Influence of Retinoids on Growth and Metastasis of Hamster Melanoma in Athymic Mice

Rosemary L. Schleicher,2 Richard C. Moon, Minu K. Patel, and Craig W. Beattie3

ABSTRACT

The effects of 2-hydroxyethyl retinamide, N-(4-hydroxyphenyl)-all-trans-retinamide, and 13-cis-retinoic acid on the growth and metastasis of a malignant hamster melanoma cell line HM1-F5 was determined in a double blind study using 4- to 5-week-old male NIH Swiss and BALB/c derived athymic nu/nu mice. Mice were fed retinoids (0.75 and 1.0 or 1.5 mmol/kg diet) or a placebo diet ad libitum beginning on the day of s.c. inoculation of 5 x 10⁵ HM1-5 cells. Tumor incidence, latency, and growth rate were similar in both strains of mice. All placebo-treated mice had lung metastasis on the day of autopsy, although the total number of metastases was lower in NIH Swiss derived athymic mice. While mean tumor incidence and latency were not significantly altered by any retinoid treatment, tumor growth rate (volume) and final tumor weight were significantly reduced (P < 0.05) by 0.75 mmol/kg 13-cis retinoic acid and 1.5 mmol/kg N-(4-hydroxyphenyl)-all-trans-retinamide. In contrast, at 1.0 or 1.5 mmol/kg diet, 2-hydroxyethyl retinamide had no significant effect on tumor growth rate. 13-cis retinoic acid, 0.75 mmol/kg, 2-hydroxyethyl, 1.0 mmol/kg, and N-(4-hydroxyphenyl), 1.0 mmol/kg significantly reduced the mean number of metastatic lesions in NIH Swiss derived mice, but N-(4-hydroxyphenyl) all-trans-retinamide also reduced metastatic incidence while 2-hydroxyethyl retinamide and 13-cis retinoic acid had no effect. A concentration of 1.5 mmol/kg diet of 2-hydroxyethyl and N-(4-hydroxyphenyl) all-trans-retinamide significantly reduced the overall number of gross lung metastases in BALB/c and Swiss mice, and mean numbers of metastases in Swiss mice. Analysis of correlation indicated that the inhibitory effect of high-dose N-(4-hydroxyphenyl) and 2-hydroxyethyl retinamide on metastasis was not associated with (independent of) any inhibitory effect on primary tumor invasiveness or growth rate. Our observations suggest that agents such as retinoids have an antimetastatic potential.

INTRODUCTION

Retinol (vitamin A) and its derivatives exhibit antineoplastic effects in vivo. Murine tumor growth in allogenic and syngeneic hosts (1-6) as well as human tumor xenografts in athymic mice (7-9) are significantly reduced by retinoids. The mechanism by which retinoids exert their antineoplastic activity is unknown. The antiproliferative, differentiating activity of retinoids on a wide variety of murine (5, 10-12) and human (7, 8, 13-17) tumor cells in vitro and apparent lack of effect on NK cell activity in athymic mice (8, 9) suggest that mechanisms other than enhancement of host immunity play a role in retinoid inhibition of tumor growth (for reviews, see Refs. 18 and 19). This may be particularly true in metastatic spread where factors other than host immunity alter metastatic incidence (20-24). Retinol apparently inhibits the formation of metastasis following i.v. inoculation of tumor cells in athymic mice (9), but a rigorous assessment of whether metastatic spread is altered by retinoids following s.c. inoculation of tumor cells and independently of primary tumor growth rate or host immune status has not been made.

We have used a highly invasive malignant melanoma of hamsters (HM1-F5) which rapidly metastasizes to lung in adult athymic mice (25) to determine whether retinoids alter metastatic incidence. Since strain differences in response to different retinoids may exist, we conducted identical experiments on NIH BALB/c and Swiss derived athymic mice. This study suggests that retinoids inhibit metastasis possibly independent of effects on primary tumor latency and growth.

MATERIALS AND METHODS

Animals. Four- to 5-week-old male NIH BALB/c and Swiss derived athymic mice were obtained from the animal production area of the National Cancer Institute-Frederick Cancer Facility (courtesy, Dr. Clarence Reeder). Mice were maintained under specific pathogen-free conditions throughout the study.

Tumor. A malignant hamster melanoma cell line HM1-F5 which is locally invasive and metastasizes selectively to the lung of hamsters and athymic mice was used in these studies. Characteristics of this cell line have previously been reported (25). Briefly, cells were removed from culture with trypsin-EDTA (26) and washed with trypsin-EDTA-free media. Mice were inoculated s.c. with 5 x 10⁵ cells in the right flank and killed 38 days later.

At necropsy, lungs were immediately dissected free of the pleural cavity, washed in saline, and perfused with 10% neutral formalin. After 24-h fixation, each lung was examined under a dissecting microscope and the number of gross tumor nodules counted and recorded by three independent observers. The correlation between observers was virtually complete.

Retinoids. 2HER, 4HPR, and 13-cis were dissolved in ethyl alcohol:triacsin containing Tenox (antioxidant) and σ-tocopherol prior to mixing with powdered Wayne Lab Blox (Allied Mills) as previously described (27). New diet was mixed twice/month and retinoid levels were monitored at that time following extraction (28). Since sterilization can reduce 4HPR activity by approximately 30% (Moon, R. C. et al., unpublished observations), retinoids were mixed subsequent to sterilization in laminar flow hoods. Control groups received sterilized diet supplemented with ethanol, triacsin containing Tenox, and σ-tocopherol (vehicle for retinoids). Mice ingested approximately 0.75 mmol 13-cis and 1.0 or 1.5 mmol 2HER or 4HPR/kg diet during the course of an experiment.

Statistics. Retinoid effects on mean tumor latency and weight were calculated by ANOVA followed by the Student-Neuman-Keuls test for differences between groups (29). Total number of lung lobes with metastasis in each treatment group were analyzed by χ² (29). Metastatic incidence was analyzed using a generalized categorical data analysis of weighted least squares approach in which three factors presence of
metastasis, mouse strain, and treatment were analyzed simultaneously (30). x2 goodness of fit suggested that the model was appropriate (29, 30). Tumor growth was estimated from semilogarithmic plots of tumor volume. Differences in growth rate were then determined by analysis of covariance using a computerized format (BMDP/V.2, UCLA).

A correlation analysis CORPAIR (BMDP8D, UCLA) was used to determine whether the antimitotic effects of 2HER and 4HPR resulted from a lower metastatic rate subsequent to a generalized inhibitory effect on primary tumor growth or were exerted independently of an inhibition in tumor growth rate (volume). Tumor growth was analyzed with and without log transformation. A value of 0.009 cm3 was selected as a volume for those tumors too small to measure accurately (<2–3 mm diameter).

RESULTS

Retinoid Toxicity. Body weight of tumor-free NIH Swiss derived athymic mice (10/group) was not significantly altered when either 1.0 mmol/kg diet 2HER and 4HPR or 0.75 mmol/kg diet 13-cis was incorporated into their diet over a 5-week test period. Food consumption of individual mice was not measured in the present study. While it is possible that the extent of response to a particular diet may be dependent on the amount of food (or chemopreventive agent) consumed by mice, all received an equal inoculum of cells and were exposed to equal risk of developing a tumor. Thus, any observed antiproliferative activity can be attributed to the retinoid consumed. All mice received either control or retinoid containing diet at the time of inoculation of HM1-F5 cells. The effect(s) of retinoids on tumor behavior were assessed in two independent studies. In an initial pilot study to assess potential toxicity in tumor-bearing mice, mean body weight of 2HER- and 4HPR-treated mice was slightly higher (~15%) than control at the time of necropsy. The small decrease (10%; N = 5; P < 0.1) in body weight of mice fed 13-cis was uniformly accompanied by skin scaling, crusting of the eyes, and general malaise. Increasing the dose of 13-cis increased the severity of these side effects and resulted in a generalized morbidity. In contrast to 13-cis, neither 1.0 nor 1.5 mmol/kg diet 2HER and 4HPR had a significant effect on body weight or gross appearance.

Tumor Growth. Mean tumor latency in NIH Swiss derived male athymic mice in the pilot study was not altered by 1.0 mmol/kg 2HER, 4HPR, or 0.75 mmol/kg diet 13-cis (Table 1). Tumor growth over the first 30 days following inoculation of 5 × 10^5 HM1-F5 cells s.c. was significantly reduced by 13-cis but not 4HPR or 2HER whose growth curves paralleled that of mice on placebo diet (data not shown). Although metastatic incidence was not significantly reduced from that in the placebo group by either 13-cis or 2HER in this initial study, the mean number of gross lung metastasis (per lung) was lower in all groups in the order 4HPR > 2HER > 13-cis (Table 1). The relative toxicity, significant decrease in primary tumor growth, absence of a significant decrease in metastatic incidence, and highest mean number of lung metastasis of the three treatment groups (Table 1) prompted us to delete 13-cis from subsequent studies with a higher dose of 2HER and 4HPR in significantly larger groups of athymic mice.

In the latter study, tumor incidence was 100%. Tumor latency and relative growth rates were not significantly different in NIH BALB/c and Swiss derived athymic mice (Table 2; Fig. 1) receiving the control diet. Addition of 1.5 mmol/kg of 2HER or 4HPR to the diet of each strain 1 week prior to the inoculation of HM1-F5 cells had no effect on tumor latency (Table 2). 2HER inhibited tumor growth (volume) slightly, but individual variance about later time points precluded significance (Figs. 2 and 3). Tumor weight was not significantly reduced by 1.5 mmol/kg diet 2HER. In contrast, tumor growth rate (volume) was inhibited by 1.5 mmol/kg 4HPR in Swiss and BALB/c mice (Figs. 2 and 3). The inhibitory effect of 4HPR on tumor volume was reflected by a significant decrease in final tumor weight in both strains of mice (Table 2).

Metastasis. The total number of lung metastasis in NIH Swiss derived mice was one-half that observed in BALB/c derived mice (Table 3). 2HER and 4HPR significantly reduced the overall number of gross lung metastasis in both strains and mean number of metastases per lung in Swiss derived mice (Table 3). The relative decrease in mean number of metastases

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor latency time (days)</th>
<th>Tumor wt, g (%)</th>
<th>Tumor wt, g (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>11.0 ± 0.4</td>
<td>4.0 ± 0.3</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>2HER</td>
<td>11.5 ± 0.4</td>
<td>3.5 ± 0.4</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>4HPR</td>
<td>10.3 ± 0.5</td>
<td>2.5 ± 0.3</td>
<td>3.4 ± 0.4</td>
</tr>
</tbody>
</table>

* Percentage decrease from placebo. ** Mean ± SE. *** P < 0.02 versus placebo (ANOVA). **** P < 0.05 versus placebo (ANOVA).
in 2HER and 4HPR-treated Swiss derived mice was greater than that in BALB/c mice, perhaps reflecting the smaller overall lung tumor burden in this strain.

This strain difference in response to 2HER and 4HPR was also reflected in the number of NIH Swiss mice with a decrease in overall tumor burden (number of mice with more than 15 lesions) and increase in the number of mice without any evidence of gross (and microscopic) lung metastasis (Table 3).

When lungs were divided into individual lobes and reexamined (Table 4), the inhibitory effect of 4HPR on metastatic incidence was even more pronounced. The number of mice with total metastatic involvement of a lung lobe by tumor was significantly decreased in BALB/c derived mice as well as the total number of lung lobes with at least one gross metastasis present. 4HPR also increased the total number of lung lobes without grossly observable metastasis in NIH Swiss derived mice. The question of whether the antimetastatic effects of 2HER and 4HPR were independent of a general inhibition of primary tumor growth in our second study (Tables 2–4; Figs. 1–3) was addressed using analysis of correlation. The inhibitory effects of 2HER and 4HPR on metastatic incidence were independent of any effect on primary tumor growth as the values were outside the 95% confidence interval.

This result was also suggested by the lack of significant effect of 2HER on tumor volume and weight in both NIH BALB/c and Swiss derived mice (N = 22) (Tables 2 and 3). The significant decrease in metastatic incidence with 4HPR in both strains of athymic mice was also independent of final tumor weight and degree of invasiveness because HM1-F5 tumors in all mice (control diet, 2HER, and 4HPR treated) had infiltrated the area of hind limb underlying the injection site and adjacent body wall musculature (25).

**DISCUSSION**

The mechanism(s) by which synthetic retinoids inhibit the growth of transplantable tumors remains unknown. Our observations confirm that selected retinoids inhibit primary tumor growth (7, 8), but more importantly, they also appear to reduce metastatic incidence in athymic mice in the absence of any significant effect on primary tumor latency or subsequent growth. Thus, the present study provides the first experimental evidence that retinoids exhibit antimetastatic activity when tumors progress through the entire metastatic process (31) and confirm previous observations of a decrease in metastasis following i.v. inoculation of tumor cells (8).

Since the antimetastatic and growth inhibitory effects may be dependent on the strain of mouse selected as well as structural modifications in individual analogues, our studies were carried out in two strains of athymic mice. Tumor latency and growth were similar in NIH BALB/c and Swiss derived (nu/nu) mice; however, the overall metastatic incidence in Swiss derived nu/nu mice was one-half that in BALB/c derived mice.

### Table 3 Incidence of gross metastasis in lungs of NIH BALB/c and Swiss derived athymic mice

<table>
<thead>
<tr>
<th></th>
<th>BALB/c</th>
<th>Swiss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>2HER</td>
</tr>
<tr>
<td>No. of mice</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Total no. of lesions</td>
<td>207</td>
<td>119</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>7.6 ± 1.6a</td>
<td>6.3 ± 1.9</td>
</tr>
<tr>
<td>No. of metastasis/lung</td>
<td>3/12</td>
<td>3/11</td>
</tr>
<tr>
<td>No. of mice with more than 15 lung metastasis</td>
<td>0/12</td>
<td>3/11</td>
</tr>
</tbody>
</table>

*a Mean ± SE.

b P < 0.05 versus placebo (ANOVA).

c P < 0.01 versus placebo (ANOVA).

d P < 0.05 versus respective placebo group (30).
There are several tenable explanations for this observation: (a) mechanical factors could lead to xenograft tumors behaving more aggressively in one strain than another (22, 26). This is unlikely because all injections were at the same site, all tumors were locally invasive (32), and had similar degrees of vascularization and virtually identical latency and growth rates (23, 24); (b) relatively small differences in mouse age at the time of tumor cell inoculation could substantially alter the level of NK cell activity and in turn metastatic incidence (33). This is also unlikely as all mice were inoculated at an identical age (5 weeks) when NK cell activity has been reported to be essentially at adult levels (34) and NK cell activity in Swiss derived athymic mice is reportedly 10% higher than age-matched BALB/c derived mice (35). At present, it would appear that a combination of intrinsic tumor cell factors and genetic and environmental host factors is the most reasonable explanation for the decrease in metastasis in randomly outbred Swiss derived nu/nu mice. A decrease in host immunocompetence in the presence of a heavy tumor burden has previously been observed in immunocompetent mice and man (36–38). Our observations also suggest that the significant decrease in primary tumor growth and metastatic incidence in mice receiving 4HPR is directly related to the structure (activity) of the analogue and retinoid sensitivity of the HM1-F5 cells. Previous reports have demonstrated that retinoids inhibit tumor cell growth in vitro (5, 7, 8, 10, 11, 15, 16). The antiproliferative effect in vitro appeared dependent on the number of retinoid responsive cells in the heterogeneous cell lines used and the potency of the analogue tested. This may also be true in vivo. The significant decrease in late log phase growth of HM1-F5 cells in BALB/c and Swiss derived nu/nu mice fed 4HPR suggests that these cells may be differentially responsive to 4HPR even though they have been repeatedly selected for their ability to metastasize to lung (25). This hypothesis of inherent structural activity and a heterogenous population of cells expressing differential sensitivity to retinoids dictating the resultant activity of a compound receives support from the relatively minor effect of 2HER on HM1-F5 growth (final tumor weight) at the doses tested under identical conditions.

The antimitastatic activity of these compounds appears to be independent of a generalized reduction on the growth rate of a primary tumor as suggested by the significant antimitastatic effect of 4HPR in the presence of a heavy metastatic burden, no significant change in tumor latency, and less than 50% reduction in final tumor weight. The reduction in tumor growth with 13-cis and apparent lack of effect on metastatic incidence support this observation, although the toxicity of 13-cis at the dose selected complicates the interpretation of its effect. More importantly, the significant reduction in the overall number of metastasis per lung in the absence of an inhibitory effect on tumor latency, growth or final weight, and lack of toxicity in 2HER-treated mice lends credence to our observation.

In sum, our data support the hypothesis that selected retinoids are able to exert antiproliferative and antimitastatic activity in an immunocompromised host.

### Table 4: Effect of 2HER and 4HPR on metastatic incidence based on examination of individual lung lobes

<table>
<thead>
<tr>
<th>BALB/c</th>
<th>Swiss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo 2HER</td>
<td>4HPR</td>
</tr>
<tr>
<td>No. of mice with total involvement of a lung lobe</td>
<td>5/12</td>
</tr>
<tr>
<td>Total no. of lung lobes with metastasis</td>
<td>36/60</td>
</tr>
</tbody>
</table>

* a P < 0.01 4HPR versus placebo by χ².
* b P < 0.05 4HPR versus placebo by χ².
* c P < 0.002 4HPR versus placebo by χ².

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