Dietary retinoic acid supplementation stimulates intestinal tumour formation and growth in multiple intestinal neoplasia (Min)/+ mice

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Chemopreventive activity by retinoic acid (RA) has been demonstrated previously in rat colon. The spontaneous tumourigenesis in the Min/+ mouse, which harbours a germline mutation in the tumour suppressor gene adenomatous polyposis coli (Apc), is characterized by inactivation of Apc, nuclear accumulation of β-catenin and the enhanced expression of specific genes activated by T cell factor (TCF)/β-catenin signalling. Recently it was reported that β-catenin interacts with retinoic acid receptor in a retinoid-dependent manner, reducing β-catenin/TCF regulated transcription. Our hypothesis was therefore that dietary supplementation with all-trans RA may inhibit the Apc-driven tumourigenesis in Min/+ mice. Surprisingly, in two different experiments the results showed that dietary RA significantly stimulated both the formation and growth of small intestinal tumours. In the first experiment Min/+ mice were exposed to 50 mg 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine/kg body weight at day 3–6 after birth and then treated with 50 mg/kg dietary RA in 1–3 weeks from the age of 2 weeks. In the second experiment the mice were not treated with carcinogen, and the diet was supplemented with 5 or 10 mg/kg RA from the age of 4 weeks until termination of the experiment at 11 weeks. Immunohistochemical studies revealed no differences in β-catenin, cyclin D1 or proliferating cell nuclear antigen staining following RA treatment. There was no intestinal toxicity in mice fed 10 mg/kg RA, indicating that the increased tumourigenesis in Min/+ mice is a specific effect of all-trans RA.

Introduction

Retinoids are essential to many biological processes and include all vitamin A (retinol) derivatives, such as the natural all-trans, 9-cis and 13-cis retinoic acid (RA), retinyl esters (mainly palmitate) and the synthetic retinoids (1). Retinol is provided in the diet as retinyl esters or as a provitamin carotenoid, mainly as β-carotene. β-Carotene and other pro-vitamin A carotenoids may be metabolized to retinol by a dioxygenase (2). β-Carotene is first converted to retinal, which may be reversibly converted to retinol or irreversibly converted to all-trans RA. All-trans RA undergoes isomerization to 9-cis RA or 13-cis RA (3). Retinoids have been shown to suppress carcinogenesis in several experimental animal models, including skin, breast, oral cavity, bladder, prostate, lung, liver and pancreas (1). In the colon of F344 rats induced with the carcinogen azoxymethane (AOM), 9-cis RA and several synthetic retinoids reduced aberrant crypt foci (ACF) formation (4,5). In addition, 9-cis RA and 4-hydroxyxyretinamide decreased the yield of AOM-induced colon tumours (5). In the Sprague–Dawley rat, all-trans RA reduced the number of ACF induced by AOM, but the size of the remaining ACFs were increased (6). The synthetic retinoid 2-carboxyphenylretinamide enhanced colon cancer in AOM-induced F344 rats (7).

RA regulates gene transcription by binding to members of the nuclear hormone receptor superfamily; the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (8). There are three subtypes (α, β and γ) of both RAR and RXR, and several isoforms. RARs bind both all-trans RA and 9-cis RA, whereas RXRs only bind 9-cis RA (9). DNA-binding RAR–RXR heterodimers regulate gene transcription in response to RA in collaboration with co-repressors and co-activators (1,10). More than 500 genes have shown to be regulated by RA, either by direct transcription or indirectly by other mechanisms (11). RXR may also form homodimers, or form a heterodimeric partner to other members of the superfamily, including vitamin D receptor, peroxisomal proliferator-activator receptor, thyroid hormone receptor, farnesoid receptor and liver X receptor (1,12). Another cross-modulation by retinoids is repression of the transcription factor activator protein-1 (c-fos and c-jun) by RAR (10).

The multiple intestinal neoplasia (Min)/+ mouse model spontaneously develops multiple adenomas in the small intestine (13). These mice have a heterozygous mutation in the adenomatous polyposis coli (Apc) gene and are a murine model of human familial adenomatous polyposis (14). Min/+ mice provide a good animal model for studying initiation and progression of intestinal tumourigenesis (15). Intestinal tumour formation in Min/+ mice is mainly associated with loss of the wild-type Apc allele (16) but also truncating Apc mutations (17). Inactivation of Apc leads to accumulation of the proto-oncogene β-catenin in intestinal tumours of Min/+ mice (18,19). Accumulated β-catenin can migrate to the nucleus and activate gene transcription after binding to DNA-binding transcription factors in the lymphoid enhancer factor (LEF)/T cell factor (TCF) family. Several target genes important for the development and progression of intestinal carcinogenesis have been reported, such as c-myc (20), cyclin D1 (21,22), E-cadherin (23) and cyclo-oxygenase-2 (24). All-trans RA has been shown to reduce transcriptional activation by LEF/TCF in several cell lines (25) and RA interacted directly with β-catenin and reduced β-catenin–TCF-mediated transcription. In addition, over-expression of β-catenin in MCF-7 cells increased the activity of the RA-responsive
RARβ promoter (25). Our hypothesis was that dietary all-trans RA supplementation could inhibit the β-catenin/TCF signaling and reduce intestinal tumourogenesis in Min/+ mice by enhancing competition between RAR and TCF for β-catenin binding.

In the present study we investigated whether dietary all-trans RA could inhibit tumour formation and/or tumour growth in 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-treated and untreated Min/+ mice.

Materials and methods

Mice breeding

Min/+ mice were bred at the Norwegian Institute of Public Health, Oslo, Norway, from mice originally purchased from The Jackson Laboratory (Bar Harbor, ME). Procedures to secure inbreeding (<12 generations) were followed. C57BL/6 J-Min/+ males and C57BL/6 J-/+/ (wild-type) females (Bomholt, Denmark) were mated and the Min/+ offspring were identified by an allele-specific PCR assay as described previously (26). The mice were housed in plastic cages in a room with a 12 h light/dark cycle, controlled humidity (55 ± 5%) and temperature (20–24°C). Water and feed were given ad libitum. The mice were weighed once a week.

All-trans RA treatment

All-trans RA (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) was mixed into the AIN-76A Purified Diet (Harlan Teklad, Madison, WI) diet by first taking all-trans RA and adding an equal amount of AIN-76A powder. After mixing, the amount of AIN-76A added was doubled and mixed successively until the desired RA concentration was achieved. In the final mixture the powder diet was blended (Bosch concept 7000, Bosch Home Appliances Corporation, Huntington Beach, CA) for 30 min to ensure that RA was homogenous in the powder diet. The homogeneity was tested by taking 1 g sample from three different places in the blender and mixing with 3 ml absolute ethanol (Arcus produkker, Oslo, Norway). The sample was centrifuged for 10 min at 3000 r.p.m. (Beckman model TS-6 Centrifuge, Palo Alto, CA) and the RA concentration in the supernatant was analysed at 350 nm (Perkin Elmer Lambda Bio 40 UV/VIS Spectrometer, Shelton, CT). AIN-76A diet with RA was protected from light and stored at 4°C until feeding and for no more than 1 month. AIN-76A without RA was stored at 4°C.

Experimental design

In experiment 1, PhIP of >98% purity was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). It was dissolved in concentrated HCl and thereafter evaporated. The PhIP±HCl was dissolved in 0.9% saline, and adjusted to pH 3.5 with NaOH.

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RA could inhibit tumour formation and/or tumour growth in 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-treated and untreated Min/+ mice.

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Results

In two different experiments we investigated whether dietary supplementation with all-trans RA could influence the tumourgenesis in Min/+ mice. In experiment 1, the effects of 1–3-week period administration of high dose RA in PhIP-exposed Min/+ mice were investigated. In experiment 2, the effect of continuous administration of low doses dietary RA in untreated Min/+ mice was studied.

The effect of dietary RA in PhIP-treated Min/+ mice (experiment 1)

Min/+ mice were exposed to a single s.c. injection of 50 mg/kg PhIP and fed AIN-76A diet supplemented with 50 mg/kg RA in 1–3 weeks from the age of 2 weeks. In these mice a 1.4-fold increase in the number of small intestinal tumours was seen (P = 0.013) (Table I). The observed 1.8-fold increase in the number of colonic tumours did not achieve statistical significance (P = 0.104).

Table I. Effects of all-trans RA on the number and size of intestinal tumours in PhIP-exposed Min/+ mice

<table>
<thead>
<tr>
<th>Part of the intestine</th>
<th>Treatment</th>
<th>Incidence</th>
<th>Mean number of tumours/mouse ± SEM</th>
<th>Mean tumour size ± SEM (number of tumours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>PhIP + 0 mg/kg RA</td>
<td>14/14</td>
<td>99.1 ± 6.8</td>
<td>0.50 ± 0.012 (n = 1388)</td>
</tr>
<tr>
<td></td>
<td>PhIP + 50 mg/kg RA</td>
<td>19/19</td>
<td>142.0± 13.0</td>
<td>0.78± 0.15 (n = 2688)</td>
</tr>
<tr>
<td></td>
<td>PhIP + 0 mg/kg RA</td>
<td>8/14</td>
<td>0.9 ± 0.3</td>
<td>2.5 ± 0.66 (n = 12)</td>
</tr>
<tr>
<td></td>
<td>PhIP + 50 mg/kg RA</td>
<td>11/19</td>
<td>1.5 ± 0.4</td>
<td>2.6 ± 0.66 (n = 29)</td>
</tr>
</tbody>
</table>

*P = 0.013 one-way ANOVA.

*P ≤ 0.001 one-way ANOVA on Ranks.
small intestinal tumour population characterized by a larger increase in tumour size compared with controls (Table II). In treatment in a dose-dependent manner ( intellect

The dietary RA treatment in PhIP-exposed mice induced a small intestinal tumour population with larger tumour area than in control tumours (data not shown). Changes in tumour size were not observed in the colon of PhIP-treated mice (data not shown).

Since we observed an unexpected increase in the number and size of small intestinal tumours by dietary RA in PhIP-exposed Min/+ mice, we further investigated effects of low doses of dietary RA in untreated Min/+ mice in a second experiment.

The effect of dietary RA in untreated Min/+ mice (experiment 2)

In Min/+ mice fed AIN-76A diet supplemented with 0 (controls), 5 or 10 mg/kg RA (week 4–11), a dose-dependent increase of small intestinal and colonic tumours was seen (Table II). The diet added 10 mg/kg of RA caused a 2-fold increase in the number of small intestinal tumours and a 5-fold increase in the number of colonic tumours compared with control animals (Table II). Dietary RA treatment induced a small intestinal tumour population characterized by a larger tumour area than the tumours of the controls, which is illustrated by the tumour size distributions shown in Figure 1. The size of the small intestinal tumours was increased by RA treatment in a dose-dependent manner (r = 0.123, P < 0.001; data not shown): 10 mg/kg RA resulted in a 1.2 fold increase in tumour size compared with controls (Table II). In Min/+ mice fed RA supplemented diet most of the small intestinal tumours were located in an area ~15–30 cm from the ventricle, as in the controls (data not shown). The size of the tumours increased evenly along the small intestine, but the difference was largest in the distal part. In the colon, no statistically significant changes of tumour size were observed (data not shown).

Five wild-type Min/+ mice fed 10 mg/kg dietary RA did not have any intestinal tumours.

Immunostaining of β-catenin, cyclin D1 and PCNA

Small intestinal tumours showed less β-catenin staining at the cell membranes than normal tissue. In contrast to normal tissue, the lesions contained cells with nuclear β-catenin. There was no difference in the distribution of β-catenin in tumours from untreated control mice and mice treated with 10 mg/kg dietary RA (data not shown). Furthermore, immunohistochemical analysis of cyclin D1 expression indicated that cyclin D1, as observed previously in Min/+ mice (H.K.Knutsen, manuscript in preparation) (27), is expressed in a subset of epithelial cells in the adenomas. Again, no differences between controls and RA mice were observed (data not shown).

We then performed PCNA staining and counted cells with positive nuclei from 21 crypts in the distal part of the small intestine from two mice fed 10 mg/kg dietary RA and two mice fed control diet in order to get an indication of eventual altered cell proliferation rate. There was no statistically significant difference in the number of stained nuclei (Table III).

Final body weights

In experiment 1, the mean final body weights, 23.5 ± 0.9 and 23.2 ± 1.1 g for PhIP-treated mice and PhIP-treated mice fed short-term 50 mg/kg dietary RA, respectively, did not differ.

Table II. Effects of all-trans RA on the number and size of intestinal tumours in untreated Min/+ mice

<table>
<thead>
<tr>
<th>Part of the intestine</th>
<th>Treatment</th>
<th>Incidence</th>
<th>Mean number of tumours/mouse ± SEM</th>
<th>Mean tumour size ± SEM (number of tumours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>0 mg/kg RA</td>
<td>10/10</td>
<td>18.0 ± 2.80</td>
<td>0.69 ± 0.07 (n = 180)</td>
</tr>
<tr>
<td></td>
<td>5 mg/kg RA</td>
<td>15/15</td>
<td>21.5 ± 1.92</td>
<td>0.73 ± 0.05 (n = 325)</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg RA</td>
<td>10/10</td>
<td>41.7 ± 9.5</td>
<td>0.83 ± 0.04 (n = 417)</td>
</tr>
<tr>
<td>Colon</td>
<td>0 mg/kg RA</td>
<td>3/10</td>
<td>0.30 ± 0.15</td>
<td>4.7 ± 0.17 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>5 mg/kg RA</td>
<td>8/15</td>
<td>0.73 ± 0.21</td>
<td>2.2 ± 0.10 (n = 11)</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg RA</td>
<td>8/10</td>
<td>1.50 ± 0.34</td>
<td>1.8 ± 0.04 (n = 15)</td>
</tr>
</tbody>
</table>

aP = 0.030 one-way ANOVA on Ranks (10 mg/kg versus control).
bP ≤ 0.001 one-way ANOVA on Ranks (10 mg/kg versus control and 5 mg/kg).

Table III. Percent of cells with PCNA positive nuclei within one crypt in the distal part of the normal small intestine in Min/+ mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of crypts</th>
<th>Number of cells</th>
<th>Mean % PCNA positive cells/crypt ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>42</td>
<td>1468</td>
<td>48.6 ± 1.3</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>42</td>
<td>1404</td>
<td>46.7 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26.6 ± 1.4</td>
</tr>
</tbody>
</table>

aNot significantly different from vehicle, one-way ANOVA.
In experiment 2, a tendency of lower mean final body weights was seen in the treatment groups, 26.0 ± 1.5, 24.0 ± 1.1 and 23.0 ± 1.0 g for mice fed vehicle, 5 and 10 mg/kg dietary RA, respectively (P = 0.131).

Histopathological examination
Haematoxylin–eosin stained sections were analysed for any visual signs of intestinal toxicity. The small intestine from mice fed 10 mg/kg RA was equal to the small intestine of control mice and indications of inflammation reactions were not observed.

Discussion
All-trans RA is reported to decrease the activity of the β-catenin-LEF/TCF signalling pathway by direct interaction of RAR with β-catenin, generating competition between RAR and TCF for β-catenin binding (25). Accordingly, it has been reported that RA represses cyclin D1 expression dependent on β-catenin/TCF signalling in the colorectal cancer cell line SW 480 with mutated APC (28). Our hypothesis was that dietary all-trans RA-supplementation might activate RAR, sequester β-catenin, and thereby inhibit β-catenin-LEF/TCF signalling and reduce intestinal tumour growth in Min/+ mice. Surprisingly, and in contrast to what we hypothesized, the present results showed that dietary all-trans RA supplementation enhanced formation and growth of small intestinal tumours in this model.

The enhanced intestinal tumourigenesis caused by all-trans RA-treatment was characterized by an increased number of tumours in the distal part of the small intestine, the same area as the spontaneous tumours originated. Moreover, the small intestinal tumours in Min/+ mice fed RA were on average significantly larger than in control mice, indicating that the tumours in mice fed RA grow faster than in control mice. The size distribution of small intestinal tumours from mice treated with RA showed a general shift towards larger tumour classes compared with controls. One might speculate whether there are tumours induced by RA that grow faster, or whether RA stimulates growth of all tumours. Previous investigations have shown that PhIP-exposed Min/+ mice are more susceptible to intestinal tumour initiation at days 3–12 after birth (29). In fact, most of the intestinal tumours in Min/+ mice are initiated before the mice are 1 week of age (30). Results from the experiment where the mice received 10 mg/kg dietary RA at 4 weeks of age (experiment 2), indicated that formation of tumours induced by RA occurred after the time where most of the spontaneous tumours had already been formed. Therefore, it seems unlikely that tumours formed in Min/+ mice after 4 weeks of age could overtake, i.e. grow to be larger than tumours formed in Min/+ mice <1 week of age. Altogether, this indicates that all-trans RA in addition to initiate the formation of new tumours also stimulated growth of all small intestinal tumours, irrespective of when they were formed.

The presently observed stimulatory effect of RA is in conflict with the general chemopreventive effect of retinoids reported in rodent models of colorectal cancer. Carotenoids, such as fucoxanthin, lycopene and lutein have been shown to inhibit ACF formation in the colon of B6C3F1 mice exposed to the carcinogen 1,2-dimethylhydrazine dihydrochloride (31). In the colon of F344 rats treated with the carcinogen AOM, 9-cis RA and several synthetic retinoids reduced ACF formation (4,5). In addition, 9-cis RA and 4-hydroxyphenylethynimide decreased the yield of AOM-induced colon tumours (5). Increasing amounts of β-carotene (1–20 mg/kg diet) have shown to decrease the number of ACF and colon tumour incidence in AOM treated F344 rats fed high fat and low or high-fibre diet (32). In the Sprague–Dawley rat, all-trans RA reduced the number of ACF induced by AOM, but interestingly, the size of the remaining ACFs were increased (6). However, there is one example of stimulated colon carcinogenesis; the synthetic retinoid 2-carboxyphenylethynimide enhanced colon cancer in AOM-induced F344 rats (7). In mouse cancer models affecting other organs stimulatory effects of all-trans RA have been reported. All-trans RA significantly increased hepatocarcinogenesis in female B6D2F1 mice (33) and skin tumourigenesis in SENCAR mice was promoted by topical application of all-trans RA (34).

The present data do not give specific information about the mechanisms that initiate additional tumour formation and stimulate tumour growth in RA-treated Min/+ mice. Histopathological examination of the small intestinal mucosa did not show any sign of local toxicity or inflammatory reaction. Neither was the general cell proliferation as assessed by counting PCNA positive cells of the normal intestinal epithelium increased. Furthermore, RA did not function as an intestinal carcinogen in wild-type mice. Hence, our observations indicate that the stimulatory effect of RA on tumour formation is linked to the Apc gene germline mutation and the processes that cause spontaneous intestinal tumour formation in Min/+ mice, which is mainly loss of the wild-type Apc allele by chromosome loss or recombination (16,17,35). Our results indicate unaltered β-catenin signalling to the nucleus in the presence of all-trans RA, as no changes in the immunohistochemically detected expression of β-catenin or the downstream gene cyclin D1 was observed.

Most probably the effects of all-trans RA are due to a direct transcriptional regulation. RA regulates at least 532 genes, directly or indirectly (11). The target genes in the mouse intestines are largely unknown and it is not clear what type of receptor isoforms that might perform the effects observed in this system. What kind of genes that are regulated depends on the promoter regions available, the expression of retinoid receptors and the availability of other factors, such as co-activators or co-repressors (12,36). The genes ultimately affected are highly cell-specific. Further studies on the expression of retinoid receptors and the genes that are regulated in Min/+ mice needs to be performed in order to understand the effects that RA executes in these mice. Possible pathways by all-trans RA might involve activator protein-1 as the expression of c-fos increased in rat colon following all-trans RA-treatment (6). The binding of all-trans RA to RAR and its transcriptional regulation is dependent on RXR as a heterodimeric partner, and may therefore also affect other heterodimeric partners of RXR, such as vitamin D receptor, peroxisomal proliferator-activator receptor and liver X receptor.

Our results show that dietary all-trans RA increased the number and growth of intestinal adenomas in Min/+ mice. This might have implications for possible use of systemic retinoid treatment in familial adenomatous polyposis patients. The most probable mechanism of action of all-trans RA is its ability to regulate gene transcription of a number of different genes, which in turn might have numerous effects on intestinal epithelial growth. However, further studies are required in order to define the mechanisms that are involved in all-trans
RAs ability to enhance carcinogenesis of intestinal cells in Min/+ mice.

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References


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