Inhibition of Growth of Established N-Methyl-N-nitrosourea-induced Mammary Cancer in Rats by Retinoic Acid and Ovariectomy

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ABSTRACT

Retinoids are effective in the prevention of N-methyl-N-nitrosourea-induced mammary carcinoma; retinoids and hormonal therapy exert synergy in cancer prevention. In this study, we examined the effects of the dietary supplementation with all-trans retinoic acid (RA) alone or in combination with ovariectomy on the growth of established N-methyl-N-nitrosourea-induced mammary carcinomas in rats. In the first experiment, animals (n = 13) were entered in each of the following treatment groups when their tumors reached 2 cm in diameter: 1) control diet; 2) RA 300 mg/kg diet; 3) ovariectomy (OVX); 4) RA 300 mg/kg diet plus OVX. Animals were sacrificed after 28 days of therapy. In the RA-supplemented animals, tumor progression was less than in the control group without signs of toxicity as assessed by total and individual tumor surface area and weight, and animal weight. OVX produced tumor regression that was not enhanced by the addition of RA. In a second experiment, RA 65- and 130-mg/kg diets were dissolved in corn oil with antioxidants prior to mixing to the diet to improve biodosibility. This resulted in overall stabilization of tumor growth by RA addition to the diet at either of the 2 doses utilized; the addition of RA 65 mg/kg diet did not modify tumor regression induced by OVX. In conclusion, the dietary supplementation with RA decreased the progression or stabilized the growth of the majority of tumors and only rarely (6%) induced tumor regression; no additive or synergistic effects were found with the combination of RA and ovariectomy.

INTRODUCTION

Both natural and synthetic retinoids have been used in large doses in vivo to prevent rat mammary carcinogenesis induced by DMBA or by MNU; the appearance of tumors is delayed, and the incidence and severity of tumors are decreased. Retinoids inhibit the growth of transformed rodent and human breast cancer cell lines in tissue culture (3-7). However, the effect of retinoids on the growth of established carcinogen-induced mammary cancer in rats in vivo has not been studied extensively yet. In one study in DMBA-induced mammary cancer in rats, the addition of large doses of retinyl acetate to the diet of rats failed to induce any significant tumor regression, but retarded tumor growth (8).

Carcinogen-induced mammary tumors in rats have been shown to be hormone-dependent (9). Castration of rats prior to administration of MNU prevents carcinogenesis (10); ovariectomy or antiestrogens induce objective temporary regression of a majority of established tumors (10-12). Retinoid treatment alone or hormonal deprivation alone produces a partial prevention of MNU-induced mammary carcinogenesis; the combination of retinoid administration and ovariectomy (13) or prolatin suppression (14) led to a synergistic effect on tumor prevention. In established human breast cancer cell lines, the combination of retinoids and antiestrogens was found to have additive inhibitory effects in 2 studies (6, 15), but not in another (16). The effects of a combination of retinoid administration and of hormonal therapy on the growth of established carcinogen-induced mammary tumors in rats has not been examined yet.

We have recently reported that the uptake and metabolism of retinol were deficient in MNU-induced mammary carcinomas in rats (17); in contrast, it was found that retinoic acid was taken up and metabolized actively in these tumors. Retinyl acetate, which was previously found to be poorly effective in inducing tumor regression (8) in DMBA-induced tumors, would be expected to be poorly taken up and metabolized as retinol. In this study, we examined the effects of the administration of retinoic acid alone or in combination with ovariectomy on the growth of established MNU-induced mammary carcinomas in rats.

MATERIALS AND METHODS

Animals and Induction of Tumors. Virgin female Sprague-Dawley rats were obtained at 45 days of age from Charles River Canada Inc., St.-Constant, Québec, were housed 2 per cage, and were fed with regular rat chow and water ad libitum. Animals received an intrajugular injection of 50 mg/kg MNU (Isopac, Sigma Chemical Co., St. Louis, MO) at 50 days of age (18). Animals were weighed weekly and examined twice weekly to identify and measure tumors with calipers. When a tumor reached 2 cm in its longest diameter, animals were allocated randomly to either one of the experimental treatment groups.

Treatment Groups. Two experiments were performed. In the first study (experiment A), the tumor-bearing animals were allocated to 1 of the following 4 treatment groups: (a) control group on regular diet; (b) diet supplemented with RA, 300 mg/kg diet; (c) regular diet and bilateral OVX; and (d) RA, 300 mg/kg diet, and OVX. Upon entering a treatment group (day 0), animals were fed with a powdered chow wetted with water; batches of RA-supplemented diet were prepared fresh weekly by blending the all-trans RA powder with the diet.

In the second study (experiment B), a vehicle of 50 ml/kg diet of corn oil (Mazola) containing 2% CHCl3, 0.1% Tenox 20, and 0.1% DL-α-tocopherol was used to dissolve all-trans-RA powder to mixing with the powdered diet from Wayne to increase bioavailability as described by Moon et al. (19). Experiment B included 5 experimental groups: (a) regular diet with corn oil vehicle; (b) RA, 65 mg/kg diet; (c) regular diet and OVX; (d) RA 65 mg/kg diet and OVX; and (e) RA, 130 mg/kg diet.

The stability of added all-trans-RA to the diets was verified by high-pressure liquid chromatography. Diets containing either 300 mg/kg of RA powder as in experiment A or 65 mg/kg of RA dissolved in the corn oil vehicle as described in experiment B were extracted with 99% methanol after storage for 0, 3, and 7 days. The methanolic extract was concentrated and an aliquot was injected onto an ultraspHERE ODS (Beckman Instruments) column; RA was separated from its oxidation products by elution of the column with a mobile phase of acetonitrile:water (60:40) containing 10 mM ammonium acetate at a flow rate of 1.7 ml/min. In this system, RA had a retention time of 7.8 min. RA powder as in experiment A or 65 mg/kg of RA dissolved in the corn oil vehicle as described in experiment B were extracted with 99% methanol after storage for 0, 3, and 7 days. The methanolic extract was concentrated and an aliquot was injected onto an ultraspHERE ODS (Beckman Instruments) column; RA was separated from its oxidation products by elution of the column with a mobile phase of acetonitrile:water (60:40) containing 10 mM ammonium acetate at a flow rate of 1.7 ml/min. In this system, RA had a retention time of 7.8 min. RA was found to be stable with either method of preparation since recovery varied between 98 and 103% for up to 7 days of storage.

During the treatment period, the dimension of the individual tumors in the largest diameter (L) and its perpendicular diameter (W) were...
RESULTS

In experiment A, 13 tumor-bearing animals were entered in each treatment group; the number of tumors present at day 0 and at day 28 is shown in Table 1. The number of tumors varied between 1 to 3 per animal and this was randomly distributed among the treatment groups. No significant differences were present in the present tumor size (sum of surface area of all tumors of a given animal), in individual tumor size (mean surface area of individual tumors in a given treatment group), or in animal weight between groups on day 0. The total tumor size increased significantly in the control group (P < 0.001) and in the RA group (P < 0.05) at day 28 compared with day 0. Tumor progression was inhibited by RA treatment compared with the control group as reflected in total tumor surface area (P < 0.001) and weight (P < 0.01), and in individual tumor size (P < 0.05) and weight (P < 0.05); RA treatment did not affect animal or uterine weight. Ovariectomy produced tumor regression as seen in total tumor size compared with day 0 (P < 0.05) and with the control group and the RA group at day 28 (P < 0.001); individual tumor size and weight were decreased as compared with control diet (P < 0.01) and with RA (P < 0.05). Animal weight was increased (P < 0.001) and uterine weight was also measured.

Statistical Analysis. The comparisons of total tumor sizes and animal weights from day 0 to day 28 and between treatment groups were performed by 2-way analysis of variance with repeated measures on one factor and a posteriori comparisons according to the method of Bonferroni (20). The differences in individual tumor size, total or individual tumor weights, or uterine weights at sacrifice were performed by one-way analysis of variance with a posteriori comparisons according to the method of Bonferroni (20). The distribution analysis of overall response rate was analyzed by χ² statistic.

DISCUSSION

Our results indicate that dietary supplementation with RA inhibits the growth of established MNU-induced mammary

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Table 1. Effects of dietary supplementation with RA and ovariectomy on the growth of MNU-induced mammary cancers

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total tumor size and wt</th>
<th>Individual tumor size and wt</th>
<th>Animal wt (g)</th>
<th>Uterine wt (g), day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>Total area (cm²)</td>
<td>Area (cm²)</td>
</tr>
<tr>
<td>RA 300 mg/kg diet</td>
<td></td>
<td></td>
<td>3.31²</td>
<td>7.56²</td>
</tr>
<tr>
<td>O VX</td>
<td></td>
<td></td>
<td>1.68</td>
<td>5.30</td>
</tr>
<tr>
<td>RA 300 mg/kg + O VX</td>
<td></td>
<td></td>
<td>0.49</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.93</td>
<td>1.78</td>
</tr>
</tbody>
</table>

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*a Number of animals per group.

*b Number of tumors per group.

*c Mean values with SD in parentheses.

*d Significantly different from day 0 (see text for P values).

*e 300 mg RA powder mixed per kg of diet.

*f Significantly different from control group.

*g Significantly different from RA group.
INHIBITION OF BREAST CANCER IN RATS BY RA AND OVARIECTOMY

Table 2: Effects of dietary supplementation with RA dissolved in corn oil and ovariectomy on the growth of MNU-induced mammary cancers

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>RA 65 mg/kg</th>
<th>OVX</th>
<th>RA 65 mg/kg + OVX</th>
<th>RA 130 mg/kg</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td></td>
<td>Wt (g)</td>
<td>Wt (g)</td>
<td></td>
<td>Wt (g)</td>
<td>Wt (g)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Individual</td>
<td>Animal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>area (cm²)</td>
<td>area (cm²)</td>
<td>weight</td>
<td>weight</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>2.57⁰</td>
<td>4.29⁰</td>
<td>7.42</td>
<td>2.17</td>
<td>2.95</td>
</tr>
<tr>
<td>11</td>
<td>2.67</td>
<td>2.80</td>
<td>5.22</td>
<td>2.26</td>
<td>2.37</td>
</tr>
<tr>
<td>11</td>
<td>2.41</td>
<td>2.71</td>
<td>5.13</td>
<td>2.21</td>
<td>2.48</td>
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<tr>
<td>10</td>
<td>2.45</td>
<td>1.97⁰</td>
<td>3.11</td>
<td>2.33</td>
<td>1.59</td>
</tr>
<tr>
<td>8</td>
<td>2.73</td>
<td>2.72</td>
<td>5.63</td>
<td>1.98</td>
<td>1.98</td>
</tr>
</tbody>
</table>

*Significantly different from control group.
Significantly different from RA group.
Significantly different from RA group.

Table 3: Overall percentage of response rate

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Regression</th>
<th>Stable</th>
<th>Progression</th>
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<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>0</td>
<td>17</td>
<td>83</td>
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<tr>
<td>RA</td>
<td>33</td>
<td>6</td>
<td>50</td>
<td>44</td>
</tr>
<tr>
<td>OVX</td>
<td>24</td>
<td>7</td>
<td>21</td>
<td>8</td>
</tr>
<tr>
<td>OVX + RA</td>
<td>23</td>
<td>78</td>
<td>4</td>
<td>17</td>
</tr>
</tbody>
</table>

*Groups of animals with similar treatments from the 2 experiments were pooled. Since no differences were found in tumor growth parameters between the 3 doses and preparations of RA groups, the data were also pooled.

Response was assessed by comparing total surface area of tumors before and after 28 days of therapy. Response criteria are defined in "Materials and Methods."

Tumors in rats. The growth pattern of these tumors can be quite heterogeneous, but in general the addition of RA to the diet resulted in an overall stabilization of tumor growth in a majority of animals or in a progression of a lesser extent than in control animals; we observed that in RA-supplemented animals, tumors continued to grow during 3–10 days after the initiation of the diet and then remained stable or regressed slowly. In a small percentage of cases, an overall tumor regression of more than 50% was observed after 28 days of treatment. We elected to use RA as the retinoid in this experiment based on our previous findings that RA was taken up and metabolized more efficiently than retinol by these tumors (17). No published data reported the utilization of all-trans-RA in mammary carcinogenesis prevention studies; 13-cis-RA at 1 mmol/kg diet was found to be noneffective (1). However, RA was found to inhibit the stimulatory effects of insulin on thymidine incorporation into DNA of slices of MNU-induced tumors in vitro (21); RA had no effect on basal thymidine incorporation. In our first experiment, we utilized the dose of 1 mmol (300 mg/kg) diet of RA powder and found a decrease in progression of tumors without signs of toxicity as assessed by animal weight. In the second experiment, we utilized a vehicle of corn oil containing 0.1% Tenox 20 and 0.1% dl-α-tocopherol per kg of diet. Same vehicle was added to the control diet.

As previously largely demonstrated (9–11), we found that ovariectomy induced an important tumor regression in most animals. However, in contrast with the findings in chemoprevention (13, 14) or on the growth of breast cancer cell lines in vitro (6, 15), we found no additive or synergistic effect of the combination of RA and ovariectomy. Retinoids do not appear to influence ovarian function directly as assessed by uterine weight (Tables 1 and 2) or by alterations in estrous cycles (23); prolactin levels were found to be unchanged in MNU-induced tumors (14) and increased in DMBA-induced tumors (8) by retinyl acetate. It is possible that RA and ovariectomy utilize common mechanisms in regulating established tumor growth while different mechanisms are implicated in tumor prevention. Recently work conducted mostly in cell lines in vitro have shown that the regulation of growth of breast cancer by estrogens is exerted at least in part by a modulation of the autocrine production of growth factors such as transforming growth factor α, transforming growth factor β, and insulin-like growth factor I (24). It would be of interest to determine whether retinoids exert similar effects, since they can modify epidermal growth factor receptors (25), or modulate the activity of growth factors (26, 27).

Retinoids have been studied most extensively in chemoprevention studies (1); however, they have been shown to inhibit the growth of certain transplantable or autochthonous tumors such as melanomas, chondrosarcomas, sarcomas, and embryonal carcinomas in vivo as reviewed recently (26, 28). In a phase II trial in metastatic breast cancer in humans, 13-cis-retinoic acid was found to stabilize tumor growth in 8 of 18 patients temporarily, but no significant tumor regression was observed (29). Evidence for therapeutic effects of retinoids in humans is limited to basal cell carcinoma, mycosis fungoids, myelodysplastic syndromes, and promyelocytic leukemia (28, 30, 31, 32). It is hoped that the synthesis of newer less toxic retinoids such as arotinoids (2) or the combination of retinoids and other chemotherapeutic drugs may eventually provide an improvement in therapy.
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REFERENCES

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