Chapter 6

B  Chemomodulation of mammary carcinogenesis by retinoic acid
Introduction

Retinoids have been shown as effective chemopreventive agents in chemically induced mammary carcinogenesis. 7,12 dimethylbenz(a)anthracene (DMBA) treated Sprague Dawley female rats and retinylacetate are known to exhibit a significant reduction in mammary cancer incidence (287). Several retinoids have been evaluated for chemopreventive activity against both DMBA and N-methyl-N-nitrosourea (NMU) induced mammary carcinogenesis as well as mammary cancer development in both mammary tumor virus positive and mammary tumor virus negative mice (316,317). McConnick et al. have shown that significant inhibition of carcinogenesis may be obtained if the retinoids are included in the diet only for limited period, two weeks before until one week after carcinogenesis administration (318). However, a continuous treatment is necessary if retinoid supplementation begins after treatment with the carcinogen (319).

Mammary carcinogenesis is known to be modulated by hormonal alteration (320,321). Antiestrogen treatment or ablation of the ovary surgically, is known to inhibit the appearance of mammary cancer in carcinogen treated animals (321,322). Earlier studies have shown that combination of antiestrogen tamoxifen and retinoids resulted in reduced tumor incidence in carcinogen treated animals. Similarly ovariectomized mice when given retinoids were found to have more effect in NMU-induced mammary carcinogenesis than the treatment alone (323). The precise mechanism of retinoid action in epithelial differentiation and cancer chemoprevention is yet not well defined.
Ornithine decarboxylase (ODC) is first and the rate limiting step in polyamine biosynthesis (143). Although retinoids have been implicated in the regulation of proliferation and differentiation (92,95). Their precise function remains to be elucidated. The rate of polyamine biosynthesis appears to correlate well with the rate of cell growth. Inhibition of polyamine levels leads to reduced cellular proliferation. Earlier studies have shown that retinoids decrease expression of ODC mRNA in human skin cells (294). Retinoids are known to control the expression of ornithine decarboxylase (295).

The present work reports the inhibitory action of retinoic acid on N-methyl-N-nitrosourea induced mammary carcinogenesis in female Sprague Dawley rats. Influence of combined effect of ovariectomy and retinoic acid on NMU-induced tumors has also been worked out. The role of polyamines in the present model system has also been evaluated.

Materials and methods

Chemicals: All trans-retinoic acid (RA), dansyl chloride, dithiothreitol, L-ornithine, methylbenzothonium hydroxide, N-nitrosomethylurea (NMU), putrescine, spermine and spermidine were purchased from Sigma Chemical Co. (St. Louis MO, USA). Standard animal food pellets were provided by Hindustan Lever Ltd. (Delhi, India). Pre-coated silica gel TLC plates were purchased from E.Merck Ltd. (Bombay, India).

Animals: Sprague Dawley rats were purchased from National Institute of Nutrition (NIN) Hyderabad, India and maintained at standard conditions of light and humidity
at the animal house facility of Jawaharlal Nehru University, New Delhi, India and fed with standard food pellets.

**Study groups:** 55-day old virgin, female Sprague Dawley rats were used for this study. The animals were provided standard diet (with or without retinoic acid) and tap water *ad libitum*. The animals were maintained in an air-conditioned room. The retinoic acid (120 mg/kg) was mixed with food pellet by pulverization. They were divided into the following groups:

**Group I (n = 25)** Animals of this group acted as control, did not receive any treatment and were kept on normal diet during the observation period.

**Group II (n = 25)** Each animal of this group was given NMU intraperitonially under mild ether anesthesia. These animals received normal diet.

**Group III. (n = 25)** The animals of this group were given NMU as in group II and they received a diet containing 120 mg/kg of retinoic acid for a month and then switched to normal diet for the remaining period.

**Group IV (n = 25)** Animals were ovariectomized one week before NMU injection. NMU injection was same as in group II and group III. They were put on a normal diet throughout the observation period.
Group V. (n = 25) Animals were ovariectomized one week before NMU injection like group IV. They were put on retinoic acid diet for a month and then switched over to normal diet for the remaining observation period.

The animals were palpated once a week for the detection and documentation of tumors in their mammary glands. All animals surviving 180 days after the initiation of the experiment were sacrificed. Tumors were harvested and used for measurement of polyamines. Tumor induction and Tumor isolation methods followed were as described in chapter 2.

**Tumor incidence:** The chemical carcinogen injected rats were weighed and observed every week after the carcinogen injection. The tumor induction was monitored by palpating the mammary glands. The tumor presence was recorded. The terms used for the documentation of the results are: 1) Tumor incidence, the percentage tumor induced animal in a group. 2) Tumor load, average number of tumor induced per animal. 3) Latency period, the time period between carcinogen injection and tumor induction. 4) Total number of tumors induced in all tumor induced rats. 5) Observation period, total time period the animals were kept under observation after carcinogen treatment.

**Polyamine estimation:** A 10% tissue homogenate in 2% perchloric acid was prepared and kept for 24h at 4°C to extract polyamines. The dansyl derivatives were prepared according to Seiler 1970 (196). The debris was precipitated by spinning at 12000 x g for 10 min. Supernatant (200 μl) was neutralized with 200 μl of super saturated
sodium bicarbonate and then dansylated overnight by adding 400 μl of dansyl chloride (5 mg/ml acetone) at room temperature in dark. Approximately after 16h, untreated dansyl chloride was removed by incubating the mixture with 100 μl of L-proline (150 mg/ml in ice cold water) for 30 min at room temperature. The dansyl derivatives of polyamines were extracted in 500 μl of toluene and separated by thin layer chromatography (TLC) on 0.22 mm thick precoated silica gel G plates using ethyl acetate cyclohexane (2:3 v/v) as solvent. Quantification of polyamines was accomplished using Camag TLC Scanner with TLC II software program Cats3 (Camag, Sonnenmattstr, Switzerland). The concentration of unknown samples was determined against standard polyamines.

Results

The number of tumor bearing rats in both control and experimental groups recorded 180 days after the carcinogen treatment are shown in Table 1. Control animals (group I) did not develop any mammary tumors during the observation period. About 80% of the animals in group II (NMU treated animal on normal diet) developed mammary tumors towards the end of the observation period. Among the animals put on diet containing retinoic acid (group III) NMU induced mammary tumors appeared very slowly (the latency period increased). There was 40% decline in tumor incidence. Thereby showing that rat receiving retinoic acid in diet showed significant reduction in tumor incidence.

Surgical ablation of the ovaries one week before NMU treatment (group IV) inhibited the appearance of mammary cancers in carcinogen treated animal by 72% (tumor incidence 8% compared to 80% in carcinogen only treated group).
Combination of treatment i.e retinoic acid with ovariectomy resulted in a much higher reduction in the number of tumors among tumor bearing animals than either treatment alone. However no significant difference in tumor incidence was noticed in group IV and V. The latency period was also increased in group V, delaying the appearance of tumors.

The effect of retinoic acid on polyamine levels in different groups is shown in Table 2. Retinoic acid when given to control animals did not alter the putrescine, spermidine and spermine content significantly in mammary epithelium (data not shown). However NMU induced tumor group II showed a 4 fold, 20 fold, 3.5 fold increase in putrescine, spermidine and spermine content respectively over the normal control mammary epithelium. Treatment with retinoic acid in NMU treated rats resulted in significant inhibition of polyamines when compared to levels in NMU treated groups. Ovariectomized rats on normal diet and ovariectomized rats one week before NMU treatment (group IV) resulted in significant decrease in putrescine, spermidine and spermine content when compared to group II.

Combined treatment of retinoic acid and ovariectomy (group V) resulted in the inhibition of polyamines levels but the levels were same as that of ovariectomized rats (group IV). No additive inhibitory effect was observed as far as polyamines level were concerned.

Discussion

The present study demonstrates that when retinoic acid is administered to rats during post-carcinogenesis phase of NMU-induced mammary carcinogenesis, there
is an appreciable decline in the number of tumor bearing rats but also there is a
decline in the mean number of tumors per animals (tumor burden). Modulation of
mammary carcinogenesis by hormonal alteration as achieved by surgical ablation of
ovaries is also observed. Combined treatment of ovariectomy with retinoic acid is
significantly more effective in reducing the total number of tumors per animals though
no difference in tumor incidence was observed. Latency period was prolonged
significantly with this combined treatment.

Retinoids are known to exert influence on DNA synthesis (297,298) cell
division (299), RNA synthesis (300), protein synthesis (301), post-translational
glycosylation of proteins (302) etc. However which of the above mentioned effect is
a key to the elucidation of retinoid action remains to be seen. Retinoids are known
to control the expression of ornithine decarboxylase at transcriptional levels (295). In
the present study we worked on the possibility that retinoids mechanism of action may
be mediated by polyamines. Reduction of polyamines content by retinoic acid in
NMU induced tumors shows that retinoid action may be mediated by polyamines in
mammary cancer. These results are in contrast with the in vitro effect of growth
inhibitory concentrations RA on polyamines in the human adenocarcinoma cell line
MCF-7. Retinoic acid was found to increase putrescine levels and inhibit spermidine
and spermine levels in this cell line. It is obvious from these results that both in vivo
and in vitro effect of retinoids is mediated by polyamines. The entire sequence of
events is however not understood.
Comparison of tumor incidence in retinoic acid treated, ovariectomised rats and ovariectomised + retinoic acid treated N-nitrosomethylurea (NMU)induced rats. Retinoic acid (120 mg/kg) was given in the diet after carcinogen treatment for one month and then animals were switched over to normal diet till the observation period. The ovariectomy operation was done one week before carcinogen treatment. Control animals were given normal animal diet. Tap water was given ad-libitum to all the groups. Tumor burden is the average number of tumors per animal in tumor induced animals. Tumor incidence is percentage of tumor induced animal. Latency period is the period between carcinogen injection and appearance of tumor in animals.
Table 2 Polyamines levels in mammary epithelium and N-nitrosomethyl urea induced tumors treated retinoic acid

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Polyamine (nmol/mg protein)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Putrescine</td>
</tr>
<tr>
<td>I</td>
<td>Mammary epithelium (Control)</td>
<td>1.4 ± 0.01</td>
</tr>
<tr>
<td>II</td>
<td>NMU-induced Tumors</td>
<td>5.52 ± 0.10</td>
</tr>
<tr>
<td>III</td>
<td>NMU-induced Tumors treated with RA</td>
<td>3.12 ± 0.05</td>
</tr>
<tr>
<td>IV</td>
<td>Mammary-epithelium from ovariectomised rats</td>
<td>0.96 ± 0.01</td>
</tr>
<tr>
<td>V</td>
<td>Mammary-epithelium from ovariectomised rats treated with RA</td>
<td>0.94 ± 0.04</td>
</tr>
</tbody>
</table>

RA Retinoic acid (120 mg/kg of animal diet)

\(a\) Mean ± SD values of triplicate samples

Effect of retinoic acid and ablation of ovary (ovariectomy) or both on the polyamine levels in mammary epithelium of untreated and 60 day NMU induced mammary tumors. Rats received 120 mg/kg of retinoic acid in diet for one month after carcinogen treatment and ablation of ovary was done one week before the carcinogen treatment. Each sample represents mammary epithelium or tumors from 4-5 rats.