A close-up of colon cancer
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Gender disparity in colonic tumorigenesis depends on male hormone tumor promotion, not female hormone protection

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Abstract

Colorectal cancer develops from adenomatous polyps, which arise from hyperplastic crypts termed aberrant crypt foci (ACF). Men are more likely than women to develop colonic cancers and precursor lesions, a phenomenon which is likewise observed in ApcPirc/+ rats that harbor a mutation in the Apc gene and spontaneously develop intestinal polyps. Here we set out to investigate the contribution of sex hormones in development of colorectal tumorigenesis. Using both ApcPirc/+ rats and a chemical model to assess ACF formation in the rat colon, we find that in female animals, depletion of endogenous hormones by means of ovariectomy does not affect prevalence of ACFs or polyps. Additionally, no effect is observed upon supplementation of one or a combination of female hormones. Depletion of male hormones by means of orchidectomy markedly protects from polyp development in ApcPirc/+ rats, which by supplementation with dihydrotestosterone (DHT) are reverted to levels found in control animals. Interestingly, no protection from ACF formation was observed upon orchidectomy and in contrast to ApcPirc/+ animals, supplementation with DHT exhibits protective effects on ACF numbers. We thus find that gender disparity in development of intestinal tumors is due to tumor promoting effects of male hormones. Androgens influence progression of established lesions, but do not influence the rate by which ACFs develop. This work highlights the importance of hormonal status in development of colorectal cancer.

Introduction

Colorectal cancer is one of most frequently occurring neoplasms and mostly develops in the absence of overt risk factors. This so called sporadic colorectal cancer (CRC) has intensively been studied a number of epidemiological studies have identified risk factors for development of CRC, of which mage gender constitutes a major one (1, 2). Large screening studies that employed colonoscopy in asymptomatic individuals have corroborated male sex as a risk factor in development of CRC and intestinal adenomas (3, 4). These studies showed that men are at higher risk in all age groups. Importantly, the increased risk in men is not limited to developing full-blown carcinomas, but was detected for tumor precursor lesions known as adenomatous polyps or adenomas (4, 5). The first clue as towards the etiology of this difference was the observation that nuns experience excess not only of known hormonal cancer (breast, ovary and endometrium), but also of colon cancer, suggesting a role for sex hormones (6). Additional evidence came from two large randomized controlled trials (RCT) in the Women's Health Initiative (WHI), examining effects of hormonal replacement therapy (HRT). A first study showed that treatment with equine estrogen plus medroxyprogesterone acetate (MPA) substantially reduced the overall risk of developing colorectal cancer compared to placebo (odds ratio = 0.63) after five years follow-up (7). No such protection was found in the second RCT in which women
who had previously undergone hysterectomy were treated with equine estrogen alone (odds ratio = 1.08) (8).

Though treatment with a combination of female hormones thus may be protective, the exact etiology of how sex hormones potentially influence gender differences in CRC and at what stage of the adenoma to carcinoma sequence these influences take place remains largely unknown.

The notion that gender disparity in CRC results from biological gender differences and not alternative factors such as behavioral, toxicologic or environmental differences is further supported by observations in a rat model for CRC (9). These rats have a germline truncating mutation in the Apc gene (Apc\Pirc\+), the first gene to be mutated in the majority of human CRC (10, 11). As a consequence of loss of the second Apc allele, Apc\Pirc\+ rats develop multiple tumors throughout the small intestine and colon. Similar to humans, male Apc mutant rats have increased tumor burden at all polyp stages and develop polyps at an earlier age (9). In contrast to Apc\Pirc\+ rats, extensively studied Apc\Min\+ mice that carry a similar truncated Apc allele do not exhibit strong gender disparity in development of intestinal tumors. Additionally, Apc\Min\+ mice develop polyps in the small intestine primarily and only rarely in the colon. Therefore Apc\Pirc\+ rats usfully model human disease.

In addition to this genetic model, male rats were shown to be more susceptible using a model of chemical induction of colorectal tumors with the carcinogen dimethylhydrazine (12). Thus, gender bias in CRC develops as the consequence of a biological difference, which is likely to be of reproductive and hormonal origin and is conserved in humans and rats. Furthermore, since gender differences are seen in rats at the adenoma stage, it is likely that the effects of sex differences take place at one of the earlier stages in which a carcinoma develops from an aberrant crypt (13-15).

We therefore set out to examine gender bias and the influence of sex hormones in two distinct experimental models of colonic tumorigenesis, looking both at fully developed adenomatous polyps and at aberrant crypt foci (ACFs), which are the earliest precursor lesions from which tumors develop.

**Methods**

**Animal experiments**

All experiments were performed according to the animal experimental committee guidelines from the University of Wisconsin, the University of Leiden or the University of Amsterdam. Wild type rats were obtained from Jacksons laboratories, Apc\Pirc\+ rats as described previously were bred in our own breeding facility (9).

For both azoxymethane experiments and experiments in Apc\Pirc\+ animals, five week old rats were subjected to ovariectomies, orchidectomies or sham operations. For female hormone replacement, slow release pellets with medroxyprogesterone acetate (MPA), estradiol or a combination of MPA
and estradiol were replaced and compared to vehicle pellets. For male hormone replacement, dihydrotestosterone (DHT) was placed and compared to vehicle (Innovative Research of America, Sarasota, FL, USA). All pellets were implanted subcutaneously in the nape of the neck. Pellets with female hormones were fabricated as 90 days slow release formulation and contained a total of 25 mg MPA (approximately 1 mg/kg/day) or 0.1 mg estradiol (approximately 4 μg/kg/day). Pellets with DHT were fabricated as 60 day slow release pellets that contained 50 mg DHT per pellet (approximately 3 mg/kg/day) for AOM experiments and as 90 day slow release pellets that contained 10 mg DHT per pellet (approximately 0.5 mg/kg/day). Vehicle pellets were of the same size and composition as pellets containing the designated steroid hormone, but contained no functional substance. For experiments in ApcPirc/+ rats, a new pellet was implanted after 90 days. ApcPirc/+ animals were housed for 7 months before animals were sacrificed. For azoxymethane (AOM) experiments, rats were left to acclimatize after surgery for one week prior to AOM injections. Subsequently, rats were injected twice with AOM (10 mg/kg; Sigma-Aldrich, Zwijndrecht, Netherlands) with 7 days between two injections. Six weeks after the first injection, animals were sacrificed and colons were removed and fixed for further analysis.

Tissue processing and counting
Tissue was fixed in 10% ice-cold formalin overnight. For ACF counting, fixed colons were stained with methylene blue (1% in PBS) for 20 minutes and washed in PBS. Counting was performed blinded for treatment under a dissection microscope.

Statistical analysis
All data in figures are presented as mean ± standard error of the mean. In the text, mean ± standard deviation was used. For animal experiments, Wilcoxon rank sum test was used comparing two groups and 1-way ANOVA tests were used comparing more than two groups. In subanalysis of localization or size, 2-way ANOVA tests were used. All ANOVA tests were followed by Bonferroni’s post test for multiple comparisons.

Results
To investigate distinct aspects of tumorigenesis, we used two independent models of colorectal tumorigenesis. In addition to the ApcPirc/+ rat, we made use of a second, frequently used model in which colorectal carcinogenesis is induced by the carcinogen azoxymethane (AOM) (16) (Figure 1A). Upon injections with this DNA alkylating agent, ACFs and later polyps develop. Where ApcPirc/+ rats are of value in the investigation of development and progression of mature polyps, analysis of ACFs in the AOM model informs about development of early tumor precursors, a process known as tumor initiation.
Female hormones do not influence intestinal tumorigenesis in two distinct models in rats. 

**Figure 1.** Female hormones do not influence intestinal tumorigenesis in two distinct models in rats. 

(A) Timeline of AOM experiment (above panel) and scheme showing surgical interventions and hormone suppletion. Upon injection of AOM in female rats, substitution of indicated female hormones do not alter AFC prevalence (B), ACF localization (C) or ACF size (multiplicity) (D). (E) Polyp numbers in female Apc<sup>−/−</sup> rats are not altered by substitution of indicated female hormones.
We first tested the effect of female hormones in our models. To this end we performed ovariectomies (OVX) with and without hormone replacement on the development of ACFs. As controls, animals were subjected to a sham operation, in which both ovaries were left in situ. During the operation, all animals received subcutaneous pellets containing either placebo, the progestin MPA, 17β-estradiol (E2) or a combination of both steroids, thereby mimicking the WHI studies. As expected, females that were subjected to OVX had a higher body weight than sham operated females, which normalized by addition of E2 (data not shown). We did not however find a difference in number of ACFs between OVX and sham operated females (114.0 ± 22.29 vs 126.4 ± 37.64, \( P = 0.4065 \)). In addition, no effect on ACF formation was observed in any of the groups that received hormone replacement (Figure 1B). We performed in depth analysis of localization of ACFs in the colon and size, which may serve as an early measure for ACF progression. Neither OVX nor hormone replacement influenced these characteristics (Figure 1C and D). We therefore concluded that female hormones do not protect against development of ACFs.

To assess whether female hormones may affect tumor development at a later stage of the adenoma to carcinoma sequence, we performed a similar experiment in female ApcPirc/+ rats. Rats were sacrificed at 210 days of age when a significant tumor load had developed. Throughout the experiment, we measured body weight which normally increases after OVX and decreases upon the administration of E2 to ensure proper administration of steroids (17). Females that underwent OVX and received either E2 (n=10) or the combination of E2 and MPA (n=10) had reduced body weight and were observed to be much leaner than animals treated with MPA alone (N = 10, \( P = 0.006 \)) or placebo (N = 8, \( P = 0.005 \)). Judging tumor numbers however, we did not find significantly differences among groups (Figure 1E). Additionally, analyzing average tumor size and localization, no differences were observed (data not shown). Thus considering our data on ACFs and polyps in rats, we concluded that female hormones do not influence intestinal tumorigenesis.

We next analyzed tumor number of male littermates of female ApcPirc/+ rats that were used in our experiments. Counting polyps, we did corroborate previous finding that male rats develop more tumors, confirming our rationale for using rats to examine gender disparity (male rats, N = 32, mean polyp number 39.4, \( P = 4.7e-14 \)) (Figure 2A). We therefore hypothesized that the observed gender disparity may be due to effects of male hormones rather than female hormones. In order to test this hypothesis we randomized by litter male ApcPirc/+ rats at weaning (30-35 days) to undergo either castration (orchidectomy, ORX, N = 24) or a sham operation (N = 34). At approximately 210 days of age, males that underwent ORX had a significantly lower colon tumor number compared to sham operated males (12.5 ± 6.9 vs. 22.6 ± 7.1 \( P = 3.8e-6 \)) (Figure 2B). Interestingly, the tumor load in males that underwent ORX had lowered to similar levels as in female animals, strongly suggesting that development of intestinal adenomas is critically influenced by male gonadal hormones and that gender differences are primarily caused by distinct levels of male hormones. A number of hormonal substances are made in
Figure 2. Increased tumor development in male rats depends on testicular hormones. (A) Littermate male Apc<sup>min/+</sup> rats have increased polyp numbers. For female rats, sham operated placebo treated females from Figure 1E were used. (B) Tumor numbers in male Apc<sup>min/+</sup> rats are decreased upon orchidectomy (ORX) compared to sham operated males. Data is depicted as mean ± s.e.m. **** = P < 0.0001.

Figure 3. Tumor development, but not development of precursor lesions is promoted by dihydrotestosterone. (A) Treatment of orchidectomized male Apc<sup>min/+</sup> rats with dihydrotestosterone (DHT) causes polyp numbers to increase to levels found in non orchidectomized males. (B) Orchidectomies have no influence on ACF formation in AOM injected male rats compared to sham operations. DHT treatment causes reduction of ACF numbers. Data is depicted as mean ± s.e.m. *** = P < 0.001; ** = P < 0.01.
male gonads of which the androgen testosterone is the principal one. We therefore evaluated whether the reduction of polyps observed upon ORX is the direct effect of declined levels of serum testosterone in these animals. We performed ORX on male Apccirc/ circ and supplemented half of these animals with dihydrotestosterone (DHT), comparing them with the other half that was treated with placebo. Upon treatment with DHT, polyp numbers increased to levels that were similar to polyp numbers in rats that had functionally normal endogenous gonadal hormone production (DHT: N = 10, 22.7 ± 6.5; sham: N = 34, 22.6 ± 7.1 P= 0.96) and were significantly increased compared to ORX males treated with placebo (placebo: N = 24, 12.5 ± 6.9 P = 0.0009) (Figure 3A). To analyze whether modulating effects of androgens occur early or late in the formation of tumors and to assess effects of DHT supplementation, we again performed ORX and injected rats with AOM one week thereafter. We compared sham operated animals, receiving placebo to ORX animals, of which half received placebo and half received DHT. As expected, males that underwent ORX had lower levels of serum testosterone compared to males that underwent sham operations (sham: 6.147 ± 4.394 vmol/l vs. ORX: 0.016 ± 0.2324 vmol/l P < 0.0001), and supplementation with DHT caused testosterone levels to increased accordingly (placebo: 0.016 ± 0.2324 vmol/l vs. DHT: 1.461 ± 1.305 vmol/l P < 0.0001). Interestingly, we found no difference in ACF numbers in rats that had undergone ORX (105.7 ± 31.84 vs. 106.0 ± 41.67 P = 0.8647). Additionally, we observed that in contrast to tumor promoting functions of DHT on polyps in Apccirc/ circ rats, animals that had received DHT exhibited marked decrease of ACF numbers after injection with AOM (106.0 ± 41.67 vs. 67.93 ± 18.32 P < 0.01) (Figure 3B). Together, these date show a tumor promoting role for male hormones in polyps, which can be attributed to DHT. During ACF formation however, male hormones do not exhibit tumorigenic effects and in contrast to our polyp data, DHT exerts a protective role.

**Conclusion**

Colorectal cancer develops more frequently in men than women, a gender bias that depends on hormonal factors. We investigated influences of sex hormones on development of intestinal tumorigenesis in rats, which best mimic gender differences in human disease. Our studies reveal that while female sex hormones exhibit no influence on polyp development, androgens promote intestinal tumorigenesis. To examine differential influences on stage of the intestinal neoplasm, we used two distinct models. We induced early precursor lesions known as aberrant crypt foci (ACFs) with injection of AOM, to assess the frequency by which these earliest lesions occur, a process know as tumor initiation. Additionally, we analyzed mature polyps in Apccirc/ circ rats, which harbor a germline mutation in the Apc gene.

Investigating female rats, we found that ovariectomies (OVX) did not alter the number of ACFs, nor did female hormone replacement. Similarly, in Apccirc/ circ rats, neither OVX nor hormone replacement caused changes in tumor burden. In male rats we find that although orchidectomies (ORX) had no effect on ACF formation in the AOM model, abrogation of male hormone
production by ORX on Apc<sup>Pirc/+</sup> rats resulted in reduction of intestinal tumors. Substitution of androgens with dehydrotestosterone (DHT) in Apc<sup>Pirc/+</sup> males that underwent ORX reverted the tumor number back to levels found in sham operated littermates. Interestingly, rats that were injected with AOM and substituted with DHT exhibited reduced numbers of ACFs. Based on our experiments, we thus reason that gender differences in development of colorectal tumorigenesis are based on tumor promoting effects of male hormones and not on effects of female hormones that protect from tumorigenesis. Since these effects were only observed on tumor numbers of Apc<sup>Pirc/+</sup> rats, not on ACF numbers in AOM-treated animals, we conclude that male hormones promote growth of established precursor lesions, while not affecting the frequency by which these precursors develop. Thus male hormones promote tumor progression, not initiation.

In the field of hormonal influences on CRC, the WHI study is among the strongest epidemiological data, showing tumor reduction upon treatment with a combination of estradiol and MPA, suggesting a role for female hormones (7). In two distinct models, using ovariectomies and female hormone replacement in rats, we find no difference in ACF- or polyp numbers. A number of experimental variables may have resulted in the fact that our experiments do not corroborate the WHI, such as duration of treatment (180 days vs. 5.2 years) or the fact that we have not utilized postmenopausal rats but rather ovariectomized animals. Moreover, hormones may affect tumor aspects such as inflammation, which differ between animal models and humans, judging the number of infiltration immune cells in the lamina propria. Last, although tumor numbers were reduced in the WHI-study, mortality between women receiving hormonal replacement therapy and placebo was similar, suggesting that female hormones protect against a subset of cancers and not all (7). Potentially, this subset is not represented in our animal experiments. Our data in male Apc<sup>Pirc/+</sup> rats clearly show that male hormones exhibit tumor promoting properties, which can be attributed to dihydrotestosterone. Presumably, androgens exert tumor promoting properties through the androgen receptor (Ar), which is underlined by the observation that this protein is increased in experimentally induced colonic polyps (18). An increased risk for CRC development was found in a cohort of men who had received androgen suppression therapy for treatment of prostate cancer with either GnRH or castration (19). However, these studies had many confounders such as age and stage of prostate cancer.

Our studies identify distinct effects of androgens in Apc<sup>Pirc/+</sup> rats on the one hand, and on ACF formation in AOM injected animals on the other of which the latter corroborates results of Osawa et al. that performed the AOM-ACF model in androgen treated mice (20). Observed divergent effects of androgens may be caused by distinct roles during tumor initiation versus growth. However, in Apc<sup>Pirc/+</sup> rats, male predisposition was not only found for development of larger polyps, but also of microadenomas (9). These lesions may be regarded as earliest visible precursors, but are different from sporadic ACFs in humans or AOM induced ACFs in rats, since these harbor mutations in the oncogene RAS (11, 21). As both human and Apc<sup>Pirc/+</sup> polyps have mutations in tumor suppressor APC mostly (10, 11), distinct effector roles of androgens in these genetically different lesions may be reflected in our results.
Our studies thus identify a tumor promoting effect for male hormones at the level of tumor growth. Further delineating the role of androgens will be important to be able to better understand and therefore treat the human disease.

Reference List