Enhanced Induction of Mucin-Depleted Foci in Estrogen Receptor β Knockout Mice

Diana Saleiro¹², Genoveva Murillo¹, Dennis B. Lubahn³, Levy Kopelovich⁴, Kenneth S. Korach⁵, and Rajendra G. Mehta¹

Abstract

The role of the estrogen receptor β (ERβ) in the colon has received considerable interest, yet in vivo models are needed to better define its protective actions. In the present study, wild-type (WT), ERα, and ERβ knockout (αERKO and βERKO) mice were injected with azoxymethane, a colon chemical carcinogen. Fourteen weeks after azoxymethane exposure, the incidence of aberrant crypt foci (ACF) was assessed by methylene blue staining. βERKO mice showed significantly higher incidence (P < 0.001) of ACF (15.0 ± 2.5) compared with αERKO (3.4 ± 1.0) and WT (4.6 ± 1.0) mice. The colons in several βERKO mice had increased thickness and loss of normal morphology. It has been reported that ERβ plays a role in the maintenance of the colonic crypt architecture; this may explain the loss of crypt organization in the colonic epithelium of βERKO mice. The presence of mucin-depleted foci (MDF) has been shown, both in humans and in rodents, as an early event in colon cancer. Therefore, to surpass the limitations with ACF scoring, we performed Alcian blue-neutral red staining to assess the presence of MDF. This assay allowed the assessment of precancerous lesions on all the βERKO mice colons (38.3 ± 4.0; P < 0.001), comparing to WT and αERKO mice (6.6 ± 1.5 and 10.0 ± 1.9, respectively), and served to confirm the ACF results. Together, these data support the use of MDF staining as a biomarker for precancerous lesions and the protective role of ERβ in colon carcinogenesis. Cancer Prev Res; 3(9): 1198–204. ©2010 AACR.

Introduction

The incidence and mortality rates of colorectal cancer are similar among the genders (1); however, women who have a history of hormone replacement therapy show a reduction of colon cancer risk (2). Similarly, both parity and oral contraceptive use seem to exert preventive effects against colon carcinogenesis in females (3–5). These protective effects have been attributed, in part, to the high levels of endogenous (pregnancy) and/or exogenous (oral contraceptive and/or hormone replacement therapy) estrogens (6, 7). Yet, the mechanism(s) through which estrogens are protective against colon cancer is still unclear.

Estrogens bind to two distinct nuclear estrogen receptors (ER), ERα and ERβ (8). The expression of the two ER subtypes is tissue specific, with ERα levels in the colon being lower than ERβ. No differences have been reported for ERα expression between normal and malignant human colon. Although ERα has a more regulatory role in mammary carcinogenesis, ERβ is the predominant functional form in the human colon (9, 10). Interestingly, a selective loss of this protein has been observed during the progression of several types of cancer, including colon (11, 12). These results support the notion that estrogen-mediated protection of colon cancer may be regulated by ERβ. Although the mechanism by which ERβ mediates its effects is not well known, the silencing of the ERβ by methylation has been considered as one of the early events in colorectal and breast carcinogenesis (13–15). Although these observations are consistent with the hypothesis that ERβ may be a potential tumor suppressor (16), further in vivo studies are needed to better clarify the biological function(s) of the two ER subtypes during colon tumorigenesis. The ability to produce animals with disrupted ER genes has been proven to be a useful tool to discern the physiologic role of each ER subtype in various tissues (17). Therefore, to better understand the functional significance of ERs in colon carcinogenesis, we used wild-type (WT), αERKO (18), and βERKO (19) mice to investigate whether the absence of ERα or ERβ affects the incidence of azoxymethane (AOM)-induced aberrant crypt foci (ACF). The ACF were first described by Bird (20) as one of the early events occurring in colon cancer progression, and since then, they have been characterized as potential colonic precancerous lesions in rodent models (21, 22). More
recently, mucin-depleted foci (MDF), dysplastic crypts characterized by the absence or low production of mucin, were identified as colonic premalignant lesions present in rodent models after AOM or 1,2-dimethylhydrazine dihydrochloride treatments (23, 24). These reports showed that MDF had more dysplastic changes and overexpression of β-catenin than ACF. Moreover, MDFs have also been found in human colons of patients with familial adenomatous polyposis and with sporadic colorectal cancer (25). These findings support the significance of MDF as a possible biomarker in the identification of colon cancer (26, 27). In the present study, we evaluated the incidence of both ACF and MDF in the colons of AOM-treated genetically engineered mice to discern the role of ERs in colon carcinogenesis progression.

Materials and Methods

Animals and carcinogen treatment
The breeding pairs of αERKO and βERKO mice were obtained from Dr. Dennis Lubahn (University of Missouri, Columbia, MO) and are maintained at IIT Research Institute. The knockout status was confirmed by genotyping. The background-WT C57BL/6 mice were purchased from Charles Rivers laboratories and used as WT controls. Animals were quarantined for 7 days and housed four mice per cage, with a 12-hour light-dark cycle and a relative humidity of 50%. Drinking water and diet were supplied ad libitum. This study was approved by the IIT Research Institute Animal Use and Care Committee.

For this study, 24 16-week-old mice were divided into three groups according to their ER genotype. The groups consisted of eight WT, eight αERKO, and eight βERKO mice, divided as four females and four males per each group. All animals were injected s.c. with the colon carcinogen AOM (10 mg/kg body weight) weekly for 4 weeks (Sigma Co.). AOM was dissolved in normal saline and kept on ice throughout the procedure. Animals were weighed weekly from the beginning to the end of the study (15 wk).

Identification of ACF

At week 15 following the initiation of the study, the animals were sacrificed by CO2 asphyxiation. The colons were evaluated for ACF by the procedure previously established in our laboratory using methylene blue staining (28). The number and size of ACF per colon were determined under the microscope Olympus DP70 at a magnification of ×10 and ×20. The ACF were distinguished from surrounding nonaberrant crypts by their increased size, elongated luminal opening, increased distance from luminal to basal surface of cells, thickened epithelial cell lining, and enlarged pericryptal area, relative to surrounding normal crypts.

Identification of MDF

After ACF determination, colons were stained with Alcian blue-neutral red (AB-NR) for MDF evaluation (29).

The colons were stained with AB solution for 10 minutes and then incubated in NR (0.5% aqueous solution) for 10 seconds. The AB-NR-stained colons were scored under the Olympus DP70 microscope (×10 and ×20 magnification). The colonic epithelial normal mucosa appeared as a reddish background (NR staining) dotted with blue spots, representing the opening of normal crypts full of mucus stained with AB. The MDF were distinguished from this blue-dotted background as a reddish spot in which the crypts do not produce mucin. Besides this characteristic, MDF can be identified, according to a criteria described by Caderni et al. (23, 27), as elevated focal lesions with a multiplicity (i.e., the number of crypts per focus) of more than three crypts often distorted and smaller than the normal surrounding crypts. The size of each MDF was determined with the MicroSuite Biological Suite 5.0 (Build 1044)-Olympus program. The area of each MDF was determined and then divided by the area of one crypt to determine the multiplicity of the MDF.

Quantitative immunohistochemistry

Tissues were sectioned (4 μm thick) and processed for immunohistochemistry as previously described (30). The antibody anti-Ki-67 rabbit monoclonal (1:50; Thermo Fisher Scientific) was used to assess the proliferation status on βERKO, αERKO, and WT mice colon sections. For all sections, controls were obtained by using the same procedure, except that they were not exposed to primary antibody.

Chromogen abundance was quantified by quantitative immunohistochemistry as previously described by Matkowskyj et al. (31, 32). Fifteen random images of the fully stained sections were saved in TIFF format, and the amount of chromogen per pixel was determined by subtracting the value for chromogen abundance of the control slide from that in the homologous region of the experimental slide. For all images, the relevant stained region was isolated from the rest of the image using Photoshop (Adobe Systems) and used for chromogen quantification using the Tifalyzer program (31, 32).

Quantitative histochemical detection of apoptosis

Colonic sections (4 μm thick) were evaluated for the presence of apoptotic cells by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay using the FragEL DNA fragmentation detection kit (Calbiochem, EMD) according to the manufacturer’s instructions. The quantification of chromogen corresponding to the apoptotic cells was determined as described for immunohistochemistry quantification.

Statistical analysis

One-way ANOVA was used for statistical comparisons among different genotypes. Bonferroni Multiple Comparison test was used to determine statistical differences between groups using the GraphPad InStat software. Differences were considered statistically significant when P value was <0.05.
Results

AOM injections increased ACF and MDF incidence in colons of βERKO mice

Intact C57BL/6, βERKO, and αERKO mice were injected with AOM at 16 weeks of age and monitored for occurrence of ACF after a 15-week study. No weight loss was observed in the three genotypes. When assessing the mean number (±SEM) of ACF per colon, it was observed that the WT and the αERKO groups had 4.6 ± 1.0 and 3.4 ± 1.0 ACF, respectively, whereas the mean number in the βERKO group was significantly higher (15.0 ± 2.5, \( P < 0.001 \); Fig. 1A). We also calculated the average number of ACF per cm of colon, for each genotype, observing a significant increased number of ACF in the βERKO mice (\( P < 0.001 \); Table 1). As expected, AOM treatment induced multiple ACF of various sizes (Fig. 2A-C), which were similarly distributed regardless of genotype with the greatest number of ACF located in the distal colon. ACF composed of four or more crypts (\( \geq 4 \) crypts/ACF), also known as large ACF (Fig. 2C), have been more closely correlated with subsequent development of colon cancer (23). The βERKO mice showed a significantly higher incidence (\( P < 0.01 \)) of large ACF compared with the αERKO and WT groups (Table 1). In addition, colons from three of eight βERKO mice presented increased thickness and loss of normal morphology as revealed by methylene blue staining, which did not allow the scoring of ACF in these βERKO colons. To surpass the limitations with ACF scoring, the incidence of MDF in the AOM-treated mice was evaluated after AB-NR staining. This assay allowed us to complete the assessment of MDF (Fig. 2D-F) in colons of all the mice, including those colons which previously could not be scored for ACF. For MDF, the βERKO mice showed an average of 38.3 ± 4.0 MDF per colon, which was significantly higher (\( P < 0.001 \)) than the mean number observed on the WT and αERKO colons (6.6 ± 1.5 and 10.0 ± 1.9, respectively; Fig. 1B). The same statistical difference was obtained between βERKO versus WT and αERKO groups when the mean number of MDF per centimeter of colon was analyzed (Table 1). We also determined the number of large MDF, which have been characterized as having a multiplicity equal or higher than 12 crypts per focus (\( \geq 12 \) crypts/MDF; ref. 23), per colon (Fig. 2F). The data showed a greater number of large MDF in the βERKO colons compared to the WT and αERKO groups when the mean number of MDF per centimeter of colon was analyzed (Table 1). No statistically significant differences (\( P > 0.05 \)) were observed between WT and αERKO groups in all the parameters analyzed, as well as between females and males of each group.

Table 1. ACF and MDF incidence in colons of AOM-treated WT and ERKO mice

<table>
<thead>
<tr>
<th>Mice genotype</th>
<th>Colon length (cm)</th>
<th>ACF/cm colon</th>
<th>Large ACF, ( \geq 4 ) crypts/ACF</th>
<th>MDF/cm colon</th>
<th>Large MDF, ( \geq 12 ) crypts/MDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>8.3 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td>αERKO</td>
<td>8.8 ± 0.4</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.4</td>
<td>1.2 ± 0.3</td>
<td>5.2 ± 1.5</td>
</tr>
<tr>
<td>βERKO</td>
<td>8.7 ± 0.4</td>
<td>1.8 ± 0.3*</td>
<td>3.2 ± 0.9†</td>
<td>4.8 ± 0.6*</td>
<td>18.0 ± 4.4†</td>
</tr>
</tbody>
</table>

NOTE: The values are means (±SEM) for each group classified by genotype. Significant statistical differences are shown for WT versus βERKO and αERKO versus βERKO groups with \( P < 0.01 \) and \( P < 0.001 \).

\*\( P < 0.001 \).

\†\( P < 0.01 \).
Previously, it was reported that colons from βERKO mice presented greater number of proliferating cells and faster migration of labeled cells toward the surface of the colonic crypts when compared with colons from WT littermates (33). Furthermore, the colonic epithelium of βERKO mice also had shown fewer apoptotic cells and...
a significant decrease in expression of cytokeratin 20 (an epithelial differentiation marker), α-catenin, and plectin (cellular adhesion molecules). These results suggested that ERβ might play a role in the maintenance of the colonic crypt architecture (33). In the present study, we observed that after AOM exposure, the colons of βERKO mice presented an increased colonic thickness when compared with the colons of αERKO and WT mice. After AB-NR staining, it was observed that in the colons of βERKO mice, the crypt organization and morphology was completely lost in several areas, which did not occur in the colonic epithelium of the αERKO and WT littermates (Fig. 3). However, the scoring of MDF was possible in these mice due to significant color contrast between the red background stained with the NR and the dark-blue spots composed of crypts full of mucin (Fig. 2D-F and Fig. 3, βERKO).

Cell proliferation and apoptosis in ERKO and WT mice exposed to AOM

To assess if the ER genotype would influence the pattern of proliferation and apoptosis in the colonic epithelium after AOM exposure, we determined Ki-67 expression by immunohistochemistry and apoptosis by TUNEL assay. As shown in Fig. 4A, no significant differences were found for Ki-67 expression among the three different genotypes (P > 0.05; Fig. 4C). The proliferation status was also assessed by the proliferating cell nuclear antigen immunostaining, and although the number of proliferative cells was higher compared with the Ki-67 immunostaining, no statistical differences were observed between the different genotypes (P > 0.05; data not shown). The difference between proliferating cell nuclear antigen and Ki-67 may be associated with an upregulation of proliferating cell nuclear antigen in response to DNA injury associated...
with the AOM injections. Results from the TUNEL assay indicated that the colon sections from βERKO mice exhibited fewer apoptotic cells compared with WT and αERKO (Fig. 4B). The quantitative analyses of the results showed a significant difference between WT versus βERKO (P < 0.01) and αERKO versus βERKO (P < 0.05) groups, with colon sections from βERKO mice expressing the least number of apoptotic cells (Fig. 4D). These findings suggest that the proliferation pattern in colonic crypts of ERKO mice does not differ from that of the WT mice after AOM exposure; however, ERβ deficiency seems to be associated with a significant decrease in apoptosis in the colon. This observation supports a potential proapoptotic role for ERβ in the colon and could comprise, in part, a possible explanation for the higher incidence of dysplastic crypts in the βERKO mice.

**Discussion**

Several epidemiologic studies have reported that women who use hormone replacement therapy are at a lower risk of developing colon cancer compared with nonusers (2). Additionally, Weyant et al. (34) showed that endogenous estrogens protect against Apc-associated tumorigenesis and that tumor prevention by 17β-estradiol was associated with an upregulation of ERβ and a downregulation of ERα expression in the colonic enterocytes. More recently, an increased incidence of colon tumors was found in ER-deficient Min/+ mice relative to ERα Min/+ controls (35, 36). Based on these observations, estrogens have been linked to colon cancer protection through interaction with ERα. However, very few in vitro studies have been carried out to provide evidence for the protective role of ERβ against colon carcinogenesis. In this study, we compared the incidence and multiplicity of AOM-induced ACF/MDF among the colon sections of WT, ERα, and ERβ-KO mice. The results showed that βERKO mice showed significantly higher incidence of ACF and MDF per colon (Fig. 1), as well as an increased number of large ACF/MDF per colon, which are considered to be more closely correlated to carcinogenesis outcome (Table 1). Furthermore, ERβ-deficient colonies showed a decrease in apoptosis but not in proliferation rates relative to normal and ERα-deficient colonies (Fig. 4). These findings collectively suggest that ERβ may be involved in the maintenance of a proper balance between cell proliferation and cell death in the colon. In contrast, the ERS-deficient colons did not present any significant difference on ACF/MDF and large ACF/MDF occurrence, as well as, on proliferation and apoptosis levels when compared with WT controls. Additionally, the colons from βERKO mice were thicker and showed significant differences in the crypt architecture after AOM exposure when compared with WT and αERKO colonic epithelium (Fig. 3). These data support the notion of a protective role for ERβ in the maintenance and differentiation of colon epithelial cells and in preventing colon carcinogenesis, whereas ERα seems to have a less prominent role in the development of colon cancer. Furthermore, ERβ may also be essential for the normal cellular growth and architectural structure of the colon, especially by controlling the rate of cell loss, playing a different biological function from ERα, as previously proposed by Wada-Hiraike et al. (33). These results are congruent with the work of Weige et al. (37) that showed that estradiol treatment reduces the incidence of premalignant lesions in the colon through an ERβ-mediated response.

Traditionally ACF are used as a biomarker for colon carcinogenesis, however, in transgenic animals, the scoring of ACF is often compromised due to the increased thickness of the colons in those animals (e.g., Vitamin D receptor knockout mice; ref. 38). In such cases, MDF may be useful as a biomarker for precancerous lesions. In conclusion, the results described in the present study indicate a possible protective role of ERβ on colon carcinogenesis. In addition, the use of selective ERβ inducers might be an important approach for preventing and treating colon cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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