Inhibition of Pancreatic Carcinogenesis by Retinoids in Azaserine-treated Rats

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ABSTRACT

Chemoprevention by retinoids of the progression of carcinomas induced in rats by azaserine was evaluated. Wistar/Lewis rats were given 15 weekly injections of azaserine, 10 mg/kg, while fed a chow diet; after the completion of carcinogen treatment, they were fed a chow diet supplemented with four different retinoids at the level of 0.5 to 2 mmol/kg diet for 1 year. The incidence of neoplasms was determined by autopsy and histological study. The incidence of pancreatic carcinoma among male positive control group (azaserine treated, but not retinoid treated) was 42%. The incidence of pancreatic carcinoma among male rats treated with retinoids was: N-2-hydroxyethylretinamide, 6%; N-4-propionylxylophenylenetrinamide, 17%; and retinylidene dimedone, 12%. The incidence in rats fed these three retinoids was significantly (p < 0.05) below the control group incidence. Thus, these three retinoids appeared to be effective in inhibiting the progression of pancreatic carcinomas in the azaserine-induced model. A similar trend was demonstrated in females, but statistical significance was shown only in rats fed N-2-hydroxyethylretinamide. A fourth retinoid, N-4-carboxyphenylenetrinamide, was more toxic and less effective in chemoprevention. Since retinoids were fed after exposure to carcinogen, the effect was exerted during the postinitiation phase of carcinogenesis. The ratio of invasive pancreatic carcinomas to localized carcinomas (carcinoma in situ) was clearly higher among non-retinoid-treated rats than among those treated with retinoids. This is consistent with retarded progression in the retinoid-treated groups.

INTRODUCTION

Experimental evaluation of the effects of vitamin A-deficient and vitamin A-supplemented nutritional states on the differentiation of epithelia and the induction of carcinomas has demonstrated that retinoids can modulate the process of carcinogenesis in experimental animals. Vitamin A-deficient animals have been demonstrated to be at increased risk for development of carcinomas when exposed to moderate or low levels of chemical carcinogens (7). In contrast, animals which have been given retinoid supplements have been shown to have a diminished risk for development of some epithelial cancers when exposed to moderate initiating regimens of carcinogens (1, 9). Tissues in which inhibition of carcinogenesis has been demonstrated experimentally include breast, bladder, skin, and lung. Thus, administration of retinoids seems to offer an approach to inhibition of carcinogenesis during the latent period, i.e., during the postinitiation phase.

Chemically modified forms of vitamin A have been synthesized in order to alter distribution and toxicity (9). In view of the rising incidence and bleak outlook for early diagnosis and successful treatment of pancreatic cancer in humans (2), it seems important to evaluate the potential of retinoids for inhibiting the development or progression of pancreatic neoplasms. During the past several years, we have developed and characterized a rat model for the induction of pancreatic adenocarcinoma by azaserine (3, 6). This model provides one experimental system in which the chemopreventive potential of retinoids for pancreatic adenocarcinoma can be evaluated. The primary objective of this study was to test the hypothesis that specific synthetic retinoids which are less toxic than is vitamin A can prevent development of epithelial cancers of the pancreas. To accomplish this goal, we treated rats with azaserine and then compared the incidence and size of carcinomas and preneoplastic acinar cell lesions in the pancreas in groups of rats fed retinoid-supplemented or control diet during the postinitiation phase of carcinogenesis.

MATERIALS AND METHODS

The general design of this study was to feed rats a diet supplemented with one of 4 retinoids for 1 year following completion of carcinogen treatment.

Animal Care and Carcinogen Treatment. Male and female Wistar/Lewis rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were 3 weeks old and weighed 37.2 ± 6.0 (S.D.) g at the time of the first carcinogen injection. They were housed individually in hanging metal cages with wire mesh bottoms in a room with a 12-hr light–12-hr dark cycle maintained at 21 ± 1°C. All animals received Charles River RMH 3000 chow (Agway, Waverly, N. Y.) and deionized water ad libitum.

The rats were treated with 15 weekly i.p. injections of 10 mg of azaserine per kg of body weight (Calbiochem-Behring Corp., LaJolla, Calif.) in 0.9% NaCl solution. All animals were weighed weekly during the injection period and during rapid growth, and then twice monthly for the remainder of the study. Animals were checked daily for health. Hair loss and tumor appearance were closely monitored.

Retinoid Treatment. The animals were divided into 9 diet groups, with 25 azaserine-treated and 5 untreated males and 25 azaserine-treated and 5 untreated females in each group, as indicated in Table 1. The untreated rats served as controls for the effect of retinoid feeding. Six groups were fed either 1.0 or 2.0 mmol of either N-2-hydroxyethylretinamide, N-4-propionylxylophenylenetrinamide, or retinylidene dimedone per kg of diet. N-4-Carboxyphenylenetrinamide was fed only at 1.0 mmol/kg of diet because of limited solubility. Two groups were maintained on diets supplemented with vehicle mixtures only.

All retinoids in this study were supplied by NIH through Dr. Michael B. Sporn. The retinoids N-2-hydroxyethylretinamide, N-4-propionylxylophenylenetrinamide, and retinylidene dimedone were dissolved in a vehicle (Vehicle 1) composed of 49 ml ethanol:triiodoatrin (1:3, v/v; Tricapylin; Tridom Chemical Co., Hauppauge, N. Y.), 0.5 ml Tenox 20
The tissues were all fixed in Susa’s fixative for 6 to 18 hr and stored in buffered alcohol until embedding in paraffin. All sections were stained with hematoxylin and eosin.

The histopathology of the lesions was initially evaluated in a “semi-blind” fashion; i.e., the pancreases were reviewed in treatment groups as the slides were processed and received over a period of several weeks. Although the pathologist did not “officially” know the treatment for the various groups, familiarity with the project and the drastic differences in size and number of carcinomas encountered in one control group permitted its recognition as a non-retinoid-supplemented group. For this reason, it was considered desirable to rescreen these slides in a completely blind fashion, intermixing pancreases from rats of several groups and obscuring the animal identification number. The data reported in Tables 3 and 4 reflect these blind reviews.

Statistical Evaluation. Pancreas weights were compared using a rank sum test and tumor incidence was compared by the $\chi^2$ test.

RESULTS

After following the outlined protocol for the levels of retinoids to be fed to the various animals for several months, it became apparent that some groups were failing to thrive. This was determined by comparing their weekly weights to that of animals that were fed the corresponding Chow + Vehicle 1 + N-4-Carboxyphenylretinamide (single level) 0.5/246 214

<table>
<thead>
<tr>
<th>Dietary level (mmol)/no. of days at that level $^a$</th>
<th>Chow + Vehicle 1</th>
<th>Chow + Vehicle 1 + N-2-hydroxyethylretinamide (single level)</th>
<th>Chow + Vehicle 1 + 4-propionylxyloxyphenylretinamide (single level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow + Vehicle 1</td>
<td>0/358</td>
<td>0.75/192</td>
<td>0.75/192</td>
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<tr>
<td>Chow + Vehicle 1 + N-2-hydroxyethylretinamide</td>
<td>2.0/336</td>
<td>672</td>
<td>672</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>Low level</td>
<td>1.0/358</td>
<td>358</td>
<td>358</td>
</tr>
<tr>
<td>Chow + Vehicle 1 + 4-propionylxyloxyphenylretinamide</td>
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<td>1.5/26</td>
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<td>365</td>
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<td>Chow + Vehicle 2</td>
<td>0/358</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chow + Vehicle 2 + 4-carboxyphenylretinamide</td>
<td>1.0/91</td>
<td>214</td>
<td>214</td>
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$^a$ The total possible days of treatment were 358 (in the case of retinylidene dimedone (low level) possible days were 365 because of variation in sacrifice schedule). When the total days are fewer than 358, this indicates that the animals were “rested,” i.e., fed retinoid-free diet.

$^b$ Expressed as “mmol days,” which is defined as: diet level of retinoid in mmol x number of days fed the diet; e.g., animals fed 1 mmol/kg on all possible days, without “rest,” would have received 1 x 358 = 358 mmol days of treatment. When more than one dietary level was fed, then the calculated mmol days for each level has been summed.

The tissues were all fixed in Susa’s fixative for 6 to 18 hr and stored in buffered alcohol until embedding in paraffin. All sections were stained with hematoxylin and eosin.

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rate, but in other cases it was necessary to reduce the retinoid level in the diet to improve growth (Table 1). Even when such changes in the retinoid levels were necessary, differences in total dose of retinoids given to "high" and "low" level groups were maintained over the course of the feeding period (Table 1).

The pancreas weights of azaserine-treated rats were larger than the pancreas weights from rats which had not received azaserine. As shown in Table 2, the mean pancreas weights in the Vehicle 1 azaserine-treated groups were: male, 4.79 g; female, 1.97 g. The sex difference in response to azaserine (male > female) is reflected in the weights of the pancreases. Pancreas weights were significantly smaller in 11 of 14 retinoid-treated groups in comparison with the appropriate vehicle-treated controls (Table 2).

Eighty-nine rats which were not given azaserine survived for 66 weeks. There were no neoplasms in the pancreas of these rats, but 1 to 4 AACN\(^3\) were present in 16 of 45 females and 29 of 44 males. The incidence of AACN was lower among retinoid-fed than among vehicle-fed rats, but the reduction was not significant (\(x^2\) test).

The azaserine-induced histological lesions of the pancreas have been tabulated in 3 categories (Tables 3 and 4). These are adenomas, localized adenocarcinomas (CIS), and invasive adenocarcinoma. AACN are focal abnormalities of acinar cell differentiation which represent the earliest manifestation of carcinogen-induced growth abnormalities in the pancreas. They were present in the pancreases of all azaserine-treated rats; therefore, their incidence is not reported in the tables. Adenomas are defined as well-differentiated encapsulated acinar cell lesions which appear to be growing in expansile fashion. Localized carcinomas are lesions with a degree of acinar cell atypia similar to that of the lesions which are invasive adenocarcinomas. Adenocarcinomas are those lesions in which there is evidence of local invasion or metastasis to lymph...

\(3\) The abbreviations used are: AACN, atypical acinar cell nodules; CIS, carcinoma in situ.
nodes, liver, or lungs. The origin and progression of these lesions are discussed in detail elsewhere (6). In many instances, individual pancreases contain several lesions. Each pancreas has been scored only once in the category of the most advanced lesion which was found in the pancreas, and we have not reported the presence of lesions of more than one category in a single pancreas or the number of lesions per pancreas.

The incidence of pancreatic carcinoma was significantly reduced among male rats fed N-2-hydroxyethylretinamide, retinylidene dimeredone, and N-4-propionyloxyphenylretinamide. The incidence of carcinomas was always lower among the rats fed the higher level of these retinoids compared with rats fed the lower level. Conversely, the incidence of adenomas and CIS was generally higher among these retinoid-treated groups than among the positive non-retinoid control groups. The incidence of pancreatic carcinomas in the groups treated with N-4-carboxyphenylretinamide was higher than among corresponding positive controls.

Table 3 shows the incidence of liver metastasis from pancreatic carcinomas in azaserine-treated male rats fed both control and retinoid-supplemented diets. The reduction of liver metastases in the retinoid-supplemented groups can be regarded as an index of the effect of the retinoids on the progression of the pancreatic lesions, although the numbers are too small to demonstrate significance.

One unexpected finding has been an apparently significant difference in incidence of pancreatic carcinomas among the 2 non-retinoid-fed male control groups which received diets supplemented with 2 different vehicles (Table 3). Vehicle 2 contained 14% propylene glycol and 14% glyceryl monooleate which were not present in Vehicle 1. Vehicle 2 also contained a lower concentration of trioctanoin. The mean final body weights for the rat groups receiving the 2 vehicles were similar, and we do not regard caloric restriction as a possible explanation for the reduced tumor incidence in rats fed Vehicle 2.

The incidence of pancreatic carcinomas in female rats was lower among all retinoid-fed groups than among their controls,
but a significant reduction ($p < 0.05$) of incidence of invasive carcinomas was demonstrated for only one of the retinoids (Table 4). When one considers the combined incidences of adenomas, CIS, and carcinomas, as shown in Table 4 in the column labeled "all" neoplasms combined, a significant reduction in incidence of neoplasms is shown in 3 of 6 retinoid-treated groups which are compared with the Vehicle 1 control group in which the incidence was 0 of 24. Although these data also indicate a significant reduction in the incidence of all neoplasms among female rats treated with Vehicle 1 controls, then a significant reduction in incidence is demonstrated in rats treated with retinoid-supplemented group. These differences in the 2 groups with an incidence of 50%.

Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Adenomas</th>
<th>CIS</th>
<th>Carcinoma</th>
<th>All combined</th>
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</thead>
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<tr>
<td>Vehicle 1</td>
<td>24</td>
<td>50</td>
<td>8</td>
<td>12.5</td>
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<td>N-2-Hydroxyethylretinamide</td>
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<td>25</td>
<td>25</td>
<td>0</td>
<td>50</td>
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<tr>
<td>High</td>
<td>24</td>
<td>12.5</td>
<td>12.5</td>
<td>0</td>
<td>25</td>
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<tr>
<td>N-4-Propionyloxyphenylretinamide</td>
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<td></td>
</tr>
<tr>
<td>Low</td>
<td>24</td>
<td>17</td>
<td>21</td>
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<td>37.5</td>
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<tr>
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<td>24</td>
<td>29</td>
<td>4</td>
<td>4</td>
<td>37.5</td>
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<tr>
<td>Retinylidene dimedone</td>
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</tr>
<tr>
<td>Low</td>
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<td>58</td>
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<td>High</td>
<td>24</td>
<td>33</td>
<td>17</td>
<td>0</td>
<td>50</td>
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<tr>
<td>Vehicle 2</td>
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<td>48</td>
<td>4</td>
<td>87</td>
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<tr>
<td>N-4-Carboxyphenylretinamide</td>
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<td>37.5</td>
<td>8</td>
<td>0</td>
<td>46</td>
</tr>
</tbody>
</table>

None of the individual retinoid-fed groups has a significantly reduced incidence of carcinoma; e.g., for an incidence of 3 of 24 versus 0 of 24, $p = 0.07$. However, when high- and low-dose groups for N-2-hydroxyethylretinamide, N-4-propionyloxyphenylretinamide, and retinylidene dimedone are combined and compared to Vehicle 1 controls, then a significant reduction in incidence is demonstrated in rats treated with Vehicle 2 controls (3 of 24 versus 0 of 48, $p = 0.012$) and all retinoid-fed groups combined (3 of 24 versus 2 of 142, $p = 0.003$).

Significantly reduced incidence with $p < 0.05$ by $\chi^2$ test; $p = 0.14$ in the 2 groups with an incidence of 50%.

The discussion of the current experiment provides a better test of the ability of the retinoids to suppress progression of established transformed clones rather than to suppress malignant transformation. These actions are regarded by Sporn as possibly separate effects in chemoprevention (9). Carcinogen treatment continued for 15 weeks before retinoid treatment was instituted. Previous characterization of the azaserine-induced model of pancreatic carcinogenesis in rats has demonstrated that AACN (some of which are putative transformed clones) can develop within 2 months following carcinogen treatment (5 and that young rats are more responsive than older rats to the induction of AACN by 5-week regimens of azaserine treatment (4). Therefore, we can conclude that the majority of AACN were established before initiation of carcinogen feeding in the experiments reported here. It is not surprising the AACN were prominent in all retinoid-supplemented azaserine-treated rats and that it was only the incidence of more advanced neoplastic lesions which was suppressed in these groups.

The principal problem experienced in evaluating the chemopreventive potential of the 4 retinoids used in this study was the toxicity of the compounds. Inhibition of growth which was evident with the high dose levels of the more toxic retinoids may reflect a reduced caloric intake which in itself has been demonstrated to be inhibitory to carcinogenesis (10). In the azaserine model, we have demonstrated that caloric restriction reduces the incidence of neoplasms but has a greater impact on the initiation phase than on the postinitiation phase of carcinogenesis (8). While reduced caloric intake may contribute to the reduced incidence of pancreatic neoplasms in the rats fed high levels of the retinoids, we do not believe that this...

**Inhibition of Pancreatic Carcinogenesis by Retinoids**

The rat model for pancreatic carcinogenesis induced by azaserine has provided a suitable system in which to evaluate the effect of several retinoids on pancreatic carcinogenesis. The greater sensitivity of male rats to the carcinogenicity of azaserine as compared with that of females is readily apparent in the tumor incidence data. The progression of azaserine-induced lesions from small foci of atypical acinar cells to nodules, localized carcinomas, and invasive carcinomas has been well characterized (3, 6). The retinoids appear to have retarded this overall process. In males, the lesions progressed more rapidly than in females, and the principal difference between retinoid- and non-retinoid-treated males was manifested as a reduction in the most advanced lesions, i.e., carcinomas. In females, the lesions progressed more slowly, and there was evidence of a diminished incidence of earlier stages of neoplastic progression.

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is of major concern in the low-dose groups or that it should be interpreted as the sole basis for inhibition of carcinogenesis.

Evaluation of the chemopreventive potential of 4-carboxyphenylretinamide was compromised by the low incidence of pancreatic carcinomas in the Vehicle 2 azaserine-treated-positive male control group. We regard this low (4%) incidence as spurious or as an unexpected effect of components of Vehicle 2 on tumor progression. The higher incidence of pancreatic carcinomas observed in the Vehicle 1 azaserine-treated group is in the range which was anticipated on the basis of our previous experience with this model.

We have previously reported a preliminary study in which retinyl acetate reduced the number and size of the azaserine-induced lesions in the pancreas (6). That study, together with the results reported here, provides a basis for adding pancreas to the list of epithelial tissues in which chemoprevention of carcinogenesis by retinoids has been demonstrated.

In summary, 3 retinoids appear to have inhibited the progression of azaserine-induced pancreatic neoplasms in Wistar/Lewis rats. In the retinoid-fed rats, the incidence of carcinomas was decreased in males; the incidence of adenomas, CIS, and carcinomas (combined) was decreased in females; and the average pancreatic weight was reduced in retinoid-treated groups of both sexes. The effective retinoids were retinylidene dimedone, N-2-hydroxyethylretinamide, the N-4-propionyloxyphe-nylretinamide. The N-4-carboxyphenylretinamide was more toxic and less effective in chemoprevention.

ACKNOWLEDGMENTS

We thank Michael B. Sporn and James D. Yager, Jr., for suggestions and critical discussion during the design and implementation of these experiments. We are indebted to Denise Stevens and Denise Primavera for their meticulous attention to detail in day-by-day supervision of the animal colony and diet mixing and to Joan Johnson for her excellent histological preparations.

REFERENCES

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