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Enhanced induction of Mucin Depleted Foci in Estrogen Receptor β Knockout Mice

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Abstract

The role of the estrogen receptor β (ER β) 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), ER α and ER β knockout (α ERKO and β ERKO) mice were injected with azoxymethane (AOM), a colon chemical carcinogen. Fourteen weeks after AOM exposure the incidence of aberrant crypt foci (ACF) was assessed by methylene blue staining. β ERKO mice showed significantly higher incidence ($P < 0.001$) of ACF (15.0 ± 2.5) as compared to α ERKO (3.4 ± 1.0) and WT (4.6 ± 1.0) mice. The colons in several β ERKO mice had increased thickness and loss of normal morphology. It has been reported that ER β plays a role in maintenance of the colonic crypt architecture; this may explain the loss of crypt organization in the colonic epithelium of β ERKO 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, in order 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 β ERKO mice colons (38.3 ± 4.0 ; $P < 0.001$), comparing to WT and α ERKO 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 ER β in colon carcinogenesis.

Keywords

Estrogen receptors; colon cancer; aberrant crypt foci; mucin depleted foci

Introduction

The incidence and mortality rates of colorectal cancer (CRC) are similar among the genders (1), however, women who have a history of Hormone Replacement Therapy (HRT) demonstrate a reduction of colon cancer risk (2). Similarly, both parity and oral contraceptive

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No potential conflicts of interest were disclosed.

(OC) use seem to exert preventive effects against colon carcinogenesis in females (3,4,5). These protective effects have been attributed, in part, to the high levels of endogenous (pregnancy) and/or exogenous (OC and/or HRT) 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), ER α and ER β (8). The expression of the two ER subtypes is tissue specific, with ER α levels in the colon being lower than ER β . No differences have been reported for ER α expression between normal and malignant human colon. While ER α has a more regulatory role in mammary carcinogenesis, ER β is the predominant functional form in the human colon (9,10). Interestingly, a selective loss of this protein has been observed during 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 ER β . Although the mechanism by which ER β mediates its effects is not well known, the silencing of the ER β by methylation has been considered as one of the early events in colorectal and breast carcinogenesis (13,14,15). Although these observations are consistent with the hypothesis that ER β 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 physiological 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), α ERKO (18) and β ERKO (19) mice to investigate whether the absence of ER α or ER β affect the incidence of azoxymethane (AOM)-induced aberrant crypt foci (ACF). The ACF were first described by Bird as one of the early events occurring in colon cancer progression (20), 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, MDF have also been found in human colons of patients with familial adenomatous polyposis (FAP) and with sporadic CRC (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 in order to discern the role of ERs in colon carcinogenesis progression.

Materials and Methods

Animals and carcinogen treatment

The breeding pairs of α ERKO and β ERKO mice were obtained from Dr. Dennis Lubahn (University of Missouri) and are maintained at IIT Research Institute. The KO status was confirmed by genotyping. The background-WT C57BL/6 mice were purchased from Charles Rivers laboratories (Raleigh, NC) and used as wild type controls. Animals were quarantined for 7 days and housed four mice per cage, with a 12-hr 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, twenty four 16 weeks old mice were divided into three groups according to their ER genotype. The groups consisted of eight WT, eight α ERKO and eight β ERKO mice, divided as four females and four males per each group. All animals were injected subcutaneously with the colon carcinogen AOM (10mg/kg body weight) once a week for 4 weeks (Sigma Chemical Co., St. Louis, MO). 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 weeks).

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 $\times 20$ and $\times 40$. The ACF were distinguished from surrounding non-aberrant 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 min and then incubated in NR (0.5% aqueous solution) for 10 seconds. The AB-NR stained colons were scored under the Olympus DP70 microscope ($\times 10$ and $\times 20$ 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 >3 crypts often distorted and smaller than 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 in order to determine the multiplicity of the MDF.

Quantitative Immunohistochemistry

Tissues were sectioned (4 μ m thick) and processed for immunohistochemistry as previously described (30). The antibody anti-Ki-67 rabbit monoclonal (1:50) (Thermo α Fisher Scientific, Fremont, CA) was used to assess the proliferation status on β ERKO, ERKO 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; Palo Alto, CA) and used for chromogen quantification using the Tiffalyzer program (31,32).

Quantitative histochemical detection of apoptosis

Colonic sections (4 μ m thick) were evaluated for the presence of apoptotic cells by TUNEL assay using FragEL DNA fragmentation detection kit (Calbiochem, EMD, Gibbstown, NJ) 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 Analysis of Variance (ANOVA) was used for statistical comparisons among different genotypes Bonferroni Multiple Comparison test was used to determine statistical differences between groups using GraphPad InStat Software (San Diego CA). Differences were considered statistically significant when p was < 0.05 .

Results

Azoxymethane injections increased ACF and MDF incidence in colons of β ERKO mice

Intact C57BL/6, β ERKO, and α ERKO 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 ($\pm$ SE) of ACF per colon, it was observed that the WT and the α ERKO groups had 4.6 ± 1.0 and 3.4 ± 1.0 ACF, respectively, whereas the mean number in the β ERKO 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 β ERKO 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. Aberrant crypt foci composed of 4 or more crypts (≥ 4 crypts/ACF), also known as “large” ACF (Fig. 2C), have been more closely correlated with subsequent development of colon cancer (23). The β ERKO mice showed a significantly higher incidence ($p < 0.01$) of “large” ACF compared to the α ERKO and WT groups (Table 1). In addition, colons from 3 out of 8 β ERKO mice presented increased thickness and loss of normal morphology as revealed by MB staining, which did not allow the scoring of ACF in these β ERKO colons. In order 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 β ERKO 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 α ERKO colons (6.6 ± 1.5 , and 10.0 ± 1.9 , respectively) (Fig. 1B). The same statistical difference was obtained between β ERKO vs. WT and α ERKO groups when analyzed the mean number of MDF per cm of colon (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 (≥ 12 crypts/MDF) (23), per colon (Fig. 2F). The data showed a greater number of “large” MDF in the β ERKO colons comparing to the WT and α ERKO groups ($p < 0.01$) (Table 1). No statistically significant differences ($p > 0.05$) were observed between WT and α ERKO groups in all the parameters analyzed, as well as between females and males of each group.

Functional significance of Estrogen receptor β in the organization of colonic epithelium

Previously, it was reported that colons from β ERKO mice presented greater number of proliferating cells and faster migration of labeled cells toward the surface of the colonic crypts when compared to colons from WT littermates (33). Furthermore, the colonic epithelium of β ERKO 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 ER β 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 β ERKO mice presented an increased colonic thickness when compared to the colons of α ERKO and WT mice. After AB-NR staining it was observed that in the colons of β ERKO mice the crypt organization and morphology was completely lost in several areas, which did not occur in the colonic epithelium of the α ERKO 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, Fig. 3 β ERKO).

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

In order 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 amongst the three different genotypes ($p > 0.05$) (Fig 4C). The proliferation status was also assessed by the PCNA immunostaining, and although the number of proliferative cells was higher as compared to the Ki-67 immunostaining, no statistical differences were observed between the different genotypes ($p > 0.05$) (data not shown). The difference between PCNA and Ki-67 may be associated with an upregulation of PCNA in response to DNA injury associated with the AOM injections. Results from the TUNEL assay indicated that the colon sections from β ERKO mice exhibited fewer apoptotic cells as compared to WT and α ERKO (Fig. 4B). The quantitative analyses of the results showed a significant difference between WT vs. β ERKO ($p < 0.01$) and α ERKO vs. β ERKO ($p < 0.05$) groups, with colon sections from β ERKO 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 ER β -deficiency seems to be associated with a significant decrease in apoptosis in the colon. This observation supports a potential pro-apoptotic role for ER β in the colon and could comprise, in part, a possible explanation for the higher incidence of dysplastic crypts in the β ERKO mice.

Discussion

Several epidemiological studies have reported that women who use HRT are at a lower risk of developing colon cancer in comparison to non-users (2). Additionally, Weyant *et al.* demonstrated that endogenous estrogens protect against Apc-associated tumorigenesis and that tumor prevention by 17 β -estradiol was associated with an up-regulation of ER β and a down-regulation of ER α expression in the colonic enterocytes (34). More recently, an increased incidence of colon tumors was found in ER-deficient Min/+ mice relative to ER^{+/+}Min/+ controls (35,36). Based on these observations, estrogens have been linked to colon cancer protection through interaction with ER β . However, very few *in vivo* studies have been carried out to provide evidence for the protective role of ER β against colon carcinogenesis. In this study, we compared the incidence and multiplicity of AOM-induced ACF/MDF amongst the colons of WT, ER α and ER β -KO mice. The results showed that β ERKO mice demonstrate 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, ER β -deficient colons showed a decrease in apoptosis but not in proliferation rates relatively to normal and ER α -deficient colons (Fig. 4). These findings collectively suggest that ER β may be involved in the maintenance of a proper balance between cell proliferation and cell death in the colon. In contrast, the ER α -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 to WT controls. Additionally, the colons from β ERKO mice were thicker and showed significant differences in the crypt architecture after AOM exposure when compared to WT and α ERKO colonic epithelium (Fig. 3). These data support the notion of a protective role for ER β in the maintenance and differentiation of colon epithelial cells and preventing colon carcinogenesis, whereas ER α appears to have a less prominent role in the development of colon cancer. Furthermore, ER β 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 ER α , as previously proposed by Wada-Hiraike *et al.* (33). These results are congruent with the work of Weige *et al.* that showed that estradiol treatment reduces the incidence of premalignant lesions in the colon through an ER β -mediated response (37).

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 (eg. Vitamin D receptor KO mice, 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 ER β on colon carcinogenesis. And the use of selective ER β inducers might be an important approach for preventing and treating colon cancer.

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Fig. 1. Influence of ER α and ER β -deficiency on incidence of ACF and MDF in AOM-treated WT and ERKO mice. **A**, number of ACF/colon; **B**, number of MDF/colon for WT, α ERKO and β ERKO mice. The values are means ($\pm$ SE). Statistical differences are shown between WT and β ERKO and between α ERKO and β ERKO groups.

Fig. 2.

Topographical features of AOM-induced ACF and MDF presenting different multiplicity. **A–C**, ACF composed of 1 and 3 crypts (original magnification, $\times 20$) and more than 4 crypts (original magnification, $\times 40$) respectively as indicated by black arrows, observed in colons of WT mice stained with MB. **D–F**, MDF composed of 4, 10 and more than 12 crypts, respectively, indicated by white arrows, observed in colons of β ERKO mice after AB-NR staining (original magnification, $\times 20$).

Fig. 3.

Topographical features of the observed colonic epithelium of AOM-treated WT, α ERKO and β ERKO mice after AB-NR staining. The background epithelium stains in red (NR), while the colonic crypts appear as dark-blue spots due to the high production of mucin stained with AB (WT, original magnification $\times 20$; α ERKO, original magnification $\times 10$). In the ER β -deficient colons there was a loss of normal crypt organization observed (β ERKO, original magnification $\times 10$). Despite this, MDF (eg. indicated by white arrow) were easily scored with AB-NR staining and used as biomarkers for precancerous lesions in the colon. β ERKO (a) shows the higher magnification of the white rectangular region in the left β ERKO panel.

Fig. 4.

A. Expression of Ki-67 in colon sections of WT and ERKO mice (original magnification, $\times 20$). **B.** Apoptosis detection by TUNEL in WT and ERKO colonic sections (original magnification, $\times 40$). Note that the images show the top of the colonic crypts where the number of apoptotic cells is higher. **C.** Ki-67 immunostaining quantification. No significant statistical differences were observed between the three ER genotypes ($p > 0.05$). **D.** Quantitative analyses for TUNEL. Fewer apoptotic cells were observed in βERKO. Comparison of apoptotic cells in βERKO colonic epithelium with that of WT and αERKO showed statistically significant differences ($p < 0.01$ for βERKO Vs WT and $p < 0.05$ for βERKO Vs αERKO). All values are presented as mean $\pm$ SE.

Table 1

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

Mice genotype	Colon length (cm)	ACF/cm colon	"large" ACF ≥ 4 crypts/ACF	MDF/cm colon	"large" MDF ≥12 crypts/MDF
WT	8.3 ± 0.2	0.6 ± 0.1	0.5 ± 0.3	0.8 ± 0.2	3.8 ± 1.2
αERKO	8.8 ± 0.4	0.4 ± 0.1	0.4 ± 0.4	1.2 ± 0.3	5.2 ± 1.5
βERKO	8.7 ± 0.4	1.8 ± 0.3 ^{**}	3.2 ± 0.9 [*]	4.8 ± 0.6 ^{**}	18.0 ± 4.4 [*]

Note: The values are means (±SE) for each group classified by genotype. Significant statistical differences are shown for WT vs. βERKO and αERKO vs. βERKO groups with

^{*} $P < 0.01$

^{**} $P < 0.001$.