Inhibition of Ultraviolet-B Skin Carcinogenesis by All-trans-retinoic Acid Regimens That Inhibit Ornithine Decarboxylase Induction

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ABSTRACT

There is a correlation between the ability to induce the polyamine-biosynthetic enzyme ornithine decarboxylase (ODC) and the tumor-promoting ability of various carcinogens in mouse epidermis. Some agents which inhibit skin carcinogenesis also inhibit ODC induction. In this study, all-trans-retinoic acid (RA) regimens that inhibited the induction of epidermal ODC by ultraviolet-B (UVB) were tested for their ability to inhibit UVB skin carcinogenesis.

Hairless mice were irradiated once daily with UVB for 20 days, receiving a total dose of UVB (17.1 kJ/sq m). Topical RA was applied immediately (RA, one dose) or applied 0, 1, 2, 3, and 4 hr (RA, five doses) after each irradiance. The mice were maintained for 52 weeks and then sacrificed. Groups treated with RA tended to have fewer mice with tumors, fewer tumors per mouse, smaller tumor diameters, and slower growing tumors than did appropriate irradiated control groups. RA given five times was more effective than was RA given one time at inhibiting UVB skin carcinogenesis. These results show that RA treatments that inhibit epidermal ODC induction may be effective in reducing the carcinogenicity of UVB.

INTRODUCTION

UV radiation from the sun is a major causal factor in human skin cancer (1), and similar skin cancers can be induced in laboratory animals with a solar simulator or other UV source (9). The most carcinogenic wavelengths of UV radiation probably lie in the sunburn or UVB region (290 to 320 nm) of the solar spectrum (9). Exposure to UVB results in several acute changes in epidermal biochemistry and physiology similar to those found after treatment with chemical carcinogens or tumor promoters and which may be indicative of the carcinogenic process. Thus, exposure to UVB results in hyperplasia, disruption of DNA synthesis, and induction of the polyamine-biosynthetic enzymes ODC (EC 4.1.1.17) and S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50) in the epidermis (10, 12, 13).

There is a correlation between the ability to induce ODC and the tumor-promoting potensity of skin carcinogens. Epidermal ODC induction may be an obligatory event in mouse skin carcinogenesis by chemical tumor promoters (14). Agents which inhibit epidermal ODC induction by chemical carcinogens are also inhibitors of tumorigenesis. Several types of antitumor agent have been shown to inhibit the induction of epidermal ODC by tumor-promoting phorbol esters, including retinoid derivatives (3) and prostaglandin synthesis inhibitors such as indomethacin (16), which supports the hypothesis that ODC induction is an essential step in the carcinogenic process.

We have shown recently that topical RA inhibits the induction of epidermal ODC by UVB in the hairless mouse (11). The time and number of applications of topical RA required to inhibit UVB induction of ODC in this system were crucial, maximum inhibition being achieved with 5 applications of RA (3.4 nmol) applied 0, 1, 2, 3, and 4 hr after UVB (11).

Although the ability of RA to prevent chemical carcinogenesis in mouse skin and to regress skin cancers in some human subjects has been established (2), studies of its effect on UV-induced carcinogenesis in the hairless mouse have produced apparently conflicting results. Forbes et al. (8) reported that topical RA enhanced the carcinogenicity of UV radiation from a solar simulator. Epstein (5) found originally that 0.3% RA enhanced the carcinogenicity of UV from a hot quartz source but reported more recently that, by using lower concentrations of RA, there was either no enhancement or inhibition of UV carcinogenesis (6).

The present study was designed to investigate the modulating effect, if any, of topical RA on UVB skin carcinogenesis with RA treatments shown previously to be effective in reducing UVB induction of epidermal ODC in the hairless mouse (11).

MATERIALS AND METHODS

Mice

Four-to-6-week-old albino female SKH/hr 1 mice were used at the start of the study.

Chemicals

RA was obtained from Sigma Chemical Co. (St. Louis, Mo.).

UV Source

The source used was a bank of 8 Westinghouse FS40 sunlamps with a peak irradiance at 313 nm, mounted 16 cm above the mouse dorsal skin. The light from the sunlamps was filtered through cellulose triacetate to remove contaminant ultraviolet-c (i.e., wavelengths below 290 nm).

Irradiation of Mice

Groups of mice were irradiated 5 times per week for 4 weeks with gradually increasing amounts of UVB, rising from one MED (0.30 kJ/sq m) on the first day to 2 MED (0.60 kJ/sq m) on the second day and 3 MED (0.90 kJ/sq m) on all subsequent days. The irradiation time was 0.65 min/MED, and UVB was delivered at a fluence rate of 7.7 J/sq m/sec. Each mouse received a cumulative dose of 17.1 kJ/sq m.

UVB Tumor Study

Six groups of mice (30/group) were treated as follows. Group 1...
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[UVB plus acetone (one dose)] received topical acetone (0.1 ml) on the back immediately after each daily irradiance with UVB. Group 2 [UVB plus RA (one dose)] received topical RA (3.4 nmol in 0.1 ml acetone) on the back immediately after each daily irradiance with UVB. Group 3 [UVB plus acetone (5 doses)] received topical acetone (0.1 ml) on the back 0, 1, 2, 3, and 4 hr after each daily irradiance with UVB. Group 4 [UVB plus RA (5 doses)] received topical RA (3.4 nmol in 0.1 ml acetone) on the back 0, 1, 2, 3, and 4 hr after each daily irradiance with UVB. Group 5 [RA (5 doses)] received 5 treatments with topical RA (3.4 nmol in 0.1 ml acetone) on the back daily at the same time as Groups 3 and 4 but was not irradiated with UVB. Group 6 (acetone only) received acetone (0.1 ml) on the back at the same time as Group 1 but received no UVB radiation and acted as unirradiated controls. Mice were inspected visually at monthly intervals for the appearance of suspected tumors and papillomas (1 mm in diameter or larger) to determine the time of onset of tumors. At the termination of the experiment, all the mice were subject to a thorough inspection, and any probable tumors were excised for histological examination and assigned a grade score.

### Histology

Punch biopsy samples of skin were taken after the final irradiation or topical treatment to observe the short-term effect of chronic treatment with UVB and RA on the epidermis.

After 52 weeks, all the surviving mice were killed, and biopsies were taken of suspected tumors to assign a cancer grading and to observe any histological changes in representative skin samples from each group. Tissues were fixed in formalin, embedded in paraffin, and sectioned and stained with hematoxylin and eosin. On histological examination of suspected tumors and papillomas, 3 types of lesions were observed (arranged in increasing order of malignant potential).

**Grade 1, Squamous Papillomas.** In these lesions, there was acanthosis of the epidermis, orthokeratotic hyperkeratosis of the stratum corneum, and a varying degree of papillomatosis. No atypical keratinocytes were seen.

**Grade 2, Atypical Keratoses.** Actinic keratoses and digitate keratoses showed atypical keratinocytic hyperplasia, present most prominently in the lower one-half of the epidermis. The epidermis was usually acanthotic, and the keratinocytes extended into the papillary dermis in "bud-like" fashion. The degree of papillomatosis present was variable, being more prominent in the digitate keratoses. The atypical keratinocytes contained pleomorphic nuclei, many of which were hyperchromatic or pyknotic. Occasional mitotic figures were seen.

**Grade 3, Squamous-Cell Carcinomas.** In these lesions, there was a neoplasia composed of nests and islands of atypical keratinocytes present within the reticular dermis. The atypical keratinocytes showed marked nuclear pleomorphism and abundant eosinophilic cytoplasm with scattered intercellular bridges between the cells. Numerous mitoses were present, and scattered dyskeratotic cells that showed individual cell keratinization were seen. The overlying stratum corneum was hyperkeratotic and contained parakeratosis.

### RESULTS

**Time Course of Appearance and Growth of Tumors.** Tumor growth in the treated areas for the UVB plus acetone (one dose) and UVB plus RA (1 dose) groups is shown in Chart 1. Tumors grew more slowly and were fewer in number for the RA-treated groups compared to the appropriate UVB plus acetone control groups. There was a slight delay (4 to 6 weeks) in the time of appearance of tumors in the retinoid acid-treated groups, but since detection of tumors was by visual inspection and only growths larger than 1 mm in diameter were recorded, this is probably due to the statistically significant slower growth of tumors in the RA-treated mice. Linear regression analysis comparing tumor diameters with time gave good correlations as shown in Chart 1; the lines generated almost intersect at the abscissa. Similar trends were obtained for the UVB plus acetone (5 doses)-treated group (data not presented), although because of the much lower overall tumor yield (only 2 tumors were detected), the correlation for the UVB plus RA (5 doses) group was nonlinear.

**Tumors Present after 52 Weeks.** These data are summarized in Table 1. The number of mice with tumors, the total number of tumors, and the mean tumor diameters tended to be lower in the RA-treated groups (Groups 2 and 4) than the vehicle only-treated irradiated control groups (Groups 1 and 3) (Table 1). The number of mice with tumors in the UVB plus RA (5 doses) was statistically significantly lower ($p = 0.05$) than in the appropriate control group.

![Chart 1. Effect of RA on the growth of skin tumors induced by UVB. Groups of mice were inspected visually at 4-week intervals for suspected tumors larger than 1 mm in diameter, and the mean tumor diameters were determined. The straight lines were generated by linear regression analysis. Data presented are for Group 1, UVB plus acetone (one dose) (Δ) (correlation coefficient = 0.96), and for Group 2, UVB plus RA (one dose) (△) (correlation coefficient = 0.97). The tumor growth of the RA-treated mice (Group 2) was statistically significantly lower ($p < 0.0005$) than that of the controls (Group 1). The fluctuations in the tumor diameters reflect a combination of spontaneous regression of tumors, the merging of adjacent tumors, and the tumor growth. The apparent discrepancy in the mean tumor diameters at 52 weeks presented here and in Table 1 reflects the elimination of data from suspected tumors, subsequently diagnosed histologically to be small dermal cysts, from the latter.

<table>
<thead>
<tr>
<th>Group</th>
<th>% of surviving mice with tumors</th>
<th>Tumor diameter (mm)</th>
<th>Histological type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1    2    3   Total</td>
</tr>
<tr>
<td>1. UVB + acetone (one dose) $n = 27^a$</td>
<td>30</td>
<td>9.9 ± 8.1$^b$</td>
<td>2    5    1   8</td>
</tr>
<tr>
<td>2. UVB + RA (one dose) $n = 26$</td>
<td>15</td>
<td>4.5 ± 4.0</td>
<td>1    1    2   4</td>
</tr>
<tr>
<td>3. UVB + acetone (5 doses) $n = 26$</td>
<td>27</td>
<td>1.9 ± 1.1</td>
<td>8    4    0   12</td>
</tr>
<tr>
<td>4. UVB + RA (5 doses) $n = 26$</td>
<td>9$^c$</td>
<td>1.0</td>
<td>1    1    0   2</td>
</tr>
</tbody>
</table>

$^a$ Number of surviving mice.

$^b$ Mean diameter of tumors diagnosed histologically as falling into one of the 3 tumor types ± S.D.

$^c$ Significantly ($p = 0.05$) fewer mice had tumors compared to the UVB plus acetone (five doses) group by contingency table analysis.
Inhibition of UVB carcinogenesis by these criteria tended to be more pronounced for the multiple RA treatment rather than for the single RA treatment.

Fewer tumors were present in untreated areas (head, ears, tail, and limbs) in both the UVB plus RA-treated groups (Groups 2 and 4) compared to UVB plus acetone-treated groups (Groups 1 and 3), which may suggest a possible systemic component to the RA-modulating effect on UVB skin carcinogenesis. However, since the overall number of tumors found in the untreated areas was small, the significance of these observations could not be established.

Unexpectedly, the mean tumor diameter was smaller for the UVB plus acetone (5 doses) treated group compared to the UVB plus acetone (one dose)-treated group, reflecting an increased number of Grade 1 type tumors (papillomas) in the former. The cause of this is unknown but may reflect a change in the penetration of UVB radiation possibly caused by acetone effects on the stratum corneum, a change which would not be detected in the histological examinations performed.

Skin Histology. Chronic UVB exposure induced epidermal hyperplasia and acanthosis, the number of cell layers being increased from the normal 2 to 3 to 5 to 6. Chronic treatment with RA also induced hyperplasia with an increase of 2 to 3-fold in the number of epidermal layers. However, the effects of UVB and RA together were not additive, and mice receiving both showed epidermal acanthosis similar in extent to either UVB or RA treatment alone. Acetone (one dose) and acetone (5 doses) had no distinct effect on the epidermal histology.

DISCUSSION

The amounts of UVB and RA administered in the present study were chosen on the basis of several criteria. The principle factors in deciding the degree of UVB exposure were that the degree of irradiation be sufficient to induce epidermal ODC but small enough not to produce a severe phototoxic response and that a significant skin tumor yield be obtained such that enhancement and inhibition of UVB skin carcinogenesis could be detected. The amount of UVB radiation administered was gradually increased, therefore, from 1 to 3 MED over 3 days, and the mice were irradiated for 5 days/week for 4 weeks.

RA is a potent skin irritant per se, and since it was applied topically to the dorsal skin, it was necessary to use as low a concentration as possible. Verma et al. (17) demonstrated inhibition of epidermal ODC induction and skin carcinogenesis by 12-0-tetradecanoylphorbol-13-acetate with 1.7 nmol RA. RA (3.4 nmol) will inhibit UVB induction of epidermal ODC (11), and this concentration was used in the present study. RA is such a potent irritant that even 3.4 nmol applied daily were observed to produce a transient erythema and some scaling of the dorsal skin.

Despite the low tumor incidence found in the control groups resulting from the low levels of UVB used, the tumors grew significantly slower in mice receiving a single daily application of RA compared to the control group. Although the final tumor incidence was reduced in the mice receiving a single daily RA treatment, this was not significant. However, the final tumor incidence was significantly reduced in the group treated with 5 hourly applications of RA compared to the controls. Under these experimental conditions, therefore, statistically significant modulation of UVB skin carcinogenesis by RA, using treatment regimens shown previously to reduce epidermal ODC induction by UVB, has been demonstrated in the hairless mouse.

The failure of other laboratories to find a reduction in UVB skin carcinogenesis by RA may be attributable primarily to 2 factors. The skin irritancy of RA has been commented on above; in the original study of Epstein (5), about a 1000-fold higher concentration of RA was used than in our study. Under such conditions, it is difficult to eliminate the indirect effects on UVB skin carcinogenesis due to primary irritancy or any inflammatory response from the effect of the RA directly. It has been shown that physical stimuli, such as repeated laceration or abrasion, can act as tumor promoters and can induce epidermal ODC (1, 4), indicating that any treatment which induces inflammation or excessive scratching behavior should be avoided.

The time of application and the number of applications of RA to the skin are important in the inhibition of UVB induction of ODC (11). In the present study, RA modulation of the induction of skin tumors by UVB was also dependent on the time and number of applications. If the time of application of RA is crucial in relation to the time of irradiation, this may explain some of the anomalies reported in the literature. The timing of RA treatment in relation to the application of chemical tumor promoters has been shown to be critical to find inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced ODC and skin tumor promotion (3).

In the study of Forbes et al. (8), when Skh/hr 1 hairless mice were irradiated with UV radiation from a solar simulator for 28 weeks and treated topically with 3.4 nmol RA, a marked enhancement of UV carcinogenesis was found. The major differences between that and the present study were the amount and type of UV radiation used, the timing of the RA administration, the use of a methanol vehicle, and the much longer period of daily irradiation and RA treatment. Further experiments are required to identify which factors are responsible for the different results obtained, particularly at higher levels of UVB radiation, since the level used in the present study produced low tumor yields which limited the statistical evaluation of the results.

Topical RA was more effective at inhibiting UVB carcinogenesis when applied 0, 1, 2, 3, and 4 hr after UVB than when applied as a single application immediately after UVB. This was not as marked as may be expected from the degree of inhibition of ODC induction reported previously (11). This may suggest that RA or a metabolite can accumulate and persist in the skin, especially after repeated daily applications. Skin tumors arising in the UVB plus RA-treated mice grew more slowly than in UVB plus acetone-treated mice, even though RA was applied only during the 20-day irradiation period. There was no significant shift between the type of tumor found, comparing RA-treated to the appropriate acetone-treated control groups. This may indicate that the RA induces a permanent phenotypic change regulating the growth of the tumor cells or that the RA (or a metabolite) persists in the skin or circulation for a long period of time.

Since topical RA induced epidermal acanthosis, it seemed possible that this could result in a shielding effect by limiting the amount of UV radiation reaching the basal cell layers. However, the extent of the acanthosis present after RA treatment or chronic UVB exposure was similar, and there was no
significant additive effect when skin was treated with both agents observed in this study or by others (7). It seems probable, therefore, that epidermal acanthosis is not a primary factor in the mechanism of the inhibition of UVB skin carcinogenesis by retinoids.

REFERENCES

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