Topical Retinoic Acid Reduces Skin Papilloma Formation but Resistant Papillomas Are at High Risk for Malignant Conversion

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ABSTRACT

Retinoic acid (RA) was topically applied to the skin of Sencar mice during the promotion phase of specific tumor induction protocols that produce papillomas at low (12-O-tetradecanoylphorbol-13-acetate promoted, TPA) or high (mezerein-promoted) risk for premalignant progression and malignant conversion. RA consistently reduced the yield of papillomas and carcinomas in both protocols, but the frequency of malignant conversion in papillomas that emerged during RA treatment was not reduced. When TPA was reapplied after cessation of RA treatment, the number of papillomas increased 2-fold, suggesting that RA had not eliminated initiated cells. In vitro, RA prevented the emergence of transformed keratinocytes in an assay that mimics malignant conversion, suggesting that RA can suppress conversion if applied during the stage of premalignant progression. Examination of tumor markers at weeks 14 and 22 of the tumor-induction experiments in vivo indicated that papillomas evolving during RA treatment exhibited a phenotype of high progression risk, even in the TPA-promoted groups. In the majority of these tumors, the α6β4 integrin and retinoid X receptor α transcripts were detected suprabasally, indicating an advanced stage of premalignant progression. RA-treated tumors also expressed higher levels of transcripts for transforming growth factor (TGF)-β1 and localized TGF-β1 peptide in the basal portions of the tumor fronds. Because up-regulated expression of TGF-β1 suppresses papilloma formation, these studies suggest a mechanism whereby RA can prevent papilloma eruption via a TGF-β intermediate, but papillomas resistant to RA may have altered TGF-β signaling and progress to carcinomas at an increased frequency.

INTRODUCTION

In experimental mouse skin carcinogenesis, the normal skin keratinocyte evolves through a well-differentiated squamous papilloma stage with progressively dysplastic foci, finally developing into invasive squamous carcinoma. This phenotypic evolution is coincident with reproducible genetic and epigenetic changes (1). Premalignant progression is characterized by an increased proliferative compartment of the tumor papillae (9, 11, 12). The loss of TGF-β1 suppresses papilloma formation, these studies suggest a mechanism whereby RA can prevent papilloma eruption via a TGF-β intermediate, but papillomas resistant to RA may have altered TGF-β signaling and progress to carcinomas at an increased frequency.

when an oncogenic fos gene is added (5, 6). Conversely, genetic deletion of the normal fos allele prevents malignant progression of benign tumors induced by oncogenic ras, suggesting that AP-1 transcriptional activity is essential for progression (7). AP-1 activity increases during premalignant progression of squamous tumors, supporting a contributory role for this family of transcription factors in premalignant progression (8).

Previous studies have demonstrated that squamous papillomas are at variable risk to undergo premalignant progression, and the high-risk phenotype is determined early in the neoplastic process (9). To explore the biological and molecular basis for the high-risk phenotype, we have developed both specific tumor induction protocols and defined phenotypic markers to evaluate benign tumors that progress to malignancy at an accelerated rate (9, 10). Among the phenotypic changes in papillomas associated with premalignant progression are the loss of keratin 1 and increase in keratin 13 expression, an expansion of both the proliferative cell compartment and the number of cells expressing the α6β4 integrin, and loss of TGF-β1 in the basal cell compartment of the tumor papillae (9, 11, 12). The loss of TGF-β1 has been causally linked to the rapidly progressing squamous tumor phenotype in studies using keratinocytes from mice genetically deleted for the TGF-β1 allele (12). Although these markers distinguish the high- and low-risk phenotypes early in the process of benign tumor formation, tumors generated from low-risk protocols acquire similar changes at later times. Thus, the molecular control of premalignant progression is similar in low-risk tumors, but the process is accelerated in the high-risk lesions (9, 12).

Nonmelanoma skin cancers are the most common malignancies in humans. Although the major carcinogen for human skin cancer is environmental exposure to sunlight, the evolution of squamous cell carcinoma on human skin has many similarities to the induction of squamous tumors by chemicals on mouse skin (1, 13). The precursor lesion to human squamous cell carcinoma, actinic keratosis, has variable risk for premalignant progression (14, 15). Although epithelial skin cancer is rarely fatal, it is associated with pain, cosmetic complications, and substantial medical expenses. The high frequency of these tumors has promoted strategies to prevent tumor development on sun-damaged skin. Among the most discussed and tested agents for this purpose are the retinoids (16, 17). Systemic administration of 13-cis-RA has been effective in preventing skin cancer development in patients with xeroderma pigmentosum, but the treatment is limited by toxic side effects (18). Topical retinoids have met with mixed results in human trials and have also produced ambiguous findings in chemically initiated or UV-light-treated mouse skin, preventing tumor formation in some studies and enhancing tumor formation or malignant conversion in others (17, 19). In contrast, dietary RA has been effective in preventing carcinoma development from chemically induced mouse skin papillomas using both low-risk and high-risk tumor induction protocols (20–23).

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The abbreviations used are: TGF, transforming growth factor; RA, all-trans-retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.
Although a number of studies have reported the results of retinoid therapy on mouse skin tumor induction in vivo, few have addressed mechanistic questions as to how retinoids may alter tumor incidence. Most mechanistic studies have analyzed the opposing effects of retinoids on responses to phorbol ester and other tumor-promoting agents or the influence of retinoids on the interaction of the target cell with carcinogens (17, 19). Recently, Mohan et al. (24) studied the effect of topical retinoids on skin papillomas at differing risk for premalignant progression and suggested that high-risk papillomas were more sensitive to the suppression. In an earlier report, we showed that the loss of RARα and RARγ and the up-regulation of RXRα were linked to premalignant progression of chemically induced mouse skin tumors (25). These results suggested that changes in retinoid response patterns could be causally involved in premalignant progression of mouse skin tumors. We have also reported that RARα and RARγ are specifically depleted in high-risk papillomas (26). In this report, we have studied the effect of topical RA on the emergence and premalignant progression of high-risk and low-risk mouse skin papillomas and used a series of phenotypic markers to attempt to discern a mechanistic basis for the observed effects.

**MATERIALS AND METHODS**

**Chemical Induction of Tumors.** Seven to 8-week-old female SENCAR mice were initiated by topical exposure with 20 μg (Exp 1) or 5 μg (Exp 2) of DMBA in acetone. Low-risk papillomas were promoted topically with TPA, 2 μg once per week (Exp 1) or 1 μg twice per week (Exp 2) for 20 weeks. High-risk papillomas were promoted with 4 μg of mezerein (LC Services, Alexis Biochemicals, San Diego, CA) twice weekly for 20 weeks (both Exp 1 and 2) or with 1 μg of TPA twice weekly (Exp 2) for 5 weeks (10). Tumors were counted weekly and classified grossly as papillomas or carcinomas. Periodically, groups of animals were sacrificed, and tumors were excised, embedded in OCT embedding medium (Miles Laboratories), and frozen for immunostaining and in situ hybridization as described previously (9). RA (34 nM) was applied in 200 μl of acetone 5 min before each application of promoting agent and discontinued at the termination of promotion. Control groups received acetone only. In one group of TPA/RA-promoted animals from Exp 1 protocol, TPA treatment was restarted for an additional 8 weeks (weeks 22–30) after termination of combined RA and TPA promoter treatment at week 20.

**In Vitro Malignant Conversion Assay.** Primary BALB/c keratinocytes were isolated and cultured in medium containing 0.05 mM Ca2+ to maintain a basal cell phenotype (27) and 10% fibroblast-conditioned medium to inhibit spontaneous malignant conversion (28). After 4 days in culture, the cells were infected with a replication-defective retrovirus encoding the v-rasH gene to produce the papilloma phenotype in vitro. Five days after infection, cultures were exposed to 13.6 μM MNNG for 1–1.5 h to induce malignant conversion in vitro as described previously (27). In the standard protocol 5 days after MNNG treatment, cells from two dishes from each group were trypsinized and counted to assess the toxic cell loss as a consequence of MNNG treatment. Ten days after MNNG treatment (the expression period in 0.05 mM Ca2+ medium), cells undergoing malignant conversion were selected by raising the Ca2+ in the culture medium to 0.5 mM for 4 weeks. The number of dense epithelial foci greater than 0.5 cm in diameter were counted after staining with rhodamine. Some cultures were treated with 0.3 μM RA for 10 days during the expression period, and some were treated for 4 weeks during the selection period. Results are presented as the number of Ca2+-resistant foci/dish after correction for cell loss due to MNNG treatment.

**Immunostaining and in Situ Hybridization.** Frozen sections of tumors from the experimental groups were processed for immunostaining and in situ hybridization at week 22 in Exp 1 and week 14 in Exp 2. For Exp 1, this was 2 weeks after the last treatment and for Exp 2, treatments were stopped in the animals to be sacrificed 1 week prior to sacrifice. Monospecific polyclonal rabbit antibody to mouse keratin 13, the anti-cytoplasmic (30–1) antibody to TGF-β1 and monoclonal antibody LOH 3 to the α6 integrin subunit and immunostaining procedures have been described previously (9, 12). In situ hybridization was performed by the protocol of Young (29) with 35S-labeled RNA sense and antisense probes to RXRα and TGF-β1. The details of the hybridization conditions and the probes used have been reported (25). For RXRα in situ studies, tumors were analyzed at week 14 in Exp 2 as described and at weeks 8–11 from a parallel study of high- and low-risk tumor induction protocols described previously (9).

**RESULTS**

Topical RA Inhibits Papilloma Formation in Both High- and Low-Risk Tumor Induction Protocols. Fig. 1, A and B, and Table 1 show that the number of papillomas per mouse produced by TPA promotion for 20 weeks is 3–4-fold greater than the number induced by mezerein promotion. RA treatment prior to application of promoter inhibits papilloma formation by 40–70%, independent of the tumor induction protocol. RA also delays the appearance of papillomas in both groups, and after cessation of TPA and RA exposure, tumors continue to evolve but reach a plateau by 25 weeks. This implies that RA can inhibit both high- and low-risk papillomas effectively, thus reducing the number of benign tumors at risk for malignant conversion. Similar results were obtained when TPA and RA were applied for only 5 weeks where maximum papilloma yield was reduced from 1.2 per mouse to 0.06 per mouse by RA (Exp 2, data not shown). In the experiment shown in Fig. 1C, TPA treatment was resumed for 8 weeks after cessation of 20 weeks of TPA and RA coapplication. TPA application increased the papilloma number 2-fold over the acetone control within 8 weeks, suggesting that RA had suppressed tumor outgrowth but had not eliminated initiated cells.

Topical RA Reduces the Number of Carcinomas but not the Conversion Frequency. As shown in Table 1, the percentage of mezerein and TPA-treated mice developing carcinomas is similar within each experiment. However, the number of papillomas in the TPA groups is substantially greater; thus, the conversion frequency of mezerein promoted papillomas is 2–4-fold higher than with TPA promotion. The higher conversion frequency in the TPA groups of Exp 2 versus Exp 1 is consistent with a later time of sacrifice in the second experiment and may also relate to the lower dose of initiator as suggested by Di Giovanni et al. (30). RA reduced the total number of carcinomas that evolved in each promoter group in both experiments, but the reduction in the papilloma and carcinoma formation was proportional so that conversion frequency was not reduced and actually appeared to increase in both experiments in each promoter group. Thus, topical RA may selectively inhibit papillomas with a low-risk for malignant conversion while sparing those with the highest risk.

RA Inhibits Malignant Conversion in Vitro. Malignant conversion in vivo can be recapitulated in cultured keratinocytes by documenting the emergence of actively growing foci in culture medium with 0.5 mM Ca2+ from a starting population of v-rasH-initiated basal keratinocytes in 0.05 mM Ca2+ medium that are treated with mutagens (27). Two components of this assay are essential: the 10-day expression period in 0.05 mM Ca2+ medium that are treated with mutagens and high Ca2+ selection; and the 4-week selection period in 0.5 mM Ca2+ medium. To assess the impact of RA in this model, we performed experiments shown in Fig. 2 and Table 2. MNNG is a potent inducer of converted foci, with about a 6-fold increase over the spontaneous background in each of three independent studies. The addition of RA to the medium either during the expression period or the selection period reduces the number of foci to near the background level. RA does not alter the survival index (Table 2) or reduce the number of cells in the expression period (data not shown), indicating there is no combined cytotoxic effect of RA and MNNG to explain the reduced focus number.
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RA Has Variable Effects on Tumor Markers Associated with Premalignant Progression. We analyzed several immunohistochemical markers shown previously to distinguish high (mezerein-promoted) and low (TPA-promoted) risk papillomas (9). Table 3 shows that aberrant keratin 13 and suprabasal α6β4, markers of a high-risk phenotype, are expressed in more than one-half of the mezerein-promoted tumors but only in <20% of the TPA-promoted papillomas when examined at week 14 or 22. Paradoxically, RA-treated, TPA-promoted papillomas displayed suprabasal α6β4 integrin in about one-half of the persisting tumors at both time points, and K13 expression increased by week 22. This suggests that topical RA can either accelerate progression, modify these markers directly, or specifically select against low-risk papillomas. Previous studies have shown that papillomas with the high-risk phenotype represent a minority subpopulation of papillomas generated in the TPA protocols (9, 12). RA had little effect on the percentage of tumors with suprabasal α6β4 in the mezerein-treated group and reduced the population of keratin 13-positive cells in this group.

RXRa Transcripts Are Modified in RA-treated Papillomas. Predictable changes in retinoid receptors have been documented during the course of skin tumor progression (25), including the predominance of RXRa and loss of RARs during premalignant progression and in carcinomas. Furthermore, mezerein is particularly potent in

Table 1 Incidence of papillomas and carcinomas among high- and low-risk tumor induction protocols

<table>
<thead>
<tr>
<th>Experiment and treatment group</th>
<th>Mice per group</th>
<th>PAPS/Mouse</th>
<th>Total carcinoma</th>
<th>% mice with carcinoma</th>
<th>Conversion frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEZ</td>
<td>161</td>
<td>3.2</td>
<td>51</td>
<td>30</td>
<td>9.8</td>
</tr>
<tr>
<td>TPA</td>
<td>76</td>
<td>10.3</td>
<td>18*</td>
<td>22*</td>
<td>2.3</td>
</tr>
<tr>
<td>MEZ/RA</td>
<td>48</td>
<td>1.0</td>
<td>9</td>
<td>17</td>
<td>19.6</td>
</tr>
<tr>
<td>TPA/RA</td>
<td>61</td>
<td>3.1</td>
<td>5*</td>
<td>8*</td>
<td>2.7</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEZ</td>
<td>36</td>
<td>3.8</td>
<td>19</td>
<td>47</td>
<td>13.9</td>
</tr>
<tr>
<td>TPA</td>
<td>35</td>
<td>9.9</td>
<td>26</td>
<td>63</td>
<td>7.5</td>
</tr>
<tr>
<td>MEZ/RA</td>
<td>36</td>
<td>1.8</td>
<td>10</td>
<td>25</td>
<td>15.4</td>
</tr>
<tr>
<td>TPA/RA</td>
<td>33</td>
<td>3.4</td>
<td>11</td>
<td>31</td>
<td>9.7</td>
</tr>
</tbody>
</table>

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Fig. 1. Papilloma yield in female Sencar mice initiated with DMBA and promoted with TPA or mezerein alone or in combination with 34 nM RA. A, 20 μg of DMBA was topically applied for initiation, and either 2 μg of TPA once weekly or 4 μg of mezerein twice weekly was topically applied for 20 weeks for promotion. B, 5 μg of DMBA was topically applied for initiation, and either 1 μg of TPA or 4 μg of mezerein was topically applied twice weekly for 20 weeks for promotion. C, using the Exp 1 protocol, after 20 weeks of combined promotion with RA and TPA, and a 2-week interim period of no treatment, mice were restarted on 2 μg of TPA or acetone only for 8 additional weeks.

Fig. 2. RA suppresses malignant conversion in vitro. Primary mouse keratinocytes were cultured as basal cells in 0.05 mM Ca²⁺ medium and initiated by transduction with the v-rasH2 oncogene using a replication-defective retrovirus. After 5 days, initiated cells were exposed to 13.6 μM MNNG for 1-1.5 h, maintained for 10 days in 0.05 mM Ca²⁺ medium (the expression period), and then changed to medium containing 0.5 mM Ca²⁺ for 4 weeks (the selection period). RA (0.3 μM) exposure occurred either during the expression period only (column 3) or the selection period only (column 4). After 4 weeks of selection, cultures were fixed and stained with rhodamine. For more details see the report by Morgan et al. (27).

MNNG (13.6 μM) RA (0.3 μM)
Table 2 RA inhibits the development of Ca$^{2+}$-resistant foci in a malignant conversion assay in vitro

<table>
<thead>
<tr>
<th>Experiment and treatment</th>
<th>Survival</th>
<th>Corrected foci/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
<td>0.9 (12)</td>
</tr>
<tr>
<td>MNNG</td>
<td>0.8</td>
<td>0.6 (5)</td>
</tr>
<tr>
<td>MNNG/RA (E)</td>
<td>0.7</td>
<td>1.6 (8)</td>
</tr>
<tr>
<td>MNNG/RA (S)</td>
<td>0.7</td>
<td>1.4 (12)</td>
</tr>
<tr>
<td>Control</td>
<td>1.0</td>
<td>0.4 (14)</td>
</tr>
<tr>
<td>MNNG</td>
<td>0.6</td>
<td>2.1 (14)</td>
</tr>
<tr>
<td>MNNG/RA (E)</td>
<td>0.5</td>
<td>0.0 (14)</td>
</tr>
<tr>
<td>MNNG/RA (S)</td>
<td>0.6</td>
<td>0.1 (14)</td>
</tr>
</tbody>
</table>

a MNNG, 13.6 µM for 1–1.5 h; MNNG/RA (E), RA (0.3 µM) during the expression phase; MNNG/RA (S), RA (0.3 µM) during selection phase. Numbers in parentheses, number of dishes/group.

Table 3 Influence of RA on expression of tumor markers associated with premalignant progression

<table>
<thead>
<tr>
<th>Marker of premalignant progression</th>
<th>Treatment group</th>
<th>Keratin 13 &gt;50% of tumor cells</th>
<th>Suprabasal α6β4 integrin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
<td>Exp 1</td>
</tr>
<tr>
<td>MEZ</td>
<td>6/12 (50)</td>
<td>7/10 (70)</td>
<td>7/12 (58)</td>
</tr>
<tr>
<td>TPA</td>
<td>4/54 (12)</td>
<td>3/16 (19)</td>
<td>4/34 (12)</td>
</tr>
<tr>
<td>MEZ/RA</td>
<td>4/21 (19)</td>
<td>4/9 (44 )</td>
<td>15/21 (71)</td>
</tr>
<tr>
<td>TPA/RA</td>
<td>11/26 (42)</td>
<td>3/17 (18)</td>
<td>14/26 (53)</td>
</tr>
</tbody>
</table>

a Measurement defined as number positive for the marker/number of tumors examined (%) in Exp 1 (22 weeks) and Exp 2 (14 weeks). Exp, experiment.

Fig. 3. Detection of RXRa transcripts in papillomas promoted by TPA or mezerein. Papillomas induced by the protocol used for Exp 1 (see Fig. 1 legend) were excised at week 11 for TPA promotion (A–F) or week 8 for mezerein promotion (G–L), and frozen sections were processed for in situ hybridization with a RXRa riboprobe as described by Darwiche et al. (25). A, D, G, and J are brightfield micrographs, and B, E, H, and K are darkfield micrographs using the antisense probe. C, F, I, and L are darkfield micrographs using the sense probe. ×200.
Fig. 4. Detection of RXRa transcripts in papillomas promoted by TPA or mezerein and treated with RA. Papillomas induced in Exp 2 were excised at 14 weeks (treatments stopped at 13 weeks), and frozen sections were processed for in situ probing with RXRa riboprobes (25). Brightfield (A, D, G, and J) and darkfield (B, C, E, F, H, I, K, and L) images of TPA (A–C), TPA/RA (D–F), mezerein (G–I), or mezerein/RA (J–L) papillomas are shown. ×200. B, E, H, and K are antisense probes, and C, F, I, and L are sense probes.

localization of TGF-β1 peptide in the basal compartment is detected twice as frequently in TPA-promoted papillomas at 14 weeks (Exp 2) then in mezerein-promoted tumors (Exp 2), but by 22 weeks (Exp 1), basal TGF-β1 was detected in only about one-third of tumors in both groups. However, the frequency of predominant basal compartment TGF-β1 peptide staining in RA-treated groups increases substantially so that about two-thirds of papillomas are positive at both time points.

DISCUSSION

Our studies indicate that topical RA reduces the incidence of benign skin tumors induced by several tumor induction protocols, but the papillomas resistant to RA suppression have a high risk for premalignant progression. RA does not eliminate initiated cells because cessation of RA treatment, followed by resumption of promoter exposure, results in a substantial increase in papilloma yield. This reversible effect was also observed in patients with xeroderma pigmentosum, whose skin tumor incidence rebounded after cessation of oral isotretinoin therapy (18). In contrast, RA protects against malignant conversion in an in vitro assay using v-raf16-initiated mouse keratinocytes, suggesting that RA exposure during the conversion process itself may be protective. This assay, therefore, might serve as a convenient screening assay to select for retinoids with anticancer activity.

The suppression of papilloma formation seen in this study confirms and extends the observations of Mohan et al. (24), who reported previously that topical RA inhibited papilloma formation in both low-and high-risk tumor induction protocols. In that study, progression to malignancy was not evaluated in the absence of further RA treatment, but RA, in the period of premalignant progression after promotion was completed, did suppress carcinoma formation. This is analogous to our finding in vitro. Our results are also consistent with prior studies of De Luca and colleagues indicating that dietary RA at pharmacological levels could prevent the formation of high risk papillomas.
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Fig. 5. Detection of TGF-β transcripts in papillomas promoted by TPA or mezerein and treated with RA. Papillomas induced in Exp 1 were excised at 22 weeks (treatments stopped at 20 weeks), and frozen sections were processed for in situ probing with a TGF-β1 riboprobe (46). A, C, E, and G are brightfield micrographs, and B, D, F, and H are darkfield micrographs using the antisense probe. A and B are TPA-promoted tumors; C and D are TPA/RA tumors; E and F are mezerein-promoted tumors; G and H are mezerein/RA-promoted tumors. ns, nonspecific binding of probe to a heavily keratinized area of tumor. x200.

(20). However, the dietary studies suggested that malignant conversion of mezerein and TPA-promoted papillomas could be reduced (but less effectively), even if dietary supplementation was stopped at the termination of tumor promotion, well before the first cancer was detected (21). This difference might reflect the sparing effect that dietary RA has on other retinoids, as shown for liver retinol stores (32). Alternatively, the mechanisms involved in systemic retinoid anticancer effects might be different from those of topical RA. For instance, the irritation caused by topical RA might play a role in elimination of initiated clones that contribute to low-risk papillomas or could enhance progression as seen for benzoylperoxide (33).

It has been proposed that the inhibitory influence of RA on skin tumor formation is related to the ability of retinoids to suppress farnesyltransferase activity, thus preventing the p21ras protein from localizing to its active site in the plasma membrane (34). However, the biological data speak against this mechanism. RA is an effective inhibitor of UV-induced tumor formation in mouse skin and UV-induced skin cancers in susceptible humans, yet ras mutations are infrequent in these tumors (35, 36). RA does not inhibit DMBA-initiated, mirex-promoted mouse skin papillomas, yet these tumors have the same frequency of ras mutations as TPA-promoted tumors (37). Together, these results indicate that RA suppression is mediated by a pathway common to multiple tumor induction protocols, most likely in the promotion or progression stages.

Our observations suggest that topical RA differentially influences benign tumors with a high- or low-risk for premalignant progression. Selective effects of RA on papilloma subpopulations have been suggested previously. Islam and Toftgard (38) showed that small papil-

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>% of tumors with TGF-β1 in the basal compartmenta</th>
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<tbody>
<tr>
<td></td>
<td>Exp 1, week 22 Exp 2, week 14</td>
</tr>
<tr>
<td>MEZb</td>
<td>31 (13) 25 (5)</td>
</tr>
<tr>
<td>TPA</td>
<td>38 (37) 47 (15)</td>
</tr>
<tr>
<td>MEZ/RA</td>
<td>52 (19) 60 (10)</td>
</tr>
<tr>
<td>TPA/RA</td>
<td>63 (27) 65 (17)</td>
</tr>
</tbody>
</table>

a Numbers in parentheses, the number of tumors studied.
b MEZ, mezerein.

Table 4 Detection of TGF-β1 peptides in skin tumors by immunostaining

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Papillomas promoted by TPA were inhibited by topical RA selectively over larger papillomas. As already indicated, Kim and Smart (37) reported that RA inhibited papillomas promoted by TPA but not those promoted by mirex.

To address the question of tumor selection, we examined a series of markers associated with malignant progression in papillomas from control and RA-treated groups. This approach is limited because the analysis of the RA-treated tumors is performed only on papillomas that are resistant to the inhibitory influence of RA on tumor formation. Nevertheless, by evaluating early time points during and shortly after promotion, we were able to detect differences among RA-treated and untreated papillomas that may provide clues to potential mechanisms of RA action. Keratin 13 is a marker of early premalignant progression, replacing keratin 1 in progressing papillomas but decreasing with advancing dysplasia and malignancy (11). However, K13 is also directly up-regulated by retinoids in cultured keratinocytes (39, 40), and K13 expression is induced by topical RA in human skin (41). Our results confirm that K13 distinguishes high-risk from low-risk tumors, but the influence of RA is unclear. In low-risk, TPA-promoted tumors, K13-positive cells are increased by 22 weeks, and in high-risk tumors, promoted by mezerein, K13-positive cells are reduced by RA. These changes could reflect the complex regulation of K13, both by retinoids directly and by changes that occur in tumor progression. Thus, this marker does not provide a clear picture. The suprabasal localization of α6β4 integrin serves as a more consistent marker, distinguishing high-risk from low-risk papillomas, as seen here and previously (9). RA is not known to directly regulate α6β4 expression, and in this study, the majority of tumors in the RA-treated groups exhibited an α6β4 suprabasal phenotype, suggesting that papillomas in a more advanced stage of progression evolve during RA treatment.

Previous studies have revealed that RARα and RARγ transcripts and protein are lost during premalignant progression, and RXRα emerges as the predominant retinoid nuclear receptor signal detected in progressing papillomas and squamous carcinomas (25). We now find that RXRα transcripts are localized both basally and suprabasally in mezerein-promoted papillomas, whereas RXRα transcripts are predominantly basal in TPA-promoted, low-risk tumors. Suprabasal expression of RXRα transcripts was detected commonly in RA-treated tumors evolving from either promotion protocol. Previous studies have shown that RXR-specific ligands are less likely to suppress growth of papillomavirus-transformed human cervical epithelial cell lines when compared to RAR-selective ligands (42). RXR ligands are also poor inducers of differentiatiation in embryonal carcinomas cells (43). In contrast, RXRα activation is a potent signal for apoptosis in HL-60 cells (44). Presently, it is not known how each family of retinoid receptors influences skin tumor formation. Our previous studies suggest that activation of RARs in papilloma cells can inhibit growth (26). Extrapolating from this result, it might follow that expansion of RXRα expression in high-risk and RA-treated papillomas favors growth and may contribute to the emergence of this tumor population. Additional studies on the RAR and RXR families of receptors in our tumors and in cultured papilloma cells will be required to address this possibility. In one clinical study, RXRα transcripts were not changed in oral premalignant lesions from human subjects either before or after therapy with isotretinoin, but this therapy did restore transcripts for RARβ that were decreased in untreated oral lesions. RARβ is not detected in normal mouse skin (45), but the possibility that this receptor is induced by retinoids in treated skin tumors should also be explored.

Previously, a causal link between loss of tumor-associated TGF-β1 and premalignant progression was established. Supporting data included the early loss of TGF-β1 in high-risk papillomas (12) and the rapid malignant conversion of papillomas generated from TGF-β1 null keratinocytes (46). Recent studies with transgenic mice have suggested that elevated TGF-β1 expression may enhance malignant conversion while suppressing benign tumor formation (47). In earlier experiments, we also demonstrated that RA could induce TGF-β1 and TGF-β2 transcripts and TGF-β2 secretion in cultured keratinocytes and mouse skin (31). We now show that TGF-β1 is up-regulated in tumors that have been treated with RA during the promotion phase but nevertheless erupt from initiated skin to form papillomas in both low- and high-risk tumor induction protocols. Although we did not measure
tumor volume in our study, Mohan et al. (24) showed that papillomas arising from RA-treated skin have a smaller volume than control tumors from similar promotion protocols, consistent with a growth-inhibitory effect of TGF-β1. Studies on cultured cells from prostate cancer (48), breast cancer (49), and HL-60 leukemia (50) indicate that tumors from similar promotion protocols, consistent with a growth-retinoids. Because premalignant progression is strongly linked to inhibitory effect of TGF-β1. Studies on cultured cells from prostate (21, 24). Alternatively, malignant conversion in association with overexpression of TGF-β (47, 53) or in the continued presence of RA may represent tumor cell populations where TGF-β responsiveness has been abrogated by alterations in the TGF-β signaling or tumor cell matrix localization systems (12, 46, 53). This would be analogous to TGF-β type II receptor mutations seen frequently in certain progressing colon cancers (54). Presently, studies are in progress to address this hypothesis using keratinocytes that are null for the TGF-β1 gene.

Our studies indicate that limited topical treatment with RA is effective in preventing benign tumor formation and reducing carcinoma incidence. However, benign tumors resistant to topical RA suppression are at higher risk for premalignant progression in the absence of continued RA exposure. This may contribute to ambiguities observed in previous animal experiments or clinical studies on this subject that demonstrated enhanced cancer risk from retinoid chemoprevention or therapy (17, 19). Although potential differences between topical and systemic exposures to RA need further analysis, these findings must be considered in the clinical application of retinoids for chemoprevention of squamous tumor development where limited application may reduce the frequency of premalignant changes but not alter the cancer risk. Because clinical trials with retinoids are in progress and markers associated with risk for premalignant progression in our studies are relevant to human squamous cancer risk (55–57), it ought to be possible to evaluate this problem with the present resources.

REFERENCES

28. Agarwal, R., Mohan, R. R., Ahmad, N., and Mukhtar, H. Protection against malignant conversion in SENCAR mouse skin by all trans retinoic acid: inhibition of the ras

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44. Mehta, K., McQueen, T., Nearnati, S., and Andreff, M. Activation of retinoid receptors RARa and RXRa induces differentiation and apoptosis, respectively, in HL-60 cells. Cell Growth Differ., 7: 179–186, 1996.


Topical Retinoic Acid Reduces Skin Papilloma Formation but Resistant Papillomas Are at High Risk for Malignant Conversion

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