A novel shift in estrogen receptor expression occurs as estradiol suppresses inflammation-associated colon tumor formation

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Abstract

Postmenopausal women on estrogen replacement therapy (ERT) have a reduced risk of developing colon cancer compared with postmenopausal women not on ERT, suggesting a role for estradiol (E₂) in protection against this disease. To determine whether E₂ protects against inflammation-associated colon cancer when administered following the initiation of colonic DNA damage, in this study, we implanted E₂-containing pellets into mice after co-treatment with azoxymethane and two rounds of dextran sulfate sodium (DSS). Wild-type (WT) E₂-treated mice had reduced numbers and average area of adenocarcinomas compared with the control mice. These effects were lost in estrogen receptor- β (*Er β* (*Esr2*)) knockout mice. Surprisingly, apoptosis was reduced and cell proliferation was increased in sections from tumors of the WT E₂ mice compared with the WT control mice. These findings are probably due, in part, to a reduction in ER β expression in colonic epithelial cells as the cells progressed from a non-malignant to a cancerous state as enhanced apoptosis was observed in normal colonocytes expressing higher levels of ER β . Furthermore, epithelial cells within the tumors had dramatically increased ER α mRNA and protein expression compared with the non-diseased mice. We conclude that while E₂ treatment resulted in an overall suppression of colonic adenocarcinoma formation, reduced ER β expression accompanied by enhanced ER α expression caused an altered colonocyte response to E₂ treatment compared with the earlier stages of colon cancer development. These data are the first examples of decreased ER β expression concurrent with increased ER α expression as a disease develops and highlight the importance of understanding the timing of E₂ exposure with regard to the prevention of inflammation-associated colon cancer.

Key Words

- ▶ colon cancer
- ▶ estradiol
- ▶ estrogen receptor β
- ▶ estrogen receptor α
- ▶ inflammation

Endocrine-Related Cancer
(2013) 20, 515–525

Introduction

Women have a reduced risk of developing colon cancer when compared with men. This protection is lost, however, once a woman reaches menopause, suggesting

estrogen is a protective agent against colon tumor development. Data from the Women's Health Initiative (WHI) study as well as other clinical trials have confirmed

this idea, as it has been documented that postmenopausal women on either hormone replacement therapy (HRT) or estrogen replacement therapy (ERT) have a reduced risk of developing colon cancer (Rossouw *et al.* 2002, Newcomb *et al.* 2007, Hoffmeister *et al.* 2009, Delellis Henderson *et al.* 2010, Wu *et al.* 2010).

Animal studies have also investigated the role of estrogens in the reduction of colon cancer occurrence: the number of dimethylhydrazine-induced colon tumors was reduced in rats administered estradiol (E₂; Smirnov *et al.* 1999). Additionally, the multiplicity of tumors induced by azoxymethane (AOM) was suppressed by orally administered estrone, a precursor to E₂, in both estrogen receptor- α (*Er α* (*Esr1*)) knockout (ER α KO) and wild-type (WT) mice (Guo *et al.* 2004).

Not only has estrogen been linked to reduced incidence of sporadic colon cancer, but data also exists showing that the risk of developing inflammation-associated colon cancer may be influenced by E₂. Epidemiological studies have demonstrated that women have a lower risk of developing this disease when compared with men, which may be due to the presence of estrogens (Soderlund *et al.* 2010). The mechanism behind this observed protection, however, is poorly understood. It is important to investigate this subtype of colon cancer and to develop strategies against the disease, because the risk of being diagnosed with colon cancer is increased in people suffering from inflammatory bowel disease (IBD). Both ulcerative colitis and Crohn's disease are associated with an increased cancer risk (Mellemejkjaer *et al.* 2000, Bernstein *et al.* 2001, Eaden *et al.* 2001). In fact, patients with inflammation-associated colon cancer have a worse prognosis and survival rate compared to those with sporadic colon cancer (Larsen *et al.* 2007). The tumors that develop have distinct characteristics compared with sporadic colon cancer: they are commonly flat, infiltrating and often occur in multiples. Additionally, there is a higher incidence of high-grade, mucinous carcinomas in inflammation-associated colon cancer. The mechanism linking IBD and colon tumor formation is not yet fully understood, but it is probably associated with the more frequent occurrence of DNA mutations in colonic epithelia due to chronic inflammation.

The aim of this study was to investigate the timing when E₂ may be administered and be protective against inflammation-associated colon tumor formation. While the WHI study did find that HRT prevented osteoporosis, reduced fractures, and lowered the incidence of colorectal cancer, the study was ended prematurely due to an observed increase in the risk of developing invasive breast

cancer and heart disease. In a follow-up to the WHI study, it has been observed that the protective effect of HRT against colon cancer was completely lost in as few as 3 years after discontinuing HRT (Heiss *et al.* 2008). This indicates that the timing of exposure to E₂ is an important factor when considering E₂ as a chemoprotective agent. Using an experimental protocol in which we delayed E₂ treatment in ovariectomized mice until after colon cancer had been initiated, we sought to determine whether E₂ could protect against colonic carcinogenesis even after DNA damage had occurred and tumor formation had started. To do so, we used AOM and dextran sulfate sodium (DSS), an inflammatory reagent that mimics ulcerative colitis in humans. The presented data provide novel insights into the timing when E₂ exposure is beneficial for colon cancer prevention and suggest a role for E₂ in inflammation-associated colon cancer.

Materials and methods

Animals

C57Bl6/J mice heterozygous for *Er β* (*Esr2*) ER β KO (+/–) were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed at the Laboratory Animal Resources and Research facility at Texas A&M University. The mice were bred to produce WT and ER β KO offspring, and the genotype was confirmed using genomic tail DNA. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Generation and identification of colon tumors

Female WT and ER β KO mice were ovariectomized and implanted with a 20 mg cholesterol (Sigma–Aldrich)-containing pellet subcutaneously on the back of their necks (Fig. 1A). At the time of surgery, the mice were placed on a semi-purified phytoestrogen-free diet (AIN-76, Laboratory Supply, Highland Village, TX, USA) and were given access to food and water *ad libitum*. Two weeks following surgery, the mice were administered a single 12.5 mg/kg injection of AOM (Sigma–Aldrich). Inflammation of the colon was induced 1 week after AOM injection by way of two 6-day regimens of 2.5% DSS solution in the drinking water, with each regimen being separated by 2 weeks. A fresh DSS solution was administered to the mice on days 3 and 5 of each regimen period. One week following the final DSS treatment, the cholesterol-containing pellets were removed, and the mice were

Figure 1

Effect of E₂ on tumor number and area. (A) Study design. (B) Median number of tumors per mouse $\pm$ median absolute deviation. (C) Mean area of tumors $\pm$ s.e.m., $n=6$ mice in the WT and ERβKO control groups, $n=7$ mice in the WT E₂ group and $n=5$ mice in the ERβKO E₂ group. Bars without a common letter differ, $P \leq 0.05$.

randomly assigned to either the E₂ group or the control group, with half of the mice being implanted with a new 20 mg cholesterol-containing pellet and the other half with a pellet composed of 19 mg cholesterol + 1 mg E₂. After 8 more weeks, the mice were injected with bromodeoxyuridine (BrdU) and killed 2 h later. Blood was collected via cardiac puncture. The colon was resected, and individual masses were cassetted and fixed in 4% paraformaldehyde (PFA) for 4 h. The size of each mass was

assessed using calipers. Uninvolved tissues from the most distal region of the colon not containing visible masses were cassetted and fixed as well. Tumors were classified by a board-certified pathologist blinded to the treatment groups following H&E staining of the tissue sections.

Ovariectomized control mice, both WT and ERβKO, were maintained in parallel with the AOM/DSS mice. These mice were handled in the same manner as the tumor-bearing mice with the exception of not being administered AOM and DSS. At the time of killing, 1 cm sections were cut from the distal colon, cassetted, and fixed in 4% PFA. Additionally, mucosal scrapings were collected and flash-frozen in liquid nitrogen for RNA isolation.

E₂ treatment in non-AOM/DSS mice

A second set of non-AOM/DSS mice were used to observe the effects of E₂ in healthy tissues. The mice were individually housed, randomly assigned to a control or an E₂ treatment group based on age and weight, and given a phytoestrogen-free diet. All the mice were ovariectomized, and the control mice were implanted with a 20 mg cholesterol-containing pellet, while the E₂ mice were implanted with a 19 mg cholesterol/1 mg E₂ pellet subcutaneously on the back of their necks. The mice were killed on day 72. At the time of killing, blood was collected through cardiac puncture. The colon was resected, and 1 cm sections from the distal end were cassetted and fixed in 4% PFA.

Plasma E₂ concentrations

Whole blood was collected at the time of killing. Plasma was obtained by centrifuging the samples at 350 *g* for 15 min at 4 °C. Plasma E₂ concentrations were measured using the Estradiol EIA kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions. Samples were diluted 1:10, and the reaction mixture was allowed to develop for 75 min. Absorbance was read on a plate reader at 415 nm, and the final E₂ concentrations were determined by comparison with standards using the formula supplied in the kit.

Immunohistochemistry for ERβ, ERα, and BrdU

From the PFA-fixed tissues, 4 μm sections were taken. The sections were deparaffinized and rehydrated. Endogenous peroxidase was quenched using 3% H₂O₂ in methanol for 30 min, and antigen retrieval was achieved by

microwaving in 10 mM citrate buffer for 20 min. The slides were then incubated with the primary antibodies, rabbit-anti-ER β (Pierce, Rockford, IL, USA), diluted 1:50, rabbit-anti-ER α (Santa Cruz Biotechnology), diluted 1:50, or anti-BrdU (Roche), diluted 1:20, at 4 °C overnight in a humidified chamber. During each staining process, the primary antibodies were left off of a single slide to serve as a negative control. The following morning, the slides were washed and then incubated with the secondary antibodies, goat-anti-rabbit-HRP (Santa Cruz Biotechnology), diluted 1:50, or goat-anti-mouse-HRP (Abcam, Cambridge, MA, USA), diluted 1:250, for the ER β and BrdU stains respectively. For the ER α stain, the VectaStain ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used. A 1% diaminobenzidine (DAB; Sigma-Aldrich) solution in PBS was used as the chromagen, and Meyer's hematoxylin was used as the counter-stain. Lastly, the slides were dehydrated and coverslipped.

TUNEL assay

The ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA) was used for the TUNEL assay following the manufacturer's instructions with slight modifications. The PFA-fixed tissues were rehydrated and treated with 10 μ g/ml proteinase K for 3 min at 37 °C. Endogenous peroxidase activity was blocked using 0.3% H₂O₂ in methanol for 30 min. The tissues were incubated at room temperature (RT) for 20 s in an equilibration buffer and then incubated for 1 h at 37 °C in a reaction buffer plus TDT enzyme. The TDT enzyme was omitted from an individual section as a negative control for the stain. Subsequently, the slides were placed in a stopwash solution for 10 min and later in an anti-digoxigenin solution for 30 min at RT in a humidified chamber. As the chromagen, 0.5% DAB was used for 20 s, and nuclei were counter-stained in 0.5% methyl green for 5 s. Lastly, the slides were dehydrated and coverslipped.

Immunohistochemical analysis

The proliferation and apoptosis stains were analyzed in the same manner. In the uninvolved tissues (the most distal region of the colon not containing a mass), crypts were symmetrically bisected, and the right halves of 20 crypts were analyzed per mouse. The total number of positively stained cells was divided by the total number of cells in the crypt to generate the percentage of apoptotic or proliferative cells for each crypt. For the tumor sections, 250 cells from four distinct regions of each tumor were

analyzed. An analysis of the ER β and ER α stains was conducted using the image analysis software Cell Profiler in conjunction with HKCellCounter, a pipeline designed to specifically identify nuclei within a designated region and return values based on the staining intensity of each identified nucleus. The pipeline identifies nuclei based on parameters such as size and shape set by the user within a specified area of a photograph. Twenty intact crypts were analyzed for each uninvolved and non-AOM/DSS sample. Four images of distinct locations from each tumor were analyzed to determine expression levels in the adenocarcinomas. The same parameters were used for each photomicrograph to ensure consistency between the analyses.

rt-PCR for ER α

RNA was isolated using the TRIzol reagent from the tumors in the tumor-bearing mice and the mucosal scrapings in the non-AOM/DSS-treated mice. cDNA was produced from the RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) as per the manufacturer's instructions: 1 μ l of a diluted sample was mixed with a random hexamer primer, an anchored-oligo(dT)₁₈ primer, and water. The samples were then placed in a thermal cycler for 10 min at 65 °C. Next, reaction buffer, RNase inhibitor, deoxynucleotide mix, and reverse transcriptase were added to the samples, and the samples were again placed in the thermal cycler for 10 min at 25 °C followed by 60 min at 50 and 85 °C for 5 min. The samples were then stored at -20 °C.

The SYBR Green method was used to assess the mRNA content of the samples. Primers against ER α and 18s sequences were produced by Sigma-Aldrich with the following sequences: ER α -forward: 5'-GACCAGATGGT-CAGTGCCTT-3' and ER α -reverse: 5'-ACTCGAGAAGGTG-GACCTGA-3' and 18s-forward: 5'-TCAAGAACGAAAG-TCGGAGGT-3' and 18s-reverse: 5'-GGACATCTAAGGG-CATCACAG-3'. Two microliters of cDNA were mixed with 2.5 μ l of the forward and reverse primers, 9.5 μ l of SYBR Green and 11 μ l of water. The prepared samples were loaded into 96-well reaction plates and run for 45 cycles. The samples were run in triplicate. ER β RNA expression was not analyzed at this time due to the previously collected protein data for ER β .

Statistical analysis

The tumor number outcome was analyzed using a bootstrap with 1000 replications, a statistical test that

compares the medians of groups. The uninvolved and normal *in vivo* apoptosis data were analyzed using a Poisson regression model allowing for overdispersion, and statistical inference was made by testing the model coefficients corresponding to group comparisons with the control using a Wald test. One-way ANOVA was used for the remaining outcomes. ANOVA was run using Minitab 15, and the bootstrap, Poisson regression, and Wald test were carried out using the statistical software 'R'.

Results

Plasma E₂ concentrations

Plasma E₂ concentrations were measured in all the mice. Plasma E₂ concentrations of both the WT and ERβKO mice administered E₂ pellets averaged 0.8 nmol/l, which were marginally lower than the peak E₂ concentrations in a premenopausal non-pregnant woman and well below the levels observed in pregnancy (Lenton *et al.* 1982, Lof *et al.* 2009). In non-ovariectomized mice, E₂ concentrations reached peak levels during estrus at around 0.24 nmol/l (Wood *et al.* 2007). Mice not administered E₂ had mean plasma E₂ concentrations of 0.03 and 0.04 nmol/l in the WT and ERβKO groups respectively. These concentrations are less than half the concentration of plasma E₂ found in postmenopausal women (Probst-Hensch *et al.* 2000).

Tumor multiplicity and area are reduced with E₂ treatment

To determine whether administration of E₂ following the initiation of DNA damage could decrease colon tumor formation, colon tumor multiplicity and area were measured in both the WT and ERβKO mice following the study design described (Fig. 1A). The tumors were characterized by a board-certified pathologist blinded to the treatment groups. The median number of tumors per mouse was decreased by nearly 50% in the presence of E₂ in the WT mice ($P=0.05$) (Fig. 1B). In comparison, no significant change in median tumor number was observed in the ERβKO mice. In the absence of E₂, the ERβKO mice had significantly reduced tumor number compared with the WT control mice ($P=0.05$). With regard to the mean size of each tumor, E₂ treatment resulted in a 30% reduction in tumor area in the WT mice ($P=0.031$). Again, the response to E₂ treatment was not observed in the ERβKO mice. Both the control and E₂-treated ERβKO mice exhibited a significant reduction in tumor size compared with the WT control mice ($P=0.001$ and $P=0.003$) (Fig. 1C).

Cellular proliferation increases in the WT E₂-treated mice at the tumor stage

The proliferation of epithelial cells within the tumors and uninvolved colonic crypts was analyzed using immunohistochemistry for BrdU in order to investigate potential mechanisms for the reduction in tumor multiplicity and size observed in the WT E₂-treated mice. Surprisingly, E₂ treatment resulted in significantly increased proliferation in both the uninvolved tissues and the adenocarcinomas in the WT mice compared with the non-E₂-treated control mice ($P=0.043$ and $P=0.017$ respectively) (Fig. 2A and B). In the uninvolved WT tissues, E₂ roughly doubled the amount of proliferation compared with the non-E₂-treated mice. In the adenocarcinomas, there was a 30% increase in proliferation. No change in proliferation

Figure 2

Effect of E₂ on proliferation. Colonocyte proliferation was measured in the tumors and uninvolved colonic tissues of the WT and ERβKO mice using immunohistochemistry. (A) Proliferation in the uninvolved colonic tissues and (B) proliferation in the tumors. Values are mean percent of positively stained cells $\pm$ S.E.M., $n=6$ mice in the WT and ERβKO uninvolved control groups, $n=7$ mice in the uninvolved WT E₂ group, and $n=5$ mice in the uninvolved ERβKO E₂ group. In B, $n=6$ mice in the WT control group, $n=7$ mice in the WT E₂ group, $n=6$ mice in the ERβKO control group, and $n=5$ mice in the ERβKO E₂ group. Bars without a common letter differ, $P \leq 0.05$.

was observed in the ER β KO mice with E₂ treatment for either uninvolved tissues or tumors or in the non-AOM/DSS-treated mice (Fig. 3B).

Apoptosis is downregulated by E₂ at the tumor stage in the WT mice

Having observed increases in proliferation in response to E₂ treatment, levels of apoptosis were also measured in the adenocarcinomas and uninvolved tissues using a TUNEL

assay. No significant differences were observed in the percent of apoptotic cells in the uninvolved tissues in response to E₂ treatment for either the WT or the ER β KO mice (Fig. 4A). In the adenocarcinomas, E₂ treatment resulted in a 25% decrease in apoptosis in the WT mice ($P=0.039$) and no change was observed in the ER β KO mice (Fig. 4B). Contrarily, in the non-AOM/DSS-treated mice, the presence of E₂ was correlated with increased apoptosis in the colonic crypts (Fig. 3C).

ER β protein expression declines as tumors develop

In order to investigate physiological changes associated with the observed reduction in tumor area and number, ER expression levels were measured. ER β levels were measured using immunohistochemistry to observe the effects of inflammation-associated carcinogenesis on the protein levels of ER β in the WT mice (Fig. 5). Tumor sections were compared with the uninvolved tissue sections from the tumor-bearing mice as well as with normal tissue sections from the normal, non-AOM/DSS-treated mice from the same animal experiment. Normal colonic tissue sections exhibited the highest staining intensity for ER β . Uninvolved tissue sections of the colon exhibited a significant reduction in staining intensity compared with the normal tissue sections ($P=0.0007$ and $P=0.002$ for the control mice and E₂-treated mice respectively). The analysis of the tumor sections demonstrated even further reduction in staining intensity when compared with the non-AOM/DSS tissue sections, and levels were significantly lower in the tumor sections when compared with the uninvolved tissue samples from the same animal experiment ($P<0.0001$). The reduction in ER β expression was not affected by the presence of E₂.

ER α mRNA and protein expression increases as tumors develop

In addition to measuring ER β levels, ER α levels were also assessed in the tumor and non-AOM/DSS colonic tissues. ER α mRNA expression was measured using rt-PCR. mRNA collected from mucosal scrapings from the non-AOM/DSS-treated mice in the same animal experiment was compared with mRNA from the tumor samples to assess whether ER α expression was altered as the tissues progressed to a malignant state (Fig. 6A). There was a 300-fold increase in ER α expression in the tumors compared with the normal mucosal samples in the WT mice not administered E₂ ($P=0.021$). Adenocarcinomas from the ER β KO mice not administered E₂ exhibited a

Figure 3
Effect of E₂ on normal colonic tissues. Colonocyte proliferation and apoptosis were measured in the non-diseased WT and ER β KO mice. (A) Study design. (B) Percent of proliferative cells in the colonic crypts $\pm$ S.E.M. (C) Percent of apoptotic cells in the colonic crypts $\pm$ S.E.M., $n=10$ mice for each of the WT control and ER β KO E₂ groups and $n=8$ mice for the WT E₂ and ER β KO control groups. Bars without a common letter differ, $P\leq 0.05$.

Figure 4

Effect of E₂ on apoptosis. Cellular apoptosis was measured in the tumors and uninvolved colonic tissues of the WT and ERβKO mice using a TUNEL assay. (A) Apoptosis in the uninvolved colonic tissues and (B) apoptosis in the tumors. Values are mean percent of positively stained cells $\pm$ S.E.M., $n=6$ mice in the WT and ERβKO uninvolved control groups, $n=7$ mice in the uninvolved WT E₂ group, and $n=5$ mice in the uninvolved ERβKO E₂ group. In B, $n=6$ mice in the WT control group, $n=7$ mice in the WT E₂ group, $n=6$ mice in the ERβKO control group, and $n=5$ mice in the ERβKO E₂ group. Bars without a common letter differ, $P \leq 0.05$.

200-fold increase in *Erα* mRNA expression compared with mucosal scrapings from the non-DSS/AOM-treated ERβKO mice ($P=0.042$). In WT E₂-treated mice, the tumors exhibited a 14-fold increase in *Erα* mRNA expression compared with the WT E₂ normal mucosal samples ($P=0.015$). In the presence of E₂, *Erα* mRNA expression was increased 13-fold in the ERβKO adenocarcinomas when compared with the ERβKO normal mucosal samples ($P=0.022$).

Protein expression levels of ERα were measured using immunohistochemistry to observe whether or not the mRNA expression levels were representative of protein ERα expression levels (Fig. 6B, C, D, E, F, G, H, and I). Immunohistochemistry for ERα from the tumor sections exhibited strong positive staining for ERα in the WT mice

with reduced staining levels in the ERβKO mice both with and without E₂ treatment, while no staining was evident in the normal tissues for either genotype.

Discussion

Studies carried out in both humans and animal models have demonstrated the efficacy of E₂ in protecting against sporadic colon cancer, and yet little investigation has been carried out on the influence of E₂ on inflammation-associated colon cancer. Existing data obtained from

Figure 5

Effect of E₂ on ERβ protein expression in the WT mice. ERβ expression was measured in the tumors and uninvolved and normal colonic tissues of the WT mice using immunohistochemistry. (A) Quantification of staining intensity. Values are mean staining intensity of the tissues $\pm$ S.E.M., $n=7$ mice in the normal, non-AOM/DSS group, $n=6$ mice in the WT cholesterol groups, and $n=7$ mice in the WT E₂ groups. Bars without a common letter differ, $P \leq 0.0001$. (B, C, D, E, F, and G) Representative photographs of staining for ERβ in an ERβKO colon (B) and normal, non-AOM/DSS-treated WT (C), uninvolved WT control (D), WT control tumor (E), WT E₂ uninvolved (F), and WT E₂ tumor (G) tissues.

Figure 6

Effect of E₂ on ERα expression in mice. (A) *Erα* mRNA expression was measured in the tumors and normal colonic tissues of the mice using rt-PCR. Values are mean fold change from the normal, non-AOM/DSS WT control mice $\pm$ S.E.M., $n=7$ mice in the normal group, $n=4$ mice in the WT control group, $n=4$ mice in the WT E₂ tumor group, $n=4$ mice in the ERβKO control group, and $n=2$ mice in the ERβKO E₂ tumor group. Bars without a common letter differ, $P \leq 0.0001$. (B, C, D, E, F, G, H, and I) Representative photographs of immunohistochemistry for ERα protein in the WT control (B and C), WT E₂ (D and E), ERβKO control (F and G), and ERβKO E₂ (H and I) mice. For each pair, the normal tissue section is presented on the left and the tumor section is presented on the right.

studies conducted on human populations suggest that the presence of E₂ may result in a reduction of the occurrence of this disease (Soderlund *et al.* 2010). Until now, no animal studies have investigated the mechanisms involved in the reduction of inflammation-associated colon cancer incidence observed in the presence of E₂. Additionally, the exact timing when E₂ can be administered and prove protective against colon cancer remains

unknown. Previous studies conducted in our laboratory have indicated that E₂ guards against the early markers of colon tumor formation. As early as a few hours following carcinogen exposure at the point of initial DNA damage, E₂ significantly induces apoptosis in the colonic epithelium of rats when compared with control mice (Armstrong *et al.* 2011). In addition, we have demonstrated that E₂ suppresses the formation of pre-malignant lesions in the colon, which is again associated with an upregulation of apoptosis in colonic epithelia (Weige *et al.* 2009).

To date, *in vitro* studies of the protective effects of E₂ during the process of colonic carcinogenesis have only investigated continuous E₂ exposure for the duration of the studies. In contrast, this study aimed to elucidate the chemoprotective role of E₂ when the intervention is given following the initiating events in colonic carcinogenesis. The data reported herein indicate, for the first time, that E₂ is protective prior to colon tumor formation even when administered after DNA and inflammatory damage had occurred in the colon. This protection is observed in the reduction in tumor multiplicity and area.

After quantifying fewer colon tumors in response to E₂ treatment in this model, we sought to determine the physiological responses to E₂ treatment at the cellular level and how these responses change over the progression of the disease. In this study, despite E₂ causing an overall decline in tumor number and area, increased proliferation and decreased apoptosis in the colonocytes of the WT E₂-treated tumor-bearing mice compared with the cholesterol-treated control mice were observed when the tumors were studied. In the ERβKO group, there was no change in proliferation between the cholesterol- and E₂-treated mice for either the uninvolved or colon tumor samples. When apoptosis was measured in the tumors of the ERβKO mice, it was found that E₂ treatment resulted in no significant change compared with the cholesterol-treated ERβKO mice. Similarly, data from human trials determining the effects of combined estrogen and progestin therapy in postmenopausal women showed that the combined HRT reduced the incidence of colon tumors in these women; however, the tumors that were detected were at a more advanced stage (Simon *et al.* 2012). The fact that a similar phenomenon was observed in human tissues supports the results obtained from mice in this study. While the mechanism behind the high-grade tumors was not investigated in the human study, it is possible that it could be similar to what has been observed in mice.

Having observed these counterintuitive responses to E₂ treatment in the tumors and uninvolved tissues in mice, a second animal study was conducted to investigate how E₂ affects normal colonic tissues. In groups that received neither AOM nor DSS, E₂ treatment resulted in no change in proliferation for either the WT or the ERβKO mice, and the non-diseased WT mice exhibited increased apoptosis in the colon. These data coincide with the results obtained previously in our laboratory and indicate that the response to E₂ treatment may be dependent on the stage of the disease and, possibly, the experimental model used in the study.

An intriguing explanation for the surprising response to E₂ treatment observed for proliferation and apoptosis in the tumor-bearing mice could be the changes in ER expression detected in this experiment. Previous studies carried out in our laboratory and others have indicated that ERβ, the primary ER within the colon, is the form of the receptor through which E₂ exerts its protective effects (Guo *et al.* 2004, Weige *et al.* 2009). Other groups have shown that ERβKO mice are more susceptible to inflammation-associated colon cancer than the WT mice (Saleiro *et al.* 2012). Clinical and experimental models of colonic carcinogenesis have suggested that the protein expression of ERβ decreases as the tissues progress from a normal colonic epithelial to a malignant state (Konstantinopoulos *et al.* 2003, Castiglione *et al.* 2008, Di Leo *et al.* 2008, Mostafaie *et al.* 2009). In fact, humans with colon tumors not expressing ERβ are likely to be in more advanced stages of cancer and have a higher risk for death compared with those with tumors in which ERβ is present (Rudolph *et al.* 2012). ERβ expression levels in this study suggest that a similar trend occurs during the formation of inflammation-associated colon tumors. Normal colonic tissues exhibited the highest staining intensity for ERβ, while the tumors exhibited the lowest intensity, with no changes being observed in ERβ expression due to the presence or absence of E₂. The analysis of the uninvolved tissues revealed ERβ intensity levels to be in between those of the normal tissue and tumor samples. The intermediate staining intensity of the uninvolved tissue samples is unsurprising when one takes into account that while these samples were not cancerous, they were collected from the same colons as the tumors and as such cannot be considered normal, having sustained high levels of damage. The varying levels of ERβ expression between the tissues indicate that the loss of expression occurs gradually as inflammation-associated colon tumors develop.

In systems where ERα is the predominantly expressed ER, such as the mammary gland, the presence of E₂ is often

associated with an increase in cancer growth (Chisamore *et al.* 2009, Castoria *et al.* 2010, Rasmussen *et al.* 2010, Truan *et al.* 2010, Brekman *et al.* 2011). In normal colonic tissues, ERα protein expression is very low, as can be observed by the lack of a positive stain in the correlative tissues in this study. In contrast, adenocarcinomas exhibited positive protein staining for ERα and exhibited significantly increased *Erα* mRNA expression: as high as 300-fold over the control tissues, supporting the idea that ERα activity could be increased in the colon tumors. Contrarily, studies conducted utilizing the APCmin/+ mouse model of intestinal carcinogenesis suggest that the loss of ERα was detrimental to the colon (Cho *et al.* 2007, Cleveland *et al.* 2009). These observed disparities are probably the result of underlying differences between the APCmin/+ mouse model and the inflammation protocol used here.

The possibility of ERα activity exceeding ERβ activity could explain the change in physiological response to E₂ treatment observed in the tumor-bearing mice compared with the non-diseased mice, and with that observed in previous studies conducted in our laboratory to determine the effects of E₂ during earlier endpoints in the process of colonic carcinogenesis. It is possible that the protection against colon tumor formation in response to E₂ treatment observed in this study occurred prior to the decline in ERβ expression and increase in ERα expression, delaying the growth of colon tumors. Once ERα had started to be expressed, tumor physiology was altered as indicated by increased proliferation and decreased apoptosis.

In addition, previous studies carried out in our laboratory suggest that p53 may be a primary modulator of the chemoprotective effect of E₂ on colonic carcinogenesis; *in vitro*, non-malignant young adult mouse colonocytes (YAMCs) treated with E₂ exhibit increased apoptosis and an overall reduction in cell number. In YAMCs lacking functional p53, the effect of E₂ is lost (Weige *et al.* 2012). Mutations in *p53* (*Trp53*) occur in roughly 50% of colon tumors (Goh *et al.* 1995), and the loss of p53 functionality could have contributed to the change in physiological response to E₂ treatment in the tumors in this study. Due to the timing of tissue collection in the present experiment, analysis of p53 would be inappropriate due to the loss of proper p53 function observed in most colon tumors. Had the tissues been harvested at an earlier time point following AOM/DSS exposure, probably a different response to E₂ treatment and relevant p53 signaling would have been observed. However, the presented data are critical in highlighting the complex role of estrogens in the colon.

The current study has taken a critical step toward understanding the role of E₂ in colonic carcinogenesis. It demonstrates that E₂ is protective against inflammation-associated colon tumor formation, which is important because it is estimated that up to 396/100 000 people worldwide suffer from IBD, increasing their risk of developing colon cancer (Lakatos 2006). Additionally, E₂ protected against the development of tumors even when introduced into the system following the initiation of inflammation and DNA damage to the colonic tissues. This observation implies that intervention with estrogen therapy may be beneficial in patients considered at a higher risk for developing colon cancer even if they have been menopausal for some time. Of utmost interest in this study was the observed upregulation of ER α expression with a concurrent reduction in ER β expression. To our knowledge, this study is the first to definitively demonstrate a concurrent and inverse shift in the expressions of ER α and ER β in any system. Several studies have reported changes in ER expression as the result of treatments or changes in disease state. However, in these studies, either one of the ERs was affected or the expression of both the receptors was either increased or decreased (Cho *et al.* 2007, Jiang *et al.* 2008). The presented data provide new insights into the role of estrogens in the reduction of colon tumor formation in patients who suffer from colonic inflammation and suggest that an intervention can still be successful even if begun later in the tumor development process.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by ACS grant RSG-11-179-01-TBE to C D Allred.

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Received in final form 13 May 2013

Accepted 23 May 2013

Made available online as an Accepted Preprint
23 May 2013