/J and A/J. Note the significant increase (2.5-fold, $p<0.05$) in number of PCNA-stained epithelial cells in the top compartments of the crypts in A/J following AOM treatment. Statistical analysis was performed using the general linear model procedure followed by PDIFF analysis for comparing the means. Each column represents mean ± SE of their respective groups. *Significant difference ($p<0.05$) between the means of control and AOM-treated groups for the respective crypt compartment.

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% H_2O_2 in PBS for 5 min. The protocol for the ApopTag

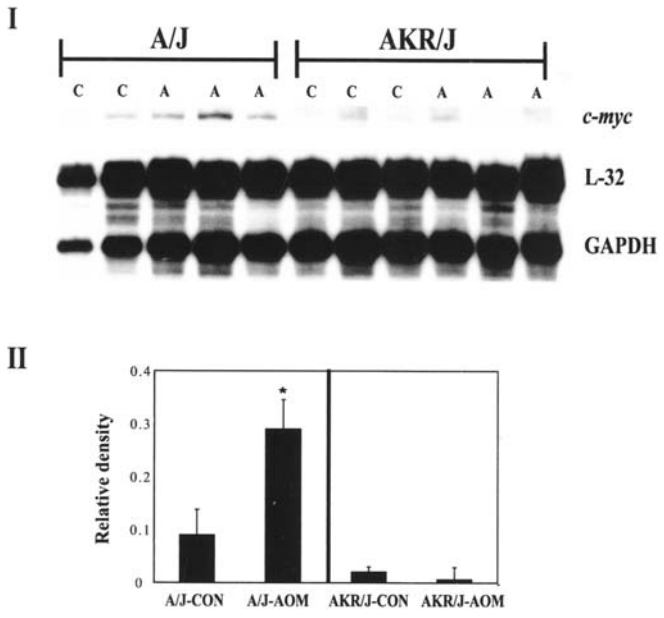

Figure 2. Quantitative analysis of *c-myc* expression. (I) Radiograph from an RPA showing *c-myc* mRNA levels in the distal colons of A/J and AKR/J. C, distal colon tissues from saline controls; and A, tissues from AOM-treated mice. As indicated, L-32 and GAPDH were used as loading controls for normalization. (II) Quantitative analysis of mRNA levels using NIH imaging software. Note the increased *c-myc* expression in AOM-exposed A/J colons. Statistical analysis was performed using the general linear model procedure followed by PDIFF analysis for comparing the means. Each column represents the mean ± SE of each treatment group. *Significant difference ($p<0.05$) in the mean mRNA levels for the respective treatment groups.

peroxidase *In situ* apoptosis detection kit (Intergen Co., Purchase, NY) was followed per the manufacturer's instructions. Briefly, digoxigenin-labeled deoxynucleotides (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 cells under light microscopy. Individual stained cells that appear shrunken without loss of membrane integrity were considered to have undergone apoptosis. For each section, ten fields of normal-appearing tissue were randomly selected and photographed at magnification x400. Apoptotic cells were then counted and expressed as a percentage of the total number of colonocytes in each field for each animal. Data were analyzed for differences in apoptosis between A/J and AKR/J mice.

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 ß-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'-GCGTGGA CAATGGCTACTCAAG-3', and reverse 5'-TATTAACTAC CACCTGGTCCTC-3' primers, which includes the GSK-3ß phosphorylation sites of *ß-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).

Western blotting and immunostaining of ß-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-*ß-catenin* monoclonal antibody at a dilution of 1:1,000, or rabbit anti-ß-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).

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-*ß-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 ß-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

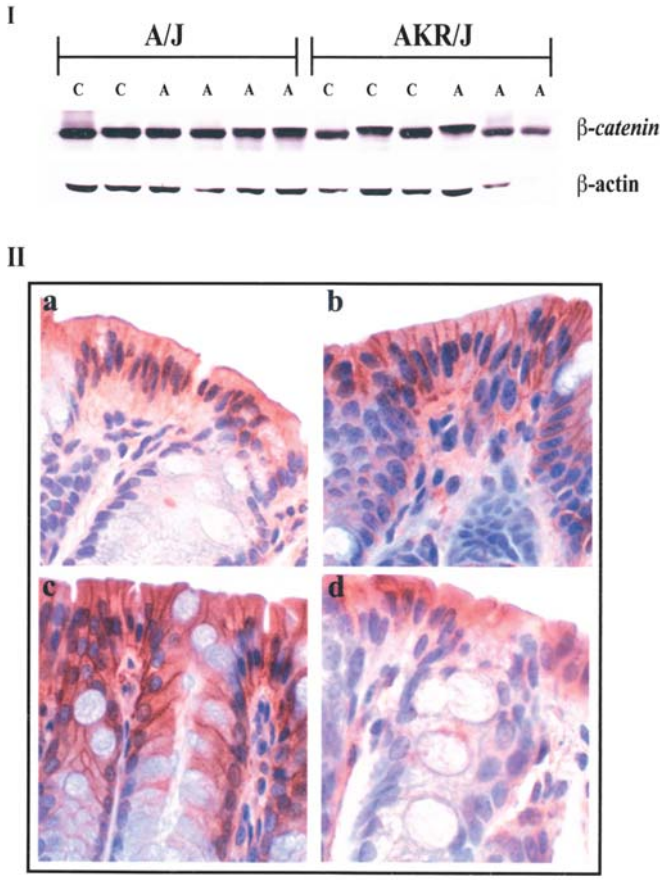

Figure 3. Expression and immunolocalization of *ß-catenin* in control and AOM-treated mouse colonic epithelium. (I) Analysis of *ß-catenin* protein levels by Western blotting. ß-actin was used as a loading control. C, distal colon tissues from saline controls; and A, tissues from AOM-treated mice. No significant changes were observed in the levels of *ß-catenin* protein in both strains after AOM treatment. (II) Immunohistochemical analysis showing *ß-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 *ß-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 *ß-catenin* was observed in any of the sections.

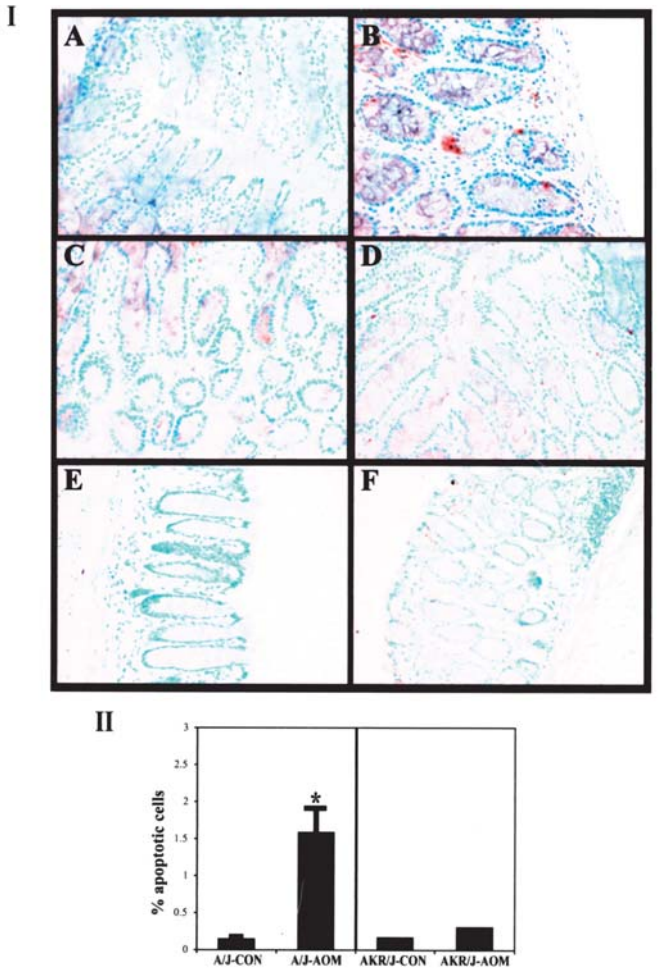

Figure 4. Enumeration of apoptotic cells using TUNEL assay. (I) Representative distal colon sections from A/J and AKR/J mice, TUNEL-stained for apoptotic colonocytes. Panels A and B, colon sections from saline control and AOM-treated A/J mice respectively (x200). Panels C and D, colon sections from control and AOM-treated AKR/J mice respectively (x200). Panels E and F, sections of the experimental negative and positive control slides, respectively. Methyl-green was used as a counter stain. TUNEL-positive cells (brown nuclear staining) were mostly restricted to the colon crypts. (II) Enumeration of the TUNEL-stained apoptotic cells. Apoptotic-cell counts from the distal colons of each animal were obtained as explained in Materials and methods. AOM-exposed A/J colons showed a significant increase (4-fold, $p<0.05$) in the number of TUNEL-positive colonocytes when compared to the saline controls. Statistical analysis was performed using the general linear model procedure followed by PDIFF analysis for comparing the means. Each column represents the mean ± SE of each treatment group. *Significant difference ($p<0.05$) between the means for the respective treatment groups.

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. As shown in Fig. 1, immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) revealed a significant increase (2.5-fold, $p<0.05$) in PCNA-positive cells within the upper third of the crypt compartment, an effect that was limited to AOM-exposed A/J colons. This finding suggests that a hyperproliferative response to carcinogen within the normal-appearing mucosa may be an important early feature of tumor initiation.

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 ß-catenin. *ß-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

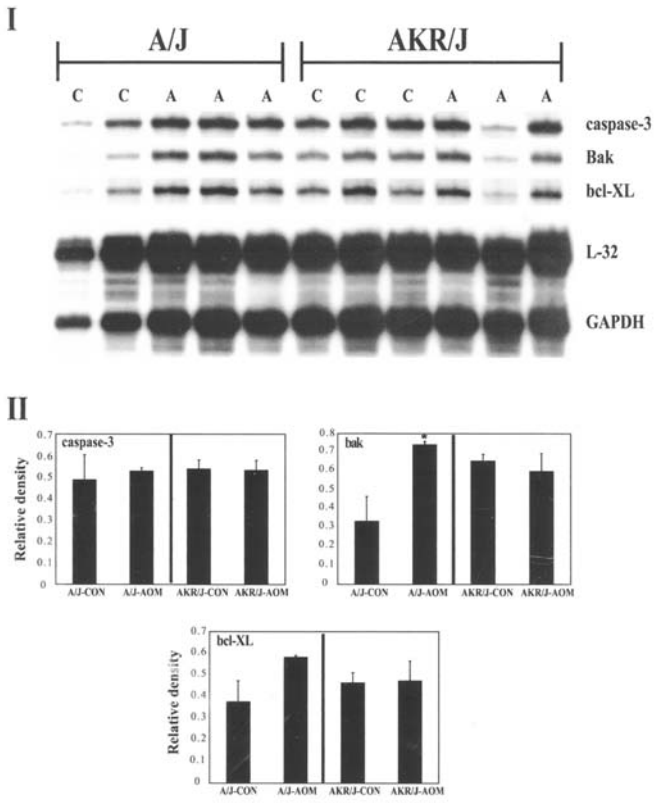

Figure 5. Expression analyses of caspase 3, bak and bcl-XL. (I) Radiograph from an RPA showing mRNA levels of caspase 3, Bak and bcl-XL in the distal colons of A/J and AKR/J. C, distal colon tissues from saline controls; and A, tissues from AOM-treated mice. L-32 and GAPDH were used as loading controls for normalization. (II) Quantitative analysis of mRNA levels using NIH imaging software. Note the significant increase (2.3-fold, $p<0.05$) in Bak expression in AOM-exposed A/J colons. Statistical analysis was performed using the general linear model procedure followed by PDIFF analysis for comparing the means. Each column represents the mean ± SE of each treatment group. *Significant difference ($p<0.05$) in the mean mRNA levels for the respective treatment groups.

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-3ß phosphorylation motif of the *ß-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 *ß-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 *ß-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-3ß 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 *ß-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 *ß-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 bcl-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 suggesting a similar metabolic efficiency between the strains.

Epithelial cell hyperproliferation is a characteristic change that commonly accompanies tumor progression (13). In fact, increased cell proliferation along the entire length of the colon and, in particular, within the upper compartments of the colonic crypts has been observed within the normal mucosa of patients with a history of adenomatous polyps and/or large adenomas (13). Using PCNA analysis, we found a significant increase in the number of positively stained nuclei within the upper third of colonic crypts in sensitive A/J mice one week after the carcinogen regimen (Fig. 1). This generalized expansion of the proliferative compartment within susceptible pre-neoplasic colons may be a consequence

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 *ß-catenin* protein suggests that *c-myc* may be transcriptionally induced within the colonic epithelium via a *ß-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 colonocytes 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

This study was supported in part by NIH grant CA-81428.

References

1. Nambiar PR, Girnun G, Lillo NA, Guda K, Whiteley HE and Rosenberg DW: Preliminary analysis of azoxymethane induced colon tumors in inbred mice commonly used as transgenic/knockout progenitors. Int J Oncol 22: 145-150, 2003.
2. Dong M, Guda K, Nambiar PR, *et al*: Inverse association between phospholipase A2 and COX-2 expression during mouse colon tumorigenesis. Carcinogenesis 24: 307-315, 2003.
3. Guda K, Giardina C, Nambiar P, Cui H and Rosenberg DW: Aberrant transforming growth factor-beta signaling in azoxymethane-induced mouse colon tumors. Mol Carcinog 31: 204-213, 2001.
4. Papanikolaou A, Wang QS, Mulherkar R, Bolt A and Rosenberg DW: Expression analysis of the group IIA secretory phospholipase A(2) in mice with differential susceptibility to azoxymethane-induced colon tumorigenesis. Carcinogenesis 21: 133-138, 2000.
5. Wang QS, Papanikolaou A, Nambiar PR and Rosenberg DW: Differential expression of p16(INK4a) in azoxymethane-induced mouse colon tumorigenesis. Mol Carcinog 28: 139-147, 2000.
6. Wang QS, Papanikolaou A, Sabourin CL and Rosenberg DW: Altered expression of cyclin D1 and cyclin-dependent kinase 4 in azoxymethane-induced mouse colon tumorigenesis. Carcinogenesis 19: 2001-2006, 1998.
7. Wang QS, Walsh A, Goldsby JS, Papanikolaou A, Bolt AB and Rosenberg DW: Preliminary analysis of azoxymethane-induced colon tumorigenesis in mouse aggregation chimeras. Carcinogenesis 20: 691-697, 1999.
8. Mori H, Sugie S, Yoshimi N, Hara A and Tanaka T: Control of cell proliferation in cancer prevention. Mutat Res 428: 291-298, 1999.
9. Pelengaris S, Khan M and Evan G: c-Myc: more than just a matter of life and death. Nat Rev Cancer 2: 764-776, 2002.
10. Wong NA and Pignatelli M: Beta-catenin - a linchpin in colorectal carcinogenesis? Am J Pathol 160: 389-401, 2002.
11. Takahashi M, Nakatsugi S, Sugimura T and Wakabayashi K: Frequent mutations of the beta-catenin gene in mouse colon tumors induced by azoxymethane. Carcinogenesis 21: 1117-1120, 2000.
12. Suzui M, Okuno M, Tanaka T, Nakagama H and Moriwaki H: Enhanced colon carcinogenesis induced by azoxymethane in min mice occurs via a mechanism independent of beta-catenin mutation. Cancer Lett 183: 31-41, 2002.
13. Anti M, Armuzzi A, Morini S, *et al*: Severe imbalance of cell proliferation and apoptosis in the left colon and in the rectosigmoid tract in subjects with a history of large adenomas. Gut 48: 238-246, 2001.
14. Papanikolaou A, Shank RC, Delker DA, Povey A, Cooper DP and Rosenberg DW: Initial levels of azoxymethane-induced DNA methyl adducts are not predictive of tumor susceptibility in inbred mice. Toxicol Appl Pharmacol 150: 196-203, 1998.
15. Sellin JH, Umar S, Xiao J and Morris AP: Increased beta-catenin expression and nuclear translocation accompany cellular hyperproliferation *in vivo*. Cancer Res 61: 2899-2906, 2001.
16. Kishimoto Y, Morisawa T, Hosoda A, Shiota G, Kawasaki H and Hasegawa J: Molecular changes in the early stage of colon carcinogenesis in rats treated with azoxymethane. J Exp Clin Cancer Res 21: 203-211, 2002.
17. Moss SF, Agarwal B, Arber N, *et al*: Increased intestinal Bak expression results in apoptosis. Biochem Biophys Res Commun 223: 199-203, 1996.