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Raloxifene and anti-estrogenic Gonadorelin inhibits intestinal tumorigenesis by modulating immune cells and decreasing stem like cells

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Abstract

Studies suggest that estrogen plays a contributing role in colorectal cancer (CRC). This project examined the preventive effects of raloxifene, a selective estrogen receptor modulator (SERM), and gonadorelin, an anti-estrogenic drug, in female Apc^{Min/+} mouse intestinal tumorigenesis. Six-week-old Apc^{Min/+} mice were fed diet containing 1 ppm raloxifene or control diet. Gonadorelin (150ng/mouse) was injected subcutaneously into one treatment group. Intestinal tumors were evaluated for tumor multiplicity and size. Mice treated with raloxifene and gonadorelin showed colon tumor inhibition of 80% and 75% respectively. Both drugs significantly inhibited small intestinal tumor multiplicity and size (75 – 65%, $P < 0.0001$). Raloxifene and gonadorelin showed significant tumor inhibition with 98% and 94% inhibition of polyps >2 mm in size. In mice fed with raloxifene or injected with gonadorelin, tumors showed significantly reduced proliferating cell nuclear antigen expression (58-65%, $P < 0.0001$). Raloxifene treatment decreased β -catenin, cyclin D1, laminin 1 β , Ccl6 and stem like cells (Lgr 5, EpCAM, CD44/CD24), as well as suppressed inflammatory genes (COX-2, mPGES-1, 5-LOX,). Gonadorelin showed significant decrease in COX-2, mPGES-1, iNOS, and stem like cells or increased NK cells and chemokines required for NK cells. Both drugs were effective in suppressing tumor growth albeit with different mechanisms. These observations show that either suppression of estrogen levels or modulation of estrogen receptor dramatically suppresses small intestinal and colonic tumor formation in female Apc^{Min/+} mice. These results support the concept of chemoprevention by these agents in reducing endogenous levels of estrogen or modulating ER signaling.

Keywords

Raloxifene; Gonadorelin; Colorectal cancer; NK cells; Stem Cells; Estrogen

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. 50,830 deaths from CRC are expected in 2013, with an estimated 142,820 new cases

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diagnosed (1). CRC incidence rates among young men and women (ages 20–49) increased by 1.5 – 1.6% from 1995 to 2005 (2-3). In younger adults, CRC incidence rates have been increasing by 1.1% per year from 2005 (2).

Epidemiological studies suggest that women have delayed development of adenomas and colon cancers compared to men (4). This disparity of CRC formation among genders has been attributed to female hormones in premenopausal women. Data have suggested an inverse relationship between post-menopausal oral hormone therapy use and risk of developing CRC. The Women's Health Initiative reported that combination of estrogen and medroxy-progesterone acetate decreased the number of colon cancers by 37% compared to placebo (5 – 6). The protective effect of this combination on colon cancers was due to progesterone, not estrogen (7 – 8). Primary analysis by Gunter et al. (2008) reported that high endogenous estrogen levels were associated with 1.5 fold increased risk of developing colorectal cancer (9). Another study reported that women with high circulating estrogen showed a 60% increased risk of colorectal cancer (10). Epidemiological and experimental studies suggest that CRC risk is associated with dietary factors, lifestyle, hormonal use/ imbalances, and genetic factors. Notably, endogenous estrogen levels have been found to be associated with increased risk for CRC (11)

Preclinical and *in vitro* CRC cell line reports are consistent with findings suggesting a positive association between endogenous estrogen level and the risk of colorectal cancer (12 – 15). In a previous study, we reported a decrease in AOM-induced aberrant crypt foci formation (colon cancer precursor lesions) in male F344 rats upon use of selective estrogen receptor modulator (SERM), raloxifene (16). Taken together, these results suggest that endogenous sex hormones play a vital role in CRC formation. In the present study, we tested whether estrogen receptor modulation or suppression of endogenous estrogen would provide better efficacy against intestinal tumorigenesis using raloxifene (SERM) and gonadorelin (a synthetic decapeptide with a structure identical with the natural gonadotrophin releasing hormone (Gn-RH) in mammals) to treat female *Apc*^{Min/+} mice. Also, we studied the effects of these agents on inflammatory molecules such as COX-2 (cyclooxygenase) and 5-LOX (lipoxygenase) as well as proliferating markers β -catenin and cyclin D along with stem like cell markers, to observe if these agents have any effects on stem like cells, because estrogen is known to modulate stem like cells in other cancers. Further we analyzed if these agents have any immune modulating effects on NK cells in female *Apc*^{Min/+} mice. NK cells have been identified as lymphoid cells capable of killing number of tumor cells both *in-vivo* and *in-vitro* without any prior stimulation (17) due to loss of MHC molecules on tumor cells, often render these cells vulnerable to NK cytotoxicity. Given that estrogens have been reported to suppress NK cell function, we sought to identify if these agents function through enhancing NK cell functions.

Materials and Methods

Chemicals

Raloxifene was provided by the Center for Cancer Prevention and Drug Development drug repository (Oklahoma City, OK). Gonadorelin was synthesized at Dr. Gali's laboratory by solid phase peptide synthesis method using standard fmoc chemistry, purified by reversed-phase high-performance liquid chromatography, and characterized by electrospray mass spectrometry (Department of Pharmaceutical Sciences, OUHSC). Primary antibodies (polyclonal) to COX-2, proliferating cell nuclear antigen (PCNA), were obtained from Santa Cruz Biotechnology (Dallas, Texas), and monoclonal antibody, fluorescent dye phycoerythrin (PE)-linked Nkp46 for NK cells from Biolegend (San Diego, CA). Primary anti-bodies, Lgr5 (monoclonal) from Abcam (Cambridge, MA), CD44 (polyclonal), CD24 (polyclonal) and EPCAM (monoclonal) were purchased from Santa Cruz Biotechnology.

Horseradish peroxidase–conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Pathway focused inflammatory genes, chemokines, chemokine receptors, and NK cell receptor PCR Array were procured from Qiagen (Valencia, CA) and Bio-Rad (Irvine, CA).

Breeding and genotyping of *Apc^{Min/+}* mice

All of the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Oklahoma Health Sciences Center (OUHSC). Six week old male *Apc^{Min/+}* (C57BL/6J) and female wild-type littermate mice were purchased initially from The Jackson Laboratory (Bar Harbor, ME) as founders. A breeding colony was established in the OUHSC rodent barrier facility. Offspring were identified by an allele-specific PCR assay according to vendor's instructions (18). All mice were housed, 3 per cage, in ventilated cages in a temperature and humidity controlled rodent barrier facility with 12hr light/dark cycle. All mice were allowed ad libitum access to the respective diets and automated tap water purified by reverse osmosis.

Experimental diets and efficacy studies in *Apc^{Min/+}* mice

A modified American Institute of Nutrition (AIN)-76A diet was used. All ingredients for the semipurified diets were procured from Bioserv (Frenchtown, NJ). Raloxifene was premixed with a small quantity of diet, and then blended into bulk diet using a Hobart mixer. Both control and experimental diets were prepared weekly and stored in a cold room. We used 0, 1 ppm raloxifene in the AIN-76A diet. Raloxifene dose was selected based on our previous observations with this drug (16).

Female *Apc^{Min/+}* mice were used in the efficacy study. The experimental protocol is summarized in Fig. 1A. Five-week-old mice were randomized into three groups so that average body weights in each group were equal (10 *Apc^{Min/+}* mice in each group). Mice were fed AIN-76A (control) diet for 1 week. At 6 weeks of age, animals were assigned to Group 1 (control diet), Group 2 (experimental diet with 1 ppm raloxifene), or Group 3 (control diet, gonadorelin injection). In Group 3, 150ng of gonadorelin was injected subcutaneously (five days a week, for three weeks (at 6th, 8th and 10th week) (Fig. 1A). Gonadorelin dose was selected based on the literature guide from European Agency for Medicinal Products (19). Food and water intake, animal activity, and physical changes were monitored regularly. Mice were evaluated weekly for signs of weight loss or lethargy that might indicate intestinal obstruction or anemia. After 100 days (14 weeks) of treatment (Fig. 1A) all mice were euthanized by CO₂ asphyxiation. After necropsy, the entire intestinal tract was harvested, flushed with 0.9% NaCl, and opened longitudinally from the esophagus to the distal rectum. To expose the tumors, the tissue was flattened on filter paper. Tissue was briefly frozen on dry ice to aid visual scoring of tumors. The number, location, and size of visible intestinal tumors were determined under a dissection microscope (18). All tumors were scored and subdivided by location (duodenal, jejunal, ileal, and colorectal) and size (>2 mm, 1–2, or <1 mm in diameter). This procedure was completed by 2 individuals who were blinded to the experimental group and the genetic status of the mice. Fresh tissues from intestinal tumors were frozen in liquid nitrogen for molecular analysis. The remainder of the intestines was fixed in 10% neutral buffered formalin, embedded in paraffin blocks, and processed by routine hematoxylin and eosin staining.

Cell proliferation, COX-2, and ER- β expression by Immunohistochemistry (IHC) and Lgr5, Epcam, CD44, CD24, and NK cells (Nkp46) expression by Immunohistofluorescence (IHF)

To evaluate the effect of raloxifene and gonadorelin on intestinal tumor tissue, proliferating cell nuclear antigen (PCNA), COX-2, and ER- β expression levels were assessed by IHC. For PCNA, COX-2, and ER- β IHC staining, paraffin sections were deparaffinized in xylene and

rehydrated through graded ethanol solutions to PBS. We then followed the procedure as previously published (18). Monoclonal/polyclonal primary antibodies against PCNA, COX-2, and ER- β were obtained from Santa Cruz Biotechnology (Dallas, Texas). The color reaction was developed with diaminobenzidine (DAB) histochemistry. Nonimmune rabbit immunoglobulins were substituted for primary antibodies as negative controls. PCNA-positive cell scoring in the polyps was performed under light microscopy at 400 magnification by 2 investigators blinded to the identity of the samples. Cells with a brown nucleus were considered positive. The proliferation index was determined by dividing the number of positive cells per polyp and multiplying by 100.

For IHF, the slides were incubated with primary antibody for NK cell receptor, tagged with phycoerythrin (PE) in the dark for 1 hr. For Lgr5, EpCAM, CD44, and CD24, the slides were incubated overnight with primary antibody. Slides were washed three times with PBS for 5 min and incubated with secondary antibody tagged with fluorescein isothiocyanate (FITC)/tetramethylrhodamine isothiocyanate (TRITC) in the dark for 1 hr. Then, slides were washed with PBS three times for 5 min and incubated with 0.5 μ g/mL, 6-diamidino-2-phenylindole (DAPI) for 5 min in the dark room. Slides were rinsed with PBS and observed for fluorescence under PE filters using an Olympus microscope IX71. Digital computer images were recorded with an Olympus DP70 camera.

Lgr5, EpCAM, CD44, CD24, 5-LOX, mPGES-1, β -catenin, cyclin D1, Laminin-1 β and GAPDH mRNA expression analysis by real-time and/or RT-PCR

Total RNA from tumor samples was extracted using Trizol reagent for total cellular RNA (Invitrogen, Grand Island, NY) per the manufacturer's instructions. Equal quantities of DNA-free RNA were used for reverse transcription reactions for making cDNA using SuperScript reverse transcriptase (Biorad, Hercules, CA). The real-time polymerase chain reaction (PCR) was carried out in a 25mL reaction volume using 3mL of a 1:10 cDNA dilution containing SYBR Green master mix (BioRad, Hercules, CA) and with respective primers for Lgr5, EPCAM, Cd44, and CD24. Glyceraldehyde phosphate dehydrogenase (GAPDH), used as an internal control, was also amplified'. For primers and PCR conditions please see supplementary table 1. PCRs for genes Lgr5, Epcam, CD44, and Cd24 were done in an iCycler iQ real-time PCR detection system. The fluorescence threshold values (Ct) were calculated using the manufacturer's software. Relative mRNA levels were assessed by standardization to GAPDH. Results were expressed as a fold difference in gene expression.

PCR (RT-PCR) was performed as described in supplementary Table 1 for 5-LOX, mPGES-1, laminin β 1, Cyclin D1 and β -catenin. PCR was done using the Taq polymerase master mix (Qiagen Inc., Valencia, CA). The PCR products were visualized and photographed under UV illumination. Relative mRNA levels were assessed by densitometry analysis using imageJ software. Results were expressed as a fold difference in gene expression.

Pathway focused inflammatory genes, chemokines and activated NK cells using real time PCR assay

A custom designed PCR array panel (Qiagen Inc. (Valencia, CA)/Biorad, Hercules, CA) with inflammatory markers (COX-2, iNOS) and chemokines (Cxcl3, Ccl6), along with activated NK cells (GP49a), was analyzed to identify which genes were targeted by raloxifene and gonadorelin. Total RNA from control and treated tissues were isolated by Trizol reagent. cDNA was generated with the iScript cDNA synthesis kit from Biorad, Hercules, CA. iTaq Universal SYBR green supermix was used for this PCR assay. Real-Time PCR run was performed per the instructions provided by the manufacturer. The data were processed and the results evaluated using CFX Manager 3.0 analysis software.

Enzyme Immunoassay (EIA) for Estrogen

Estrogen (E2) in the serum of treated and control mice are performed using commercially available kit from Cayman Chemical (Ann Arbor, Michigan). After developing the plate per the instructions provided in the kit, it is read at a wavelength between 405 and 425nm in micro plate reader. Results are expressed as nanograms per milliliter of serum. Determination was carried out in triplicates from each sample.

Statistical analyses

The Student's t test with one-tailed Welch's correction was used to evaluate the mean differences between two groups. All results were expressed as means $\pm$ SEM if not otherwise stated. Differences within the experimental groups (control vs treated) were considered significant at the $P < 0.05$ level. All statistical analysis was performed using GraphPad Prism Software 5.1 (GraphPad Software, Inc, San Diego, CA).

Results

Health Monitoring of mice

Mice in the control group (group 1) showed a significant drop in body weight gain beginning in week 8 (14 weeks age), compared to the treatment groups (Fig.1B). The weight loss in this group is mainly due to intestinal tumor burden, which leads to less nutrient absorption and anemia. In treatment groups, mice showed higher relative weight gain ($p < 0.005$), suggesting a significant protective effect from raloxifene (group 2) and gonadorelin (group 3) (Fig.1B). No animals in Groups 2 or 3 showed noticeable toxicity upon gross observation of the liver, kidney, or lung. Thus, the doses selected in this study were determined to be non-toxic.

Decreased E2 levels in treated animals

E2 Levels are significantly decreased in the serum of mice injected with the gonadorelin (Fig. 1C). Not much change was observed in serum levels of E2 in mice fed with raloxifene. (Fig. 1C).

Dietary administration of raloxifene and gonadorelin treatment significantly reduced small intestinal and colon tumors in female *Apc^{Min/+}* mice

Figure 1D, E, and F summarizes the chemopreventive effects of gonadorelin (150ng/animal) and dietary raloxifene (1 ppm) on polyp multiplicity in the intestine. Female *Apc^{Min/+}* mice fed control diet developed an average of 60 ± 6.5 (mean $\pm$ SEM) small intestinal polyps (Fig. 1D). Raloxifene and gonadorelin administration significantly reduced total intestinal polyp multiplicity and size (means $\pm$ SEM; 18 ± 4.4 and 19 ± 2.86 , Fig. 1D and E). Raloxifene and gonadorelin showed 75% ($P < 0.0001$) and 65% ($p < 0.001$) small intestinal polyp inhibition (Fig. 1D). The number of large-sized polyps (> 2 mm) was dramatically reduced with raloxifene and gonadorelin treatments (Fig. 1D). Mice receiving raloxifene (0.125 ± 0.09 , mean $\pm$ SEM) and gonadorelin (0.50 ± 0.380 mean $\pm$ SEM) had ~98% and ~94% fewer polyps with sizes greater than 2 mm, compared to control (8.625 ± 1.391 , mean $\pm$ SEM) mice (Fig.1E). The mean number of colon tumors in mice fed with control diet was 1.00 ± 0.577 (means $\pm$ SEM). Mice treated with raloxifene (0.160 ± 0.1090 , mean $\pm$ SEM) and gonadorelin (0.250 ± 0.1700 , mean $\pm$ SEM) showed colon tumor inhibition of 80% and 75% respectively (Fig. 1F).

Inhibition of PCNA, COX-2, ER- β and up-regulation in NK cell receptor protein expression by raloxifene and gonadorelin in colon tumors

The effect of gonadorelin and raloxifene on tumor cell proliferation is summarized in Figure 2A and B, as measured by PCNA over expression. Qualitative microscopic examinations of PCNA-stained sections were performed. Colon tumors exposed to gonadorelin and raloxifene showed a substantial decrease in PCNA-positive cells compared with tumors from the mice fed with control diet. Raloxifene and gonadorelin significantly suppressed proliferation in the colon tumors (Fig. 2A). The quantification of PCNA staining showed 43.1 ± 4.4 (mean $\pm$ SEM) PCNA-positive cells in control colon tumors, as compared with 18.2 ± 3.4 and 15 ± 2.8 (mean $\pm$ SEM) PCNA-positive cells in colon tumors from gonadorelin and raloxifene-treated mice, accounting for a decrease in the proliferation index by approximately 58% and 65% ($P < 0.0001$). IHC analysis of treated colonic tumors revealed a significant decrease in inflammatory marker COX-2 and estrogen receptor (ER- β) protein expression (Fig. 2A). Further, the colon tumors were analyzed for any immune modulatory effects of gonadorelin and raloxifene on NK cells by IHC staining. An increase in cells that were positively stained for NK cell receptor were observed in gonadorelin treated tumors compared to controls. (Fig. 2 C,D). The quantification of positively stained cells showed 6.8 ± 1.6 (Mean $\pm$ SEM), Nkp46 receptor stained cells in control colon tumors compared with 28.40 ± 4.1 and 10.40 ± 0.92 (Mean $\pm$ SEM) Nkp46 receptor-positive cells in colon tumors from gonadorelin and raloxifene-treated mice respectively, accounting for an increase in the number NK cells approximately 4 folds ($P < 0.002$) for gonadorelin. Though an increase in NK cells was observed by raloxifene treatment, it was not statistically significant.

Immunomodulatory and anti-inflammatory effects of raloxifene and gonadorelin in colon tumors

To analyze the effect of gonadorelin and raloxifene on inflammatory, chemokine, and proliferation markers, we measured the relative mRNA levels by both quantitative real time PCR and RT – PCR. As shown in Fig.3A and B, a significantly decreased mRNA expression of inflammatory markers COX-2 and iNOS was observed in tumors treated with gonadorelin. The mRNAs for activation and recruitment of immune modulators NK cells, Cxcr3, Cxcl3, and Ccl6, along with Gp49a receptor for activated NK cells, were analyzed and found to be significantly increased in gonadorelin treated colon tumors (Fig. 3A). We observed a significant decrease in COX-2 mRNA expression by raloxifene in colon tumors. We did not find any effects on iNOS mRNA expression (Fig. 3A). Though raloxifene treatment showed an increase in activated NK cell receptor Gp49a and Cxcl3 mRNA expression, it was statistically non-significant (Fig. 3A). However, tumors treated with raloxifene showed a significant decrease in Ccl6 mRNA levels (Fig. 3A). As shown in Fig. 3B, the raloxifene treated colon tumors showed significantly reduced mRNA expression of 5-LOX, mPGES-1, β -catenin, cyclin D1, and laminin-1 β (Fig. 3B). Gonadorelin treated colon tumors showed a significant decrease in mPGES-1 and laminin-1 β , with no effect on 5-LOX, β -catenin, or cyclin D1, suggesting it has a different mode of action in inhibiting colon tumors than does raloxifene.

Inhibition of cancer stem like cell markers by raloxifene and gonadorelin

To determine the expression of cancer stem cell markers in the colonic tumor tissues, EPCAM, Lgr5, CD24, and CD44 were analyzed using immunohistofluorescence (IHF) and real time PCR approaches. Significantly fewer positively stained cells for EpCAM and Lgr5 were observed in both raloxifene and gonadorelin treated colon tumor samples (Fig. 4A-B). IHF staining showed that cells in these colon tumors were positively labeled by CD24 and CD44, and were observed to co-localize in mice fed AIN-76A diet alone (Fig. 5A, upper panel). Markedly fewer CD24, CD44, and co-localized CD24/CD44 cells were observed in

colon tumors of treated mice (Fig. 5A, lower panel and middle panel). To examine the effect of raloxifene and gonadorelin on the mRNA expression of CSC marker genes in tumor tissues, we measured the relative mRNA levels of CSC marker genes by quantitative RT-PCR (qRT-PCR). We found that gonadorelin and raloxifene significantly decreased the expressions of CD24, CD44, EpCAM, and Lgr5 mRNAs, suggesting that the inhibition of tumor progression may be associated with the downregulation of markers of CSCs (Fig. 4 C & D; Fig. 5B & C).

Discussion

Epidemiological and experimental data suggests that high endogenous estrogen and estrogen metabolites are associated with carcinogenesis and are involved in various hormone responsive cancers, including the development and pathogenesis of colon cancer (20 – 24). The involvement of ER β receptor signaling in colon cancer is still unclear and controversial. Though various reports suggest a protective and or pro-tumorigenic role for estrogen signaling in later stages of cancer, we have observed the pro-tumorigenic role of estrogen signaling in the colon (16). To date, the tumorigenic role of estrogen signaling is not conclusive.

In the present study, we successfully used gonadorelin to produce chemical oophorectomy (as previously described) in female Apc^{Min/+} mice, a genetically predisposed animal model of human familial adenomatous polyposis (FAP). Using gonadorelin an anti-estrogenic agent in CRC, we observed a significant decrease in circulating E2 levels, small intestinal polyps and colon tumors, and improved mouse activity and body weight of mice, compared to control untreated mice (Fig. 1). In this study we also tested a SERM, estrogen receptor antagonist raloxifene. Our results showed the chemopreventive efficacy of long term feeding of this agent (1ppm) on both small intestinal polyps and colon tumors in female Apc^{Min/+} mice.

The reason for testing these two agents was to determine if ER receptor modulation or suppressing estrogen synthesis provides better efficacy in inhibiting CRC. Both of the agents significantly reduced the overall number of polyps, and decreased the incidence of large polyps and colon tumors in female Apc^{Min/+} mice, compared to untreated mice (Fig.1). Treatment with raloxifene and/or gonadorelin was also associated with decrease in cell proliferation, ER β protein expression, and a reduction of β -catenin, cyclin D1, laminin β 1, COX-2, and 5-LOX and its pathway molecules (Fig.2 A and Fig. 3 A & B). We observed an increased number and expression of NK cells and activated receptors in gonadorelin treated colon tumors (Fig. 2C & D). Though an increase in NKs was observed with raloxifene, it was not significant. Both of the agents significantly decreased stem like cells (EpCAM, Lgr5, CD24 and CD44) in colon tumors (Fig. 4 & 5). These results, together with our earlier findings (16), strongly support the chemopreventive efficacy of SERM and the anti-estrogenic compound gonadorelin in preclinical models of CRC, suggesting their translational potential against human CRC.

In the present study, the observed chemopreventive effects of raloxifene and gonadorelin were accompanied by inhibition of ER β and anti-proliferative mechanisms. ER β is reported to be involved in regulating epithelial proliferation (25). We have previously reported that raloxifene inhibits proliferation in human colon cancer cells (16). Excessive cell proliferation is often associated with tumor development and progression, and the agents modulating proliferation in neoplastic cells have immense potential in CRC prevention and therapeutic intervention (26). In the present study, both raloxifene and gonadorelin inhibited cell proliferation, as evidenced by decreased PCNA and suppressed ER β expression in colon tumors. Under Apc mutant conditions, aberrant β -catenin signaling involved with increased

epithelial proliferation is observed in CRC (27). In the present study, $Apc^{Min/+}$ mice showed increased levels of β -catenin together with increased expression of cyclin D1 and laminin β 1 in intestinal tumors which were significantly decreased by raloxifene treatment. Overexpression of cyclin D1 downstream of the β -catenin pathway, a critical oncogene, is directly associated with increased proliferative index in CRC and results in more aggressive cancer phenotype (28). Thus, downregulation of ER β , β -catenin, and cyclin D 1 in intestinal tumors from raloxifene-treated $Apc^{Min/+}$ mice also support the drug's role in inhibiting proliferation. These results suggest that modulation of estrogen receptors might affect proliferation through cyclin D1 and β -catenin regulation. Though gonadorelin showed decreased proliferation, it did not have any effect on β -catenin and cyclin D1, indicating it uses a different pathway than does SERM (Fig.3).

Epidemiological studies suggest that women have delayed development of adenomas and colon cancers compared to men (29). Observational studies demonstrating a positive association between high endogenous estrogen levels and colon cancer risk are consistent with studies done in laboratory animals as well as *in vitro* studies with human cancer cell lines (5 – 8, 30 – 33). In the present study, we created a chemical oophorectomy during early stages of tumorigenesis in female $Apc^{Min/+}$ mice, which resulted in decreased intestinal tumors. Our results are in agreement with previously reported results in which post-oophorectomy female mice were markedly less prone to tumor development than were mice that had ovaries *in situ* (5, 9, 30). We observed decreased expression of ER β upon gonadorelin treatment, suggesting that this drug has a role in estrogen synthesis and proliferation. ER β regulates estrogen production: mice with ER β mutation display aberrant regulation of estrogen production by the ovaries, which influence tumor outcomes in animal models (10, 34, 30).

Estrogen compromises mucosal repair under inflammatory conditions (30) leading to tumor growth by enhancing inflammation. In the present study, we observed decreased expression of inflammatory marker COX-2 and its metabolites (mPGES1) in mice treated with gonadorelin. COX-2 and iNOS have been shown to be involved in CRC development in humans (35 – 36). Significant inhibition of iNOS was observed in mice treated with gonadorelin, indicating the role of estrogen in inducing inflammation during CRC. Various cytokines and chemokines are associated with CRC and are modulated by inflammatory signaling. Chronic activation of pro-inflammatory responses by estrogen contributes to pro-carcinogenic processes, thus promoting tumor formation or progression. In the present study, we evaluated cytokines which might have been affected by suppression of estrogen formation and which might also have a role in NK cell stimulation. Ccl6 is reported to promote innate immune response by NK cell activation in animal models of peritonitis (37). We observed increased expression of Ccl6 with an increase in the number of NK cells in colon tumors treated with gonadorelin. However, raloxifene showed an inhibitory effect on Ccl6 (Fig. 3), suggesting a different mode of action by this agent.

Cxcr3, a chemokine receptor expressed in malignancy, is not generalized to all types of malignant tumors. It is either associated with aggressive disease, or low expression is associated with bad prognosis (38). It is expressed on activated immune cells, including NK cells. Treatment with gonadorelin resulted in accumulation of activated NK cells, evident by increased expression of Gp49a, the NK cell receptor in the tumors (Fig.3). The gonadorelin might have reversed Cxcr3 functions by attracting the Cxcr3 NK cells to the tumor site. In patients with colorectal cancer, NK cells are suggested to have an antineoplastic role (39). It is reported that sustained high levels of β -estradiol leads to a reduction in natural killer cell activity (40), which may inhibit antineoplastic role of NK cells. Our results indicate that gonadorelin stimulation which leads to decrease in estrogen might have increased natural cell activity. Also, we observed increased 5-LOX mRNA expression in gonadorelin treated

tumors. It is reported that NK cell activity is restored by 5-LOX and its products (41). Together, these results suggest that the immune-modulating effects of gonadorelin could be one of its chemopreventive mechanisms. However, additional studies are needed.

In breast cancer, estrogen is reported to modulate cancer stem cell properties both *in vivo* and *in vitro* (42). The modulation of cancer stem cells markers in colon carcinogenesis and chemoprevention remains largely unexplored. We observed a correlative response of gonadorelin and raloxifene with anti-tumor effects, an increase in NK cells, and a decrease in stem like cells/CSC markers (Fig.2, 4 & 5). Though it is documented that estrogen modulates cancer stem cells in other cancers, further research is needed to show whether these agents target stem like cells or if they simply behave like biomarkers. Still, these agents tempering effects on stem like cells are novel and have potential significance in chemoprevention.

We show that either suppression of estrogen synthesis or modulation of ER will inhibit intestinal tumorigenesis although through different mechanisms. Further studies are warranted to determine the differential mechanistic effects of raloxifene and gonadorelin on inhibition of intestinal tumorigenesis (Fig. 6A, B).

In summary, our results demonstrate *in vivo* anti-proliferative, anti-inflammatory and immunomodulatory effects of gonadorelin and raloxifene in small intestinal polyps, and colon tumors which collectively contribute to chemopreventive efficacy against spontaneous intestinal tumorigenesis in female Apc^{Min/+} mice. Together with our previous reports on estrogen antagonist's efficacy against CRC in a pre-clinical model, these findings suggest that raloxifene and gonadorelin could be an effective strategy for preventing CRC or helping patients with FAP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Experimental design for the evaluation of the chemopreventive efficacy of Raloxifene (R) and Gonadorelin (G). For details about animals and treatments, see Materials and Methods. (B) Changes in body weight over time in female Apc^{Min/+} treated with or without Raloxifene and Gonadorelin. Statistically significant differences in bodyweight between drug-treated and control groups (C) were observed. (C) EIA for serum estrogen (E2) in treated mice compared to untreated mice (D) Inhibition of total SI polyp formation in Apc^{Min/+} mice by raloxifene and Gonadorelin. Data are means $\pm$ SE of ten animals per treatment group. The control and treated groups are significantly different from one another ($P < 0.0001$). (E) Tumor sizes in the small intestine of Apc^{Min/+} mice in Raloxifene and Gonadorelin treated mice compared to control animals. (F) Average number of colon tumors per mouse in control and treated Apc^{Min/+} mice. A significant ($P < 0.0007-0.003$) inhibition of colon tumors was observed with both raloxifene and Gonadorelin. Data are means $\pm$ SE of ten animals per treatment group.

Figure 2.

(A) Serial paraffin sections of intestinal tumors from $Apc^{Min/+}$ mice were subjected to immunohistochemical analysis using an anti-PCNA, anti-COX-2 and anti-ER- β polyclonal antibody in treated and control tissues. Original magnifications, $\times 60$. (B) Significant difference was observed in proliferative index between Raloxifene and Gonadorelin-treated and control group polyps. (C). Immunohistofluorescence of intestinal tumors from $Apc^{Min/+}$ mice for NK cells treated with Raloxifene and Gonadorelin. (D) Quantitative analysis of NK cells in intestinal tumors from $Apc^{Min/+}$ mice treated with Raloxifene and Gonadorelin.

A

B

Figure 3.

(A) Real-time PCR analysis for COX-2, iNOS, Gp49a, Cxcl3, GAPDH and Ccl6 mRNA expression in intestinal tumors of treated and untreated $Apc^{Min/+}$ mice. (B) Quantitative Reverse Transcriptase PCR analysis for 5-LOX, mPGES-1, β -catenin Cyclin D1 and laminin 1 β with GAPDH as housekeeping gene.

A

B

C

D

Figure 4.

(A) Immunohistofluorescence for EpCAM positive cells in intestinal tumors from $Apc^{Min/+}$ mice treated with Raloxifene and Gonadorelin. (B) Immunohistofluorescence for Lgr5 positive cells in intestinal tumors from $Apc^{Min/+}$ mice treated with Raloxifene and Gonadorelin. (C) Real-Time PCR analysis for EpCAM mRNA expression in intestinal tumors of treated and untreated $Apc^{Min/+}$ mice. (D) Real-Time PCR analysis for Lgr5 mRNA expression in intestinal tumors of treated and untreated $Apc^{Min/+}$ mice. GAPDH was used as reference gene.

A

B

C

Figure 5.

(A) Immunohistofluorescence for CD44 and CD24 positive cells in intestinal tumors from $Apc^{Min/+}$ mice treated with Raloxifene and Gonadorelin. The last vertical row displaying the merged figure of CD44 and CD24 in Raloxifene and Gonadorelin treated tumors compared to control tumors. (B) Real-Time PCR analysis for CD44 mRNA expression in intestinal tumors of Raloxifene and Gonadorelin treated and untreated $Apc^{Min/+}$ mice. (C) Real-Time PCR analysis for CD24 mRNA expression in intestinal tumors of Raloxifene and Gonadorelin treated and untreated $Apc^{Min/+}$ mice. GAPDH was used as house-keeping gene.

Figure 6.

(A). Schema for Raloxifene action in intestinal tumors of Female $Apc^{Min/+}$ mice. (B) Schema for Gonadorelin action in intestinal tumors of Female $Apc^{Min/+}$ mice.