Long-Term Effect of 2-Hydroxyethyl Retinamide on Urinary Bladder Carcinogenesis and Tumor Transplantation in Fischer 344 Rats

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ABSTRACT

The effects of HER upon early and late stages of BBN-induced bladder cancer in rats were examined. Female Fischer 344 rats were administered HER in the diet either before and during or continuously after BBN administration and were monitored periodically for up to 2 years. The total dose of BBN was 600 mg administered over a 6-week period. In a separate experiment, the effects of HER administration to syngeneic recipients of a transplanted primary bladder cancer were examined.

No effects on neoplastic development were observed as the result of HER treatment before and during carcinogen administration. However, at the 1-year sacrifice, there was a significant increase in bladder tumor incidence in the animals receiving BBN followed by continuous retinoid treatment versus animals receiving BBN only. At the 2-year sacrifice, there was a significant increase in tumor progression in the continuous retinoid group versus the animals receiving BBN alone, based upon grading and staging of tumors, although tumor incidences were not significantly different. In the transplantation experiment, more recipients (9/20 versus 2/20) receiving continuous HER had large, anaplastic tumors following 9 months of observation than did control animals.

This study supports the view that retinoids should not be considered as only inhibitors of carcinogenesis, but rather as modifiers which vary in their effects depending upon factors yet to be understood.

INTRODUCTION

Several studies have demonstrated an inhibitory effect by vitamin A analogues (retinoids) on experimental carcinogenesis in epithelial tissues of animals, including the respiratory tract, skin, intestines, mammary gland, and urinary bladder (1-3). However, there are also conflicting data which indicate either no effect (4, 5) or enhancement of neoplastic development by retinoids (6-10). Most prior studies of urinary bladder carcinogenesis in animals have been relatively short term and limited to the early stages of carcinogenesis (11-16), whereas proposed chemoprevention or chemotherapy of human bladder cancer may involve long-term administration to patients with established disease (17).

In a recent long-term study by Hicks et al. (18), 13-cis-retinoic acid and N-ethylretinamide were observed to prolong the latent period and thus delay the progression of experimental bladder cancer in rats induced by BBN. Although tumor incidences were not affected, the progression of preneoplastic hyperplasia to tumors was delayed 7-10 weeks. We report here our findings from a similar long-term study using the same carcinogen and rat strain and a related retinoid, HER. Our results differ from those of Hicks et al. (18) in that we observed enhancement of neoplastic development in animals receiving continuous retinoid in the diet for up to 2 years following BBN exposure. In a separate group of animals, HER was administered only before and during BBN administration, and no effect upon carcinogenesis was evident.

Primary tumor transplantation into syngeneic hosts reflects some of the biological features of late tumor behavior, and studies of the influence of retinoids upon tumor transplantability have been limited to established cell lines (2). Consequently, we also examined the effects of HER on primary bladder tumor transplantation and observed apparent enhancement of tumor growth.

The disparate findings in animal experiments emphasize the uncertainties involved in retinoid chemoprevention and the need to further explore factors influencing retinoid effects on cellular differentiation and growth.

MATERIALS AND METHODS

Urinary Bladder Carcinogenesis

Animals. Six-week-old female Fischer 344 rats were obtained from Charles River Laboratories (Wilmington, MA), quarantined for 2 weeks, and housed in individual stainless steel wire bottom cages.

Chemical and Diet Preparation. BBN was synthesized by IIT Research Institute and provided through the National Cancer Institute. The BBN was diluted with 20% aqueous ethanol so that each 50-mg dose was contained in a volume of 0.5 ml. It was administered via gastric intubations at doses of 50 mg twice/week for 6 weeks for a total dose of 600 mg per animal. HER was obtained from Southern Research Institute (Birmingham, AL), also through the courtesy of the National Cancer Institute. It was administered at a level of 515 mg (1.5 mmol) per kg of diet in a gelatinized beadlet form. The diet was prepared weekly by dissolving 515 mg of HER in a 1:3 mixture of ethanol:trioctanoin and adding 0.5 ml of Tenox 20 (Eastman Chemical Products, Kingsport, TN) and 0.5 ml of DL-a-tocopheral (Sigma Chemical Co., St. Louis, MO) as antioxidants. This solution was added to 1 kg of Purina powdered rodent chow and mixed in a Hobart food blender for 30 min. Prepared diet was maintained under refrigeration, and animals were fed ad libitum. Food consumption was measured, and animals were weighed twice/week.

Treatment Groups. Two hundred and eighty rats were divided into 5 test groups according to a table of random numbers as shown in Table 1. Group A received HER 21 days prior to and throughout the 6 weeks of BBN dosing; group B received HER beginning at the last BBN dosing, which continued until sacrifice, and group C received BBN but no HER. One-half of the animals in group C (Co) were fed Purina rodent chow containing the HER solvents and antioxidants, and the other half (Cf) was fed Purina rodent chow only. Group D received plain Purina rodent...
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Table 1
Design of bladder carcinogenesis experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>BBN dose</th>
<th>HER dose</th>
<th>Schedule of HER treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>85</td>
<td>600 mg</td>
<td>515 mg/kg diet (1.5 mm)</td>
<td>3 wk. prior and throughout 6 wk. BBN dosing</td>
</tr>
<tr>
<td>B</td>
<td>85</td>
<td>600 mg</td>
<td>515 mg/kg diet (1.5 mm)</td>
<td>From last BBN dosing to sacrifice</td>
</tr>
<tr>
<td>Crt</td>
<td>40</td>
<td>600 mg</td>
<td>Vehicle alone</td>
<td></td>
</tr>
<tr>
<td>Cfl</td>
<td>40</td>
<td>600 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No. of rats examined at the following sacrifice intervals after initial BBN dose:

<table>
<thead>
<tr>
<th>wk.</th>
<th>7 wk.</th>
<th>12 wk.</th>
<th>24 wk.</th>
<th>52 wk.</th>
<th>52-104 wk.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>9</td>
<td>20</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>9</td>
<td>20</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Crt</td>
<td>4</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Cfl</td>
<td>4</td>
<td>4</td>
<td>10</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Table 2
Incidence of morphological diagnoses at 7-, 12-, and 24-week sacrifice intervals after initial BBN dose

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Normal</th>
<th>Hyperplasia</th>
<th>Hyperplasia with atypia</th>
<th>TCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 wk.</td>
<td>A</td>
<td>8</td>
<td>0</td>
<td>5 (62)</td>
<td>3 (37)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8</td>
<td>6 (75)</td>
<td>1 (12)</td>
<td>1 (12)</td>
</tr>
<tr>
<td></td>
<td>Crt</td>
<td>4</td>
<td>0</td>
<td>4 (100)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cfl</td>
<td>4</td>
<td>0</td>
<td>4 (100)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>5</td>
<td>5 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12 wk.</td>
<td>A</td>
<td>9</td>
<td>2 (22)</td>
<td>6 (66)</td>
<td>1 (11)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>9</td>
<td>1 (11)</td>
<td>6 (66)</td>
<td>2 (22)</td>
</tr>
<tr>
<td></td>
<td>Crt</td>
<td>5</td>
<td>1 (20)</td>
<td>4 (80)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cfl</td>
<td>4</td>
<td>1 (25)</td>
<td>2 (50)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>5</td>
<td>5 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24 wk.</td>
<td>A</td>
<td>20</td>
<td>3 (15)</td>
<td>6 (30)</td>
<td>5 (25)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>20</td>
<td>12 (60)</td>
<td>6 (30)</td>
<td>3 (10)</td>
</tr>
<tr>
<td></td>
<td>Crt</td>
<td>10</td>
<td>5 (50)</td>
<td>4 (40)</td>
<td>4 (40)</td>
</tr>
<tr>
<td></td>
<td>Cfl</td>
<td>10</td>
<td>0</td>
<td>4 (40)</td>
<td>5 (50)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>5</td>
<td>5 (100)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Approximately 10% animal loss in all test groups due to gavage accidents.

RESULTS

Carcinogenesis Experiment

There were no significant differences in feed consumption or weight gain among groups and no clinical or pathological evidence of vitamin A toxicity. There were no significant differences between Ca and Cβ control groups for any bladder observations; therefore, these groups were combined into a single group C for statistical analyses.

Seven-, 12-, and 24-week Sacrifice. The lesions present at these sacrifice periods were minimal (Table 2). At gross examination the urinary bladders varied from normal to minimal mural thickening or small raised masses (transitional cell papillomas).

Transplantation Experiment

An additional forty 4-week-old female Fischer 344 rats were divided into retinoid and control groups. Twenty animals (group E) were placed on a diet of Purina rodent chow containing 515 mg HER/kg feed until sacrifice, and 20 animals (group F) received chow diet alone. Two weeks later both groups were anesthetized with methoxyfluorane, and the right lateral thoracic wall was surgically prepared. Tumor inocula were all derived from a single bladder tumor in a 1-year-old female Fischer 344 rat that had been administered 600 mg of BBN as described above. The tumor was removed aseptically, placed in Hanks' solution, and cut into 1.0-mm³ pieces. Each recipient was given an injection subcutaneously over the lateral rib cage with 1.0 mm³ using a 12-gauge bone marrow aspirate needle and trocar. Histologically, the transplanted tumor was a moderately differentiated papillary transitional cell carcinoma with foci of stromal invasion. No evidence of inflammation, degeneration, or necrosis was present. Following transplantation, recipients were examined each day, and 3-dimensional tumor measurements were made at weekly intervals. All animals were sacrificed at 9 months post-transplant. Tumor tissue, lungs, livers, spleens, and all gross lesions were preserved in 10% neutral buffered formalin and embedded in paraffin, and hematoxylin and eosin sections were examined microscopically.

Pathological Examinations. Animals were killed by an overdose of chloroform according to the schedule shown in Table 1. Complete gross necropsies and histopathological examinations of all major tissues were done on all animals. Urinary bladders were inflated transurethrally with 0.5 ml of neutral buffered formalin. Fixed bladders were divided into anterior and posterior halves and embedded separately into paraffin blocks. Step sections of each half were cut at 3 different levels and stained with hematoxylin and eosin. Bladder tissue slides were coded in a randomized order and evaluated without knowledge of the treatment group.

Criteria for Histopathological Evaluation. The proliferative bladder lesions in animals killed at 7, 12, 24, and 52 weeks were histologically scored according to the criteria reported by Squire et al. (15). Atypia, squamous metaplasia, and flat, exophytic, and endophytic proliferation were each scored 0 to 5+ depending on severity or size. A morphological diagnosis of each lesion was also made based upon traditional cytological and histological criteria. Diffuse proliferative lesions characterized by an increase in cell layers were diagnosed as hyperplasia, with or without slight cytological atypia. Discrete, exophytic masses were diagnosed as transitional cell or squamous cell papillomas if composed of well-differentiated cells with no evidence of invasion and none to slight atypia. Transitional cell or squamous cell carcinomas were discrete masses diagnosed as transitional cell carcinomas or squamous cell carcinomas with foci of stromal invasion. No evidence of inflammation, degeneration, or necrosis
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Most histopathological scores in all groups were low (0–2+) in all categories. Tumor incidences and mean group scores for each histopathological alteration were compared statistically at each sacrifice interval (Student’s t-test), and there were no significant differences among groups. In addition, total group mean scores, i.e., the sum of all histopathological alterations, were compared, and there were no significant differences.

Fifty-two-week Sacrifice. Bladders from each group had either focal or diffuse mural thickening or contained discrete, raised, single, to multiple, polyloid masses projecting into the lumen. The histopathological scores and morphological diagnoses for each group are presented in Table 3. Group B had higher histopathological scores in 4 of the 5 categories of alteration, as well as in the total number of animals with tumors. The increase in combined benign and malignant tumor incidence over group C was statistically significant.

Fifty-two- or 104-week Death or Sacrifice. Mean survivals were not significantly different among groups. Both clinical and pathologic observations at 52–104 weeks indicated significant tumor enhancement by HER (Table 4). The numbers of early deaths directly attributed to bladder cancer as determined by the presence of severe hematuria, anemia, and moribund status for group A, B, and C were 3/21, 7/21, and 2/21 respectively; these animals had grade 2 to 3 invasive tumors.

The urinary bladders from all test groups were enlarged, many reaching 3 cm in diameter. Most bladder walls were thickened and contained multiple raised polyloid masses with multifocal areas of hemorrhage and necrosis. The predominant histological patterns present in all groups were papillary transitional cell tumors. Areas of solid infiltrating carcinomas were also often present. There was a significant increase in tumor progression in animals receiving continuous retinoid over other groups as measured by increased tumor grade or stage. Numbers of animals with high grade or invasive tumors was also significantly increased in group B versus group C. There was no evidence of metastasis in any group.

Transplantation Experiment

As early as 1 week post-transplantation, small palpable masses were present in several animals in both groups. At 1 month post-transplantation, 6 animals in group E (retinoid) and 2 animals in group F (control) had nodules which measured 0.5 to 1.0 cm³. Animals were sacrificed at 9 months post-transplantation. Histological examinations confirmed that all measurable masses were viable-appearing transitional cell carcinoma. In 6 group E rats and 7 group F rats, no tumor was found microscopically.

There was wide variation in tumor size, however; 9 retinoid-treated versus 2 control animals had tumors greater than 1.0 cm³ and, in general, tumor behavior and morphology correlated with size. In tumors greater than 1 cm³, the cells were more densely packed and exhibited more cytological characteristics of malignancy. In addition, multiple foci of necrosis, squamous metaplasia, and invasion of surrounding tissues were present. This included extension through the thoracic wall in 2 animals in group E (retinoid). Tumors less than 1.0 cm³ were well differentiated, papillary in nature, and appeared as small circumscribed foci in the subcutaneous fat. There was no correlation between the degree of lymphocyte or plasma cell infiltrates in or around tumors and retinoid treatment. As a result of single, very large tumors present in 1 group E (213.0 cm³) and 1 group F (370.5 cm³) rat, there was no significant difference between the mean tumor sizes. Excluding these 2 animals, the means were significantly different (P = < 0.01).

DISCUSSION

The efficacy of retinoid chemoprevention of cancer remains a controversial issue, at least in part because of conflicting experimental animal data and our ignorance concerning the mechanisms of retinoid action (20). Although inhibition and chemoprevention with retinoids have received major attention, there are also reports suggesting enhanced carcinogenesis (9). Longnecker et al. (7) recently reported increased incidence of hepatocellular carcinomas induced by azaserine in female rats with retinoids. Enhancement of tracheal (10) and cheek pouch (8) carcinogenesis in hamsters and of skin papillomas in mice (6) have been reported in retinoid-treated animals. Croft et al. (4, 5) reported varying results ranging from no effect to enhancement of N-(4-(5-nitro-2-furyl)-2-thiazolylformamide-induced urinary bladder carcinogenesis with HER, 13-cis-retinoic acid, or N-ethylretinamide.

Explanations for the different results among retinoid studies are not readily apparent. Available data do not rule out the possibility that retinoid effects may be species or tissue specific or that the differences may be attributable to the various retinoids used. The reports of Croft et al. (4, 5) suggest that retinoid...
activity may vary according to the carcinogen administered. In our study, tumor enhancement was not evident until the 1- and 2-year sacrifices, which suggests a relationship between the stage of tumor progression and retinoid response. There is also evidence to suggest that levels of carcinogen exposure may be involved. Most investigators (12–18) who reported retinoid inhibition of bladder cancer in rats induced invasive tumors at 6 months to 1 year with large doses (1200–3200 mg) of BBN. Becci et al. (13) and Hicks et al. (18) both demonstrated that retinoid inhibition appears to be greater in animals receiving high carcinogenic exposures.

With the exception of our experiment and that of Hicks et al. (18), observations of bladder carcinogenesis have been limited to the relatively early stages of tumor development in animals following large carcinogen exposures. In a 2-year study, Hicks et al. (18) reported an increase in tumor latency of 7–10 weeks in retinoid-treated animals using the same carcinogen and at a comparable dose to that reported here. However, Hicks et al. used 13-cis-retinoic acid or N-ethyl-retinamide instead of HER. In our studies, no inhibitory effect upon the rate or degree of tumor development was evident, and at the later sacrifice periods, enhancement of carcinogenesis was apparent in animals receiving continuous retinoid. Significant increases in tumor incidence were observed at 1 year and in extent of tumor progression at 2 years (Tables 3 and 4). Although tumor incidences at 2 years were not significantly different among groups, objective grading and staging of lesions clearly demonstrated more advanced tumors in retinoid-treated animals. Since tumor incidences were 100% in all carcinogen-treated groups at 2 years, a considerably reduced carcinogen dose would undoubtedly be necessary to permit any effects on tumor incidence to be realized at this late stage.

Animals receiving retinoid only before and during carcinogen administration did not differ significantly at any period from animals receiving carcinogen alone. The enhancement was, therefore, apparently an effect on tumor progression rather than the early stages of carcinogenesis.

The apparent discrepancy between our findings and those reported by Hicks et al. (18) is difficult to explain. It is unlikely that the levels of retinoids used account for the difference. Hicks et al. (18) reported minor toxic effects as indicated by decreased bone length and body weight. We saw no effect on body weight and no histological evidence of toxicity; bone lengths were not measured. However, in a concurrent toxicity study in our laboratory, a level of 2.5 mM HER/kg diet induced significantly increased liver enzyme levels, and 5.0 mM HER/kg diet caused overt liver toxicity and bone fractures in less than 1 year. Therefore, the 1.5 mM/kg diet used in our carcinogenesis experiment approached a toxic level. The differences observed may be attributed to the different retinoids used or to variations in pathological methods or interpretations. Results of animal carcinogenesis studies are reported in terms of tumor incidence or progression, and such data depend upon the pathological interpretation, classification, and nomenclature used. Ultimately, standardized pathological classifications will be required to accurately compare animal cancer chemoprevention or chemotheraphy experiments.

In our study of primary transplantation into syngeneic recipients, the evidence also indicates enhancement of tumor progression in recipients receiving retinoid. In general, these animals had larger, more anaplastic tumors than did controls. Our findings may be related to several in vitro studies which have demonstrated retinoid-induced cell activation in several cell lines (2), including rat bladder tumor cells (21). In addition, enhanced anchorage independence of kidney cells (22) and plasminogen activation and secretion (23), which have been associated with tumor transplantability and invasiveness, respectively, have been induced by retinoids.

Until the basis for the different reported results are elucidated, emphasis on retinoids as being primarily inhibitors of carcinogenesis may be unwarranted. Rather, they should be considered as modifiers of carcinogenesis (and normal cellular differentiation) which vary in their effects, depending upon factors yet to be understood.

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REFERENCES

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