Effect of retinoids on AOM-induced colon cancer in rats: modulation of cell proliferation, apoptosis and aberrant crypt foci

Ye Zheng, Paula M. Kramer, Ronald A. Lubet¹, Vernon E. Steele⁴, Gary J. Kelloff¹ and Michael A. Pereira²

Department of Pathology, Medical College of Ohio, 3055 Arlington Avenue, Toledo, OH 43614 and ¹Chemoprevention Branch, Division of Cancer Chemoprevention and Control, National Cancer Institute, Bethesda, MD 20892, USA

We have previously reported that the retinoids, 4-(hydroxyphenyl)retinamide (4-HPR) and 9-cis-retinoic acid (RA) prevented azoxymethane (AOM)-induced colon tumors and along with 2-(carboxyphenyl)retinamide (2-CPR) prevented aberrant crypt foci (ACF). In this study, we evaluated the effect of 2-CPR on AOM-induced colon tumors and the effect of the three retinoids on apoptosis and cell proliferation. Male F344 rats were administered 15 mg/kg AOM at weeks 7 and 8 of age. 2-CPR (315 mg/kg) was administered in the diet starting either 1 week before or at week 12 after the first dose of AOM. The rats continued to receive the 2-CPR until killed at week 46. Unlike the demonstrated prevention of colon cancer by the other two retinoids, both dosing schedules of 2-CPR resulted in an approximate doubling of the yield of colon tumors. In adenomas, 2-CPR, 4-HPR and 9-cis-RA were equally effective in reducing mitotic activity, while only 4-HPR and 9-cis-RA but not 2-CPR enhanced apoptosis. When administered for only the 6 days prior to killing 4-HPR but not 2-CPR decreased the Mitotic Index and increased the Apoptotic Index in adenomas. In non-involved crypts, chronic exposure to 4-HPR and 9-cis-RA in contrast to 2-CPR reduced the Mitotic Index and enhanced the Apoptotic Index. In concurrence with our previous study, both 2-CPR and 4-HPR were very potent in preventing ACF when administered in the diet starting 1 week before the first dose of AOM and continuing for the 5 weeks of the study. Hence, unlike the other two retinoids, 2-CPR, although very potent in preventing ACF, enhanced rather than prevented AOM-induced colon cancer. Furthermore, our results suggest that the effect of 2-CPR on tumor yield is different from 4-HPR and 9-cis-RA because, unlike them, it does not enhance apoptosis.

Introduction

Colon cancer is a major cause of cancer mortality and morbidity both in the USA and worldwide (1,2). Retinoids are candidates for use as cancer chemopreventive agents including the control of this disease (3–9). Previous studies in animals with retinoids have indicated prevention of cancer of colon, liver, mammary gland, prostate and skin (10–14). These studies have also demonstrated the need to develop other retinoids with greater efficacy and less toxicity. In an attempt to increase the margin of safety, synthetic vitamin A derivatives including 4-(hydroxyphenyl)retinamide (4-HPR) and 2-(carboxyphenyl)-retinamide (2-CPR) are being developed.

Aberrant crypt foci (ACF) are hyperproliferative lesions found in the colon of humans and carcinogen-treated laboratory animals with characteristics in common with colon tumors (15–18). Thus, they have been proposed as precancerous lesions (17). Furthermore, prevention of ACF in rats has been used to screen agents for chemopreventive activity in the colon (19–23). 4-HPR, 13-cis-retinoic acid (RA) and all-trans-RA have been reported to reduce the yield of ACF induced by azoxymethane (AOM) in rats (20–23). We evaluated 13 retinoids for the ability to prevent AOM-induced ACF in rats and found 2-CPR, 4-HPR and 9-cis-RA to be very potent (23). We then demonstrated that 4-HPR and 9-cis-RA also prevented AOM-induced colon cancer (23). Since 2-CPR was more potent than either of these two retinoids in preventing ACF, we evaluated in the present study whether it would also prevent AOM-induced colon cancer.

In the colon, enhancement of cell proliferation, expansion of the cell proliferation zone and inhibition of apoptosis are considered risk factors for tumor development (24–29). Thus, reduction in cell proliferation and enhanced apoptosis are two proposed mechanisms for the chemopreventive activity of retinoids (6,26,30,31). We have demonstrated a good correlation between the ability of retinoids including 2-CPR, 4-HPR and 9-cis-RA to prevent AOM-induced ACF and to reduce cell proliferation in foci and non-involved crypts (23). Therefore, along with determining whether 2-CPR would prevent AOM-induced colon cancer in rats, we also determined the ability of 2-CPR, 4-HPR and 9-cis-RA to reduce mitosis and to enhance apoptosis in adenomas and non-involved crypts.

Material and methods

Chemicals

2-CPR, 4-HPR and 9-cis-RA were obtained from the National Cancer Institute, DCPC Repository (c/o McKesson BioServices, Rockville, MD). AOM was purchased from Sigma (St Louis, MO) and AIN-76A diet from Dyets (Bethlehem, PA).

Animals

Male F344 rats were obtained at 6 weeks of age from Charles River Laboratories (Frederick, MD). They were housed in our AAALAC accredited laboratory animal facility in accordance with the US DHHS Guide for the ‘Care and Use of Laboratory Animals’. Solid-bottomed polycarbonate cages with stainless steel wire-bar lids and Bed-o-Cob bedding (Andersons, Toledo, OH) were used to house 2 rats/cage. The light cycle consisted of 12 h each of yellow light and dark. The animal rooms were maintained at 64–76°F and 55 ± 15% relative humidity. Drinking water and AIN 76A diet were supplied to the animals ad libitum.

Experimental design 1: effect of 2-CPR on the yield of colon tumors

At 8 weeks of age, the rats were randomly assigned to three treatment groups so that there was no significant difference in the starting body weights among the groups. Group 1 contained 72 animals and groups 2 and 3 contained 36
rats each. The rats in group 2 then started to receive 2-CPR (315 mg/kg) in the AIN-76A diet, while those in groups 1 and 3 continued to receive AIN-76A diet. One week later, all the rats were administrated the first of two weekly i.p. injections each of 15 mg/kg body wt AOM in saline. At week 12 after the first dose of AOM, the rats in group 3 started to receive 2-CPR (315 mg/kg diet). Six days prior to being killed, 18 rats in group 1 started to receive 2-CPR (315 mg/kg diet) until killed and another 18 rats received 4-HPR (782 mg/kg diet). Body weight was monitored weekly for the first 4 weeks and monthly thereafter throughout the study. All the animals were killed by carbon dioxide asphyxiation at 46 weeks after the first dose of AOM. At necropsy the animals were weighed and the colons removed, flushed with 0.9% saline, slit open longitudinally and tumors harvested. Peripheral pieces of tumors were rapidly frozen in liquid nitrogen and stored at –70°C for future analysis. The remainder of the tumor was fixed in 10% buffered formalin overnight and then stored in 70% alcohol until embedded in paraffin. Paraffin sections (5 µm) were stained with hematoxylin and eosin for histopathologic evaluation.

**Experimental design 2: prevention of ACF by 2-CPR and 4-HPR**

At 8 weeks of age, 8 male F344 rats were randomly assigned to each of three treatment groups, so that there was no significant difference in the body weights among the groups. Then group 1 started to receive 2-CPR (315 mg/kg diet); group 2 received 4-HPR (782 mg/kg diet) and group 3 continued to receive the AIN-76A diet. Both at 7 and 14 days later, all the rats received i.p. injections each of 24 mg/kg body wt AOM in saline. Body weight was monitored weekly throughout the experiment. The animals continued to receive the retinoids in their diet for a total of 5 weeks, at which time they were killed by carbon dioxide asphyxiation.

At necropsy the animals were weighed and the colons removed from cecum to anus, flushed with saline and slit open longitudinally. The colons were flattened between filter paper and a plastic screen, fixed in 70% alcohol and stored at 4°C. Whole mounts of colons were stained with 2% methylene blue in alcohol and evaluated under a microscope for ACF as described by Bird et al. (15,17) and modified by us (23). The number of ACF, their location in the colon and the number of crypts/focus were recorded. After evaluation for ACF, 3–4 mm transverse sections of colon were obtained 9.0–11.0 cm from the anus and embedded in paraffin blocks.

**Source of colon tumors and tissue from animals exposed to 4-HPR and 9-cis-RA**

Colon adenomas and tissue were obtained from our previously published study that demonstrated prevention by 4-HPR and 9-cis-RA of AOM-induced tumors and ACF (23). Briefly, male F344 rats at 8 weeks of age started to receive in AIN-76A diet either 4-HPR (782 mg/kg diet) or 9-cis-RA at 30 mg/kg diet for 3 weeks followed by 60 mg/kg until killed. Seven and 14 days after the start of administering the retinoids, each rat received 15 mg/kg body wt AOM by i.p. injection. After administering the retinoids for 36 weeks, the animals were killed by carbon dioxide asphyxiation.

**Evaluation of apoptotic and mitotic cells.**

Paraffin-embedded sections (5 µm) were stained with hematoxylin and eosin and evaluated under a light microscope for apoptotic and mitotic cells. Apoptotic cells were identified by cell shrinkage, homogeneous basophilic and condensed nuclei, nuclear fragments (apoptotic bodies), marked eosinophilic condensation of cytoplasm and sharply delineated cell borders surrounded with a clear halo as described by Wyllie et al. (32). Figure 1 presents examples of apoptotic cells in adenomas and crypts. In adenomas, at least 1000 epithelial cells were randomly evaluated for apoptotic and mitotic cells. The Apoptotic Index (AI) and Mitotic Index (MI) in crypts were determined in longitudinal sections that allowed evaluation of the whole crypt from top to base. Fifteen randomly chosen crypts/colon were evaluated for apoptosis and mitosis. The AI and MI were determined by dividing the total number of apoptotic or mitotic cells, respectively, by the number of epithelial cells evaluated×100.

**Determination of proliferating cell nuclear antigen-Labeling Index (PCNA-LI)**

Sections of colon adenomas were stained with mouse monoclonal anti-PCNA antibody to PCNA (Sigma) as previously described (23). Briefly, the paraffin was removed by xylene and endogenous peroxidase quenched by 3% H2O2 for 30 min. The sections were blocked by horse serum (Vector Laboratories, Burlingame, CA) and 1:300 diluted monoclonal mouse anti-PCNA antigen were applied for 1 h at room temperature. The sections were then washed and incubated with biotinylated horse anti-mouse IgG and Vectastain ABC reagent followed by 3,3′-diaminobenzidine tetrachloride for 15 min. The sections were counterstained with hematoxylin. At least 1000 epithelial cells/--------------------

**Table I. Effect of 2-CPR on AOM-induced tumors in rat colon**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatmenta</th>
<th>No. of animals</th>
<th>Body weightb,c (g)</th>
<th>Colon tumorsd,e</th>
<th>Adenomas</th>
<th>Adenocarcinomas</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM</td>
<td>63</td>
<td>429 ± 7.49d</td>
<td>0.76 ± 0.14 (46.0)</td>
<td>0.37 ± 0.08 (31.7)</td>
<td>1.13 ± 0.15 (65.1)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AOM + 2-CPR (before)</td>
<td>34</td>
<td>430 ± 6.26</td>
<td>1.09 ± 0.26 (56.3)</td>
<td>0.97 ± 0.19 (56.3)*</td>
<td>2.06 ± 0.30 (79.4)*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AOM + 2-CPR (after)</td>
<td>30</td>
<td>433 ± 6.08</td>
<td>1.60 ± 0.39 (63.3)*</td>
<td>0.67 ± 0.12 (56.7)</td>
<td>2.27 ± 0.41 (76.7)*</td>
<td></td>
</tr>
</tbody>
</table>

*a2-CPR (315 mg/kg) was administered in the diet starting either 1 week before or 12 weeks after the first dose of AOM and for a total of 46 weeks.

*bBody weight of rats at killing.

*cMeans ± SE.

*dNo tumors/animal ± SE (% animals with tumors).

*eSignificantly different from treatment group 1 by ANOVA followed by the Tukey test, P < 0.05.
tumor were evaluated for PCNA-positive cells. The PCNA-LI was determined by dividing the number of PCNA-stained cells by the total number of cells evaluated×100.

**Extraction and gel electrophoretic analysis for DNA fragmentation**

DNA from colon adenomas that have been stored at −70°C was isolated as described by Miller et al. (33) with some modification. Briefly, tumor tissue was minced and digested overnight at 37°C with proteinase K (Sigma) in 10 mM Tris–HCl, 400 mM NaCl, 2 mM disodium EDTA and 10% sodium dodecyl sulfate (pH 8.2), followed by salt extraction and ethanol precipitation. A total of 10 µg DNA/lane was loaded on 2% agarose gel followed by electrophoresis. The oligonucleosomal DNA fragments were visualized by staining with ethidium bromide.

**Statistical analyses**

The data were analyzed by a one-way ANOVA followed by comparison of pairs using the Bonferroni correction or the Tukey test with \( P < 0.05 \). SigmaStat v.2.0 software was used (Jandel, San Rafael, CA).

**Results**

**Effect of 2-CPR on AOM-induced colon tumors and ACF**

The effect of 2-CPR (315 mg/kg diet) when administered starting either 1 week before or 12 weeks after the first dose of AOM on the yield of colon tumors is presented in Table I. The body weight of the animals at killing is also presented in this table. During the duration of the study, 2-CPR did not affect the body weight of the rats. Both treatment schedules of 2-CPR significantly increased the yield of total tumors, i.e. almost doubling the sum of adenomas + adenocarcinomas. When administered starting before AOM (total duration of exposure of 47 weeks) the yield of adenocarcinomas was significantly increased, while when administered after AOM (total duration of exposure of 34 weeks) the yield of adenomas but not adenocarcinomas was significantly increased. Since, the yield of adenomas + adenocarcinomas was not different for the two dosing schedules, it would appear that the longer duration of exposure to 2-CPR enhanced the progression of adenomas to adenocarcinomas.

The increased yield of colon tumors in animals exposed to 2-CPR was unexpected since we had previously demonstrated that it prevented AOM-induced ACF (23). Therefore, we repeated the evaluation of the effect of 2-CPR on ACF including 4-HPR as a positive control since it too had previously prevented ACF (23). The effect of 2-CPR (315 mg/kg diet) and 4-HPR (782 mg/kg diet) on the yield of AOM-induced ACF is presented in Figure 2. Consistent with our previous results both retinoids were very potent, reducing the yield of ACF by 50.4 and 54.3% in response to 2-CPR and 4-HPR, respectively.

**Modulation by retinoids of cell proliferation in colon adenomas and crypts**

The modulation of the MI in colon adenomas and crypts by 2-CPR (315 mg/kg diet), 4-HPR (782 mg/kg diet) and 9-cis-RA (60 mg/kg diet) was determined as a measure of their effect on cell proliferation. In adenomas, chronic exposure to the three retinoids significantly decreased the MI by 55–63% (Figure 3A). Exposure of a short duration, i.e. for only 6 days...
Modulation by retinoids of apoptosis in adenomas and crypts

The modulation by 2-CPR, 4-HPR and 9-cis-RA of the AI in adenomas and crypts is presented in Figure 4. Chronic exposure to 4-HPR and 9-cis-RA but not 2-CPR, significantly enhanced the AI in adenomas and crypts producing a more than doubling in the Index. Exposure to 4-HPR but not 2-CPR for 6 days prior to killing also enhanced also enhanced by ~2-fold the AI in adenomas and crypts. The enhancement of apoptosis in adenomas was confirmed by the presence of the DNA fragmentation ladder, a marker for apoptosis (38,39) (Figure 5). A fragmentation ladder was presence in DNA isolated from adenomas of animals exposed to 4-HPR and 9-cis-RA, but not from adenomas of animals exposed to 2-CPR or the control diet. Thus confirming the enhanced level of apoptosis in animals exposed to 4-HPR and 9-cis-RA but not to 2-CPR.

Discussion

We had previously demonstrated in rats that retinoids including 2-CPR, 4-HPR and 9-cis-RA effectively prevented AOM-induced ACF and that 4-HPR and 9-cis-RA also prevented colon cancer (23). Of 13 retinoids previously evaluated for prevention of ACF, 2-CPR was the most efficacious. We therefore evaluated 2-CPR for the ability to prevent AOM-induced colon cancer. Unexpectedly instead of preventing colon cancer, 2-CPR increased the yield. It was equally effective in enhancing the tumor response when administered starting either 1 week before or 12 weeks after AOM. The efficacy of 2-CPR when administered after AOM demonstrated that the enhancement of tumor yield was not the result of altered metabolism or DNA binding of AOM but rather the result of activity during the promotion/progression phase of carcinogenesis. The correlation of the duration of exposure with the enhancement of the yield of adenomas and adenocarcinomas further supports the tumor promoting activity for 2-CPR. The shorter exposure of 34 weeks duration (starting 12 weeks after AOM) resulted in an increase in adenomas while longer exposure of 47 weeks (starting 1 week before AOM) increased the yield of adenocarcinomas without any further increase in the total yield of tumors. This would suggest promotion of adenomas to adenocarcinomas by the longer duration of exposure.
Increased cell proliferation and/or inhibition of apoptosis are proposed mechanisms for enhancement of carcinogenesis during the promotion/progression phase (32–37). Hence, proposed mechanisms for chemoprevention have included inhibition of the promotion/progression phase by reduction in cell proliferation and/or enhancement of apoptosis. The two retinoids, 4-HPR and 9-cis-RA that prevented AOM-induced colon cancer reduced cell proliferation (MI and PCNA-Labeling Indices) in both normal crypts and adenomas. Chronic administration of 2-CPR also reduced cell proliferation in adenomas. Furthermore, we have previously determined in ACF and non-involved crypts that 5 weeks of exposure to all three retinoids reduced the PCNA-LI and the size of the PCNA proliferative compartment (23). On the other hand, only the two retinoids 4-HPR and 9-cis-RA that prevented colon cancer enhanced apoptosis, while 2-CPR did not affect apoptosis in either adenomas or crypts. 9-cis-RA binds both RARs and RXRs, while 4-HPR and probably 2-CPR appear not to bind these receptors (40,41). Thus, it was suggested that 4-HPR induced apoptosis by an independent pathway (41). We would propose that the inability of 2-CPR to induce apoptosis resulted from its lack of binding to the retinoid receptors and to its inability to activate the 4-HPR pathway.

Three other chemopreventive agents, curcumin, phenylethyl-3-methylcaffeate and sulindac have been shown by Samaha et al. (42) to enhance apoptosis in AOM-induced colon tumors. The enhancement of apoptosis in tumors by these three agents and by 4-HPR and 9-cis-RA, would indicate sensitivity to them. The sensitivity of tumors was demonstrated by the enhancement of apoptosis after exposure to 4-HPR for only the 6 days prior to killing. Furthermore, for this limited number of chemopreventive agents enhancement of apoptosis would appear to be a predictor of efficacy in preventing colon cancer. Alternately, tumor promoters such as 2-CPR are expected to decrease apoptosis and enhance cell proliferation. However, 2-CPR did not affect apoptosis and reduced rather than enhanced cell proliferation. Samaha et al. (42) have reported that the colon tumor promoter, 6-phenylethyl isothiocyante (6-PHITC) also did not affect apoptosis in colon tumors. They suggested that 6-PHITC might render cells resistant to apoptosis. However, since apoptosis in colon tumors was not significantly affected by either 2-CPR or 6-PHITC, the mechanism by which they increased the yield of tumors is still very speculative.

The prevention of AOM-induced ACf has been proposed as a bioassay to screen candidates for chemopreventive activity (19–23). However, 2-CPR which in this study and in our previously published study (23) was very potent in preventing ACF, enhanced rather than reduced the AOM-induced tumor response. Thus, 2-CPR is an example of an agent that, although it prevents ACF, does not prevent colon tumors. 2-CPR was distinguished from the other two retinoids that prevented both AOM-induced ACF and colon cancer by its inability to enhance apoptosis in either crypts or adenomas. Thus, we propose that the ability to enhance apoptosis could be used to distinguish agents that prevent both ACF and colon cancer from those that only prevent ACF. However, the application of enhancement of apoptosis to distinguish agents that prevent cancer requires further validation with additional retinoids as well as other classes of agents.

Acknowledgement
This work was supported in part by US National Cancer Institute contracts NO1-CN-55151 and NO1-CN-55175.

Effect of retinoids on AOM-induced colon cancer in rats

References
Y.Zheng et al.


Received April 21, 1998; revised September 22, 1998; accepted October 9, 1998