CHAPTER 4

Colorectal tumor prevention by progestins is critically dependent on postmenopausal status

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ABSTRACT
Large placebo controlled trials have shown that medroxyprogesterone acetate (MPA) protects from colorectal cancer in postmenopausal women. Further in-depth animal research has thus far not been able to recapitulate these results. Here we show in a VCD-induced mouse model of menopause that the protective effect of MPA is strictly dependent on postmenopausal hormone status, and future in vivo research should be investigated using an appropriate postmenopausal mouse model.
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MAIN TEXT

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the third leading cause of cancer related death. A protective role of female hormones against the development of CRC is suggested by data from the Women’s Health Initiative (WHI). Two very large randomized controlled trials examined the effects of hormonal replacement therapy on postmenopausal (PMP) women over a five year interval. The first study showed that combined treatment with both equine estrogen (E2) and medroxyprogesterone acetate (MPA) substantially reduced the risk of colorectal cancer compared to placebo with 37%. However, protection was not found in a second randomized controlled trial among women that were treated with equine estrogen alone. Thus large clinical studies suggest that MPA is protective against CRC.

Since the reports of the WHI, the effect of female hormones on CRC was further investigated in a number of animal models of colonic neoplasia. These animal models permit an experimental approach to examine the molecular basis of CRC protection in the absence of environmental, behavioral and toxicological confounders. None of these reports however, has thus far been able to recapitulate the protective effect of female hormones, making these studies difficult to interpret and translate to human setting. In all of these studies, the postmenopausal hormone status was mimicked by performing ovariectomies which leads to attenuated production of the female hormones estrogen and progesterone, but also the male hormone testosterone. Although human ovaries in postmenopausal women do not produce female sex hormones, they are still capable of producing other substances that have oncogenic effects, such as androgens.

We therefore set out to investigate the effect of medroxy progesterone acetate (MPA), a synthetic variant of progesterone, on CRC in a specific mouse model of menopause. In this model, menopause is induced with repetitive i.p. injections with 4-vinylcyclohexene diepoxide (VCD) leading to depletion of ovarian follicles, attenuation of female hormone production but leaving male hormone production intact similar to human menopause. After menopause induction we chemically induced colonic adenomas with the carcinogen azoxymethane (AOM) and started hormonal replacement therapy with subcutaneous MPA slow release pellets (Figure 1, upper panel). Effective induction of menopause and hormone suppletion was confirmed by the absence of ovarial follicles in the VCD induced postmenopausal mice (Figure 1, lower panel).

In fertile mice MPA did not reduce adenoma number or the total tumor load (Figure 2a, b), confirming previous research reporting lack of effect of MPA on adenomagenesis in ovariectomized rodents. Surprisingly, induction of menopause resulted in a significant increase of adenoma number (2.6 vs 1.3 P<0.05) while MPA treatment fully protected from the oncogenic effects of menopause by reducing the adenoma number (0.9 vs 2.6, P<0.001, Figure 2) to comparable levels as those in fertile mice.

To examine how MPA affects mucosal homeostasis, we assessed numbers of proliferating cells in the normal appearing mucosa at the moment of sacrifice (Figure 3). We did not observe differences in
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Figure 1. Experimental design and menopause induction. Upper panel: Experimental design. Lower panel: H&E of representative ovary of a fertile mouse (left) showing presence of follicles in all stages. Top (40X magnification) primary follicle with single layer of cuboidal cells. Bottom (20X magnification) secondary follicle with multiple layers of granulosa cells. On the right a representative ovary of a VCD treated postmenopausal mouse depleted of follicles.

Epithelial proliferation as assessed by BrdU incorporation, suggesting that the effects of menopause and MPA are specific for tumor initiation and not general epithelial proliferation. To the best of our knowledge, the VCD animal model for menopause is the first to mimic human chemoprevention by MPA and these data clearly demonstrate that MPA protects from colonic adenomagenesis, but only after the menopause.

The increase in adenoma formation observed after the menopause suggests that ovary-derived factors, produced in response to VCD treatment, enhance adenoma formation. One of the factors that are produced by postmenopausal ovaria is the androgen androstenedione. Strikingly, we have recently demonstrated that androgens promote colonic adenomagenesis and therefore measured the levels of androstenedione in our animal groups. Indeed, VCD treated mice had significantly higher levels of serum androstenedione compared to fertile mice (0.024 vs 0.163 nmol/L, p<0.0001, Figure 4). MPA treatment did not affect androgen levels. This can be explained by MPA’s capacity to disrupt androgen signaling by competitive binding to the androgen receptor implicating that the inhibitory
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Figure 2. MPA protects from adenoma formation in postmenopausal mice. Upper panel: Total adenoma number in fertile mice receiving placebo pellets (N=10), fertile mice receiving MPA pellets (n=10), VCD treated postmenopausal mice receiving placebo pellets (n=9) and VCD treated postmenopausal mice receiving MPA pellets (n=8). Lower panel: Total tumor load consisting of tumor sizes added up per mouse. 1-way analysis of variance (ANOVA) test was used, followed by Bonferroni post-test for multiple comparisons. Data are mean ± SEM. *P < 0.05; **P < 0.01.

Figure 3. Epithelial proliferation is not influenced by menopause induction and MP treatment. Number of BrdU positive cells per crypt for all treatment groups. Total tumor load consisting of tumor sizes added up per mouse. 1-way analysis of variance (ANOVA) test was used, where p<0.05 was regarded as significant.
effect of MPA on androgen signaling is downstream of serum levels. In fact, androstenedione levels were slightly higher in MPA treated postmenopausal mice compared to placebo (0.186 vs 0.146, not significant), potentially as a result of negative feedback regulation. If the chemoprotective effect of MPA is truly due to a decrease in androgen signaling has yet to be investigated.

In conclusion, large clinical studies suggest that MPA functions as a chemopreventive agent in the development of CRC. Here we show that this protective effect is critically dependent on postmenopausal hormone status and that the results of the WHI may not be applicable to fertile women at increased risk of developing CRC. The lack of effect of MPA in previous animal studies can be explained by the animal models that were used. We propose that the VCD-induced mouse model of menopause is an appropriate \textit{in vivo} model for investigating the hormonal effects on postmenopausal CRC development. This may also hold true for other tumor models that are influenced by hormones such as breast, ovarian, endometrial cancer and other hormonally regulated diseases.

\section*{METHODS}

\section*{Animal experiments}

The protocol of this study was approved by the animal ethics committee of the University of Amsterdam (permit number ALC102969). C57B6/J\textit{Ol}a\textit{Hsd} (Harlan Laboratories) at six weeks of age were injected intraperitoneally in 21 subsequent days with either 4-vinylcyclohexene diepoxide (VCD, 160 mg/kg in corn oil) or vehicle only according to Hoyer et al\textsuperscript{8}. Three months after the first VCD injection colonic adenomas were chemically induced by 6 weekly injection of azoxymethane 10mg/kg in NaCl. Simultaneous with the first AOM injection, hormone replacement therapy (HRT) was started in fertile and PMP mice by placement of subcutaneous slow release pellets containing either MPA or Vehicle (7.5 mg in 90 days, Innovative Research of America). Additional pellets were placed 12 and 24 weeks after placement of the first pellet. All animals were sacrificed 25 weeks after the

Figure Legend 4. Menopause induction increases circulating levels of serum androstenedione.

Serum levels of circulating androstenedione for all treatment groups. 1-way analysis of variance (ANOVA) test was used, followed by Bonferroni post-test for multiple comparisons. Data are mean ± SEM. *P < 0.05; **P < 0.01
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first AOM injection. One hour prior to sacrifice, all animals were injected with 200 μl BrdU (10 mg/ml in PBS; Sigma-Aldrich).

**Adenoma counting**
After overnight fixation of the colons in 4% paraformaldehyde in PBS, tissue was rinsed with PBS twice and immersed in 70% ethanol overnight. Subsequently, adenomas were counted while the researcher was blinded for the treatment. Adenoma size was estimated using a ruler guide.

**Tissue processing and immunohistochemistry**
Tissue was fixed in 4% ice-cold formalin and embedded in paraffin. Sections of 4 μm were deparaffinised in xylene and rehydrated. For immunohistochemistry, endogenous peroxidase was blocked using 0.3% H2O2 in methanol. The sections were cooked in 0.01 M citrate buffer pH 6.0 for 20 min and incubated with the primary antibody in PBS with 1% bovine serum albumin and 0.1% Triton X-100. Antibody binding was visualised with Powervision horseradish peroxidase-labelled secondary antibodies, and diaminobenzidine for substrate development. All sections were counterstained with Mayer’s haematoxylin. The following antibodies were used: mouse monoclonal anti-BrdU (clone BMC9318, Roche).

**Androstenedione measurements**
Plasma testosterone levels were measured for intact male mice and castrated mice that received testosterone enanthate or placebo. One week after the twelfth injection with testosterone enanthate all mice were sacrificed and blood was drawn by cardial puncture. Blood samples were separated by centrifugation at 3000 rotation per minutes for 5 minutes and stored at -20°C until assayed. Plasma testosterone levels were measured by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and are depicted as nmol/L.

**Statistical analysis**
All data are presented as mean ± standard error of the mean. For animal experiments a 1-way analysis of variance (ANOVA) test was used, followed by Bonferroni’s post-test for multiple comparisons, where p<0.05 was regarded as significant.
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REFERENCES


