Loss of Retinoic Acid Receptors in Mouse Skin and Skin Tumors Is Associated with Activation of the ras\(^{Ha}\) Oncogene and High Risk for Premalignant Progression

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ABSTRACT

Retinoic acid receptor transcripts (RAR\(\alpha\) and RAR\(\gamma\)) are decreased in benign mouse epidermal tumors relative to normal skin and are almost absent in carcinomas. In this report, the expression of RAR\(\alpha\) and RAR\(\gamma\) proteins was analyzed by immunoblotting in benign skin tumors induced by two different promotion protocols designed to yield tumors at low or high risk for malignant conversion. RAR\(\alpha\) was slightly reduced in papillomas promoted with 12-O-tetradecanoylphorbol-13-acetate (low risk) and markedly decreased or absent in papillomas promoted by mezerein (high risk). Mezerein also caused substantial reduction of RAR\(\alpha\) in nontumorous skin. RAR\(\gamma\) was not detected in tumors from either protocol and was greatly reduced in skin treated by either promoter. Both RAR\(\alpha\) and RAR\(\gamma\) proteins were decreased in keratinocytes overexpressing an oncogenic v-ras\(^{Ha}\) gene, and RAR\(\alpha\) was undetected in a benign keratinocyte cell line carrying a mutated c-ras\(^{Ha}\) gene. Introduction of a recombinant RAR\(\alpha\) expression vector into benign keratinocyte tumor cells reduced the S-phase population and inhibited \(^{3}H\)thymidine incorporation in response to retinoic acid. Furthermore, transactivation of B-RARE-dtk-LUC by retinoic acid was markedly decreased in keratinocytes transduced with the v-ras\(^{Ha}\) oncogene (v-ras\(^{Ha}\)-keratinocytes). Blocking protein kinase C function in v-ras\(^{Ha}\)-keratinocytes with bryostatin restored RAR\(\alpha\) protein to near normal levels, reflecting the involvement of protein kinase C in RAR\(\alpha\) regulation. Both RAR\(\alpha\) and RAR\(\gamma\) are down-regulated in cultured keratinocytes by 12-O-tetradecanoylphorbol-13-acetate, further implicating PKC in the regulation of retinoid receptors. Our data suggest that modulation of RARs could contribute to the neoplastic phenotype in mouse skin carcinogenesis and may be involved in the differential promoting activity of mezerein and 12-O-tetradecanoylphorbol-13-acetate, particularly for selecting tumors at high risk for malignant conversion.

INTRODUCTION

Retinoids are essential for normal epidermal differentiation (1, 2) and are used in the prevention and treatment of some skin disorders and cancers in humans (reviewed in Refs. 3 and 4). Two families of nuclear receptors, each consisting of three receptor types (\(\alpha\), \(\beta\), and \(\gamma\)) and different isoforms, have been identified: the RARs\(^{2}\) (reviewed in Ref. 5), which bind both PA and 9-cis-retinoic acid, and RXRs (reviewed in Ref. 6), which bind 9-cis-retinoic acid.

Multistage mouse skin carcinogenesis is divided into distinct and well-characterized stages (7). Initiation with the carcinogen DMBA and subsequent use of different promotion protocols generates benign tumors with differing conversion rates to malignancy (8). For example, when TPA is applied for 20 weeks as a promoter, most of the tumors are at low risk for malignant conversion. In contrast, application of mezerein for 20 weeks produces benign tumors with a high conversion rate. Benign tumors at low or high risk for conversion can be distinguished by altered patterns of integrin (9) and TGF-\(\beta\) expression and by differing patterns of tumor cell proliferation (10). The major keratinocyte RAR transcripts (RAR\(\alpha\)1 and RAR\(\gamma\)) are down-modulated in mouse epidermal tumors when compared to normal skin, and transcripts are virtually lost in undifferentiated squamous cell carcinomas (11). In contrast, RAR\(\alpha\) and RAR\(\beta\) transcript expression increases in papillomas and carcinomas as the number of undifferentiated cells also increases. Since high dietary RA decreases carcinoma incidence and yield in low- and high-risk chemically induced skin tumors (12), retinoid receptors may be functionally important in determining tumor behavior in the mouse skin model.

One of the earliest genetic alterations detected in multistage mouse skin carcinogenesis is an activating mutation of the c-ras\(^{Ha}\) gene (13, 14). When DMBA is used to initiate carcinogenesis, c-ras\(^{Ha}\) mutations are detected in more than 90% of the epidermal tumors of both low and high risk phenotypes (15). In addition, retroviral transduction of mouse keratinocytes with a v-ras\(^{Ha}\) gene produces cells that resist Ca\(^{2+}\) - or phorbol ester-induced terminal differentiation (16, 17) and form benign tumors when grafted onto nude mice (18). Recent studies indicate that some of the phenotypic alterations in v-ras\(^{Ha}\)-keratinocytes are mediated by changes in PKC activity, particularly the activation of PKCa and the down-modulation of PKCb (19, 20). Oncogenic ras reduces the level of RAR\(\alpha\) in NIH 3T3 cells, altering the responsiveness of these cells to RA (21). However, little is known of the relationship of ras activation, retinoid receptors, and tumorigenesis.

In the present study, we investigated whether benign mouse skin tumors induced by different promotion protocols are distinguished by differential changes in RAR\(\alpha\) and RAR\(\gamma\) proteins and how modifying RAR levels in cultured papilloma cells may influence cell behavior. We also analyzed the relationship between ras\(^{Ha}\) activation and the retinoid receptor profile and the potential involvement of PKC in RAR regulation in keratinocytes.

MATERIALS AND METHODS

Cell Culture. Primary epidermal keratinocytes were isolated from newborn BALB/c mice as described previously (22). The neoplastic keratinocyte cell line SP-1 was established from papillomas produced in SENCAR mice by DMBA initiation and promotion with TPA. SP-1 cells carry an activating mutation in the c-ras\(^{Ha}\) gene and produce benign dysplastic papillomas when grafted onto the back of immune-deficient mice (17). All keratinocytes were cultured in Eagle’s minimal essential medium containing 8% Chelex (Bio-Rad Laboratories, Richmond, CA)-treated fetal bovine serum and 0.05 mM Ca\(^{2+}\) to maintain a basal cell phenotype (23). TPA and mezerein were obtained from LC Services (Woburn, MA), and bryostatin was obtained from the Pharmaceutical Resources Branch of the National Cancer Institute. For cell culture, stock solutions were prepared in DMSO (TPA and mezerein) and absolute ethanol (bryostatin) and stored at −20°C. For \textit{in vivo} application, TPA and mezerein were diluted in acetone. RA was from Sigma Chemical Co. (St. Louis, MO).

\[^{1}\] Received 6/3/96; accepted 9/4/96.

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\[^{2}\] The abbreviations used are: RAR, retinoic acid receptor; RA, all-trans-retinoic acid; RXR, retinoid X receptor; RARE, RA response element; \(\kappa\), thymidine kinase; LUC, luciferase; DMBA, 7,12-dimethylbenz(a)anthracene; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; TGF, transforming growth factor; MOI, multiplicity of infection.
Retroviral Infection of Cells. The v-ras \(^{\text{Re}}\) oncogene was introduced into primary keratinocytes by a replication-defective retrovirus containing the \(v\)-ras \(^{\text{Re}}\) gene using 4 \(\mu\)g/ml polybrene as described (18). The retroviral vector LRARSN, in which the full-length RARs cDNA is introduced into the retroviral vector LXSN, has been described previously (24). Cells were infected with the LXSN or LRARSN retroviral vectors in the presence of 4 \(\mu\)g/ml polybrene and selected in growth medium supplemented with G418 as described (24), except that 40 \(\mu\)g/ml were used instead of 400 \(\mu\)g/ml.

Chemical Induction of Tumors. Eight-week-old female CD-1 or SEN-CAR mice were initiated with 20 or 5 \(\mu\)g of DMBA in acetone. Low-risk papillomas were generated by promotion with 2 \(\mu\)g of TPA once (SENCAR) or twice (CD-1) a week until sacrifice. High-risk papillomas were generated by promotion with 4 \(\mu\)g of mezerein twice weekly until sacrifice. For analysis of RARs, tumors were excised 48 h after the last promoter treatment and snap-frozen in liquid nitrogen. Tumor samples from both mouse strains were included in the analysis and gave similar results. In some experiments, untreated or promoter-treated normal skin was snap frozen for analysis, and in one experiment, tumors and skin were excised 30 weeks after the last promoter treatment.

Immunoblot and RNA Analysis. Total SDS lysates (where indicated) were prepared from cultured cells washed twice with ice-cold PBS and scraped into SDS sample buffer (21). Nuclear protein extracts from cultured cells, treated mouse skin, or low- and high-risk tumors were prepared as described by Schreiber et al. (25). Protein concentration was determined using the BCA-Pierce protein assay (Pierce, Rockford, IL) or the Bio-Rad protein assay (Bio-Rad). Total cellular (30 \(\mu\)g) or nuclear proteins (15–20 \(\mu\)g) were boiled in SDS sample buffer [62.5 mM Tris (pH 6.8), 10% glycerol, 7.5% SDS, and 6% \(\beta\)-mercaptoethanol] and run immediately on 10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell) by standard blotting procedures. The membranes were stained with Ponceau S (Sigma) to check for equal loading and efficiency of transfer. The blots were incubated overnight at 4°C in 5% milk in TBS (50 mM Tris-HCl and 150 mM NaCl). Polyclonal antibodies against the carboxy termini of RARs and RARy (26, 27) were kindly provided by Dr. F. Chamley (Laboratoire de Genetique Moleculaire des Eucaryotes du Centre National de la Recherche Scientifique, Strasbourg, France) and used as described previously (26, 27) at 1:1000 dilution. Some blots were also probed with anti-PKC antibody specific for the catalytic subunit of PKC \(\alpha\) at a dilution of 1:500, (Upstate Biotechnology, Inc., Lake Placid, NY), keratin 14-specific antibodies (La Recherche Scientifique, Strasbourg, France) and used as described previously (11). After transfer to NYTRAN, blots were probed with \(3^{\text{H}}\)H-thymidine (Amersham). The amount of label incorporated into DNA was determined as described (31). Triplicate wells were used to determine cell number, and the radioactivity associated with individual samples was reported as cpm/cell number.

Cell Cycle Analysis. Asynchronously growing LXSN-SP-1 and LRARSN-SP-1 cells in 100-mm Falcon tissue culture dishes were treated for 24 h with DMSO or 3 \(\times\) \(10^{-8}\) m RA. Cells were trypsinized and fixed in 70% ethanol and stored at 4°C. Cells were incubated for 30 min with 50 units RNase A (Sigma) and then stained with 50 \(\mu\)g/\(\mu\)l propidium iodide (Sigma). Cell cycle analysis was performed using a FACScan (Becton Dickinson, Research Triangle, NC) flow cytometer, and the percentage of cells in G0-G1, S, and G2-M phases was determined using the CellFit program.

RESULTS

Loss of RAR\(\alpha\) and RAR\(\gamma\) Proteins during Skin Tumor Promotion. RARs proteins were abundantly expressed in nuclear extracts from normal skin and were reduced slightly in the six TPA-promoted tumors analyzed (Fig. 1A). In contrast, much lower or undetectable levels of RAR\(\alpha\) were observed in nuclear extracts of seven of seven mezerein-promoted papillomas (Fig. 1A). RAR\(\gamma\) proteins were detected in normal skin but not in any of the tumors that were examined, although RAR\(\gamma\) transcripts were detected in tumors, as shown previously, by in situ hybridization and Northern blot analysis (11). RAR\(\gamma\) transcripts were detected in tumors but were reduced relative to normal skin, especially in the high risk tumor group (Fig. 1B).

Since the papillomas were generated by different promotion protocols, we determined if TPA and mezerein might have differential effects on RAR proteins in nontumorous skin. Forty-eight h after topical treatment of SENCAR mouse skin with TPA, there was a minimal effect on RAR\(\alpha\) (Fig. 2A); in contrast, mezerein caused a marked reduction in RAR\(\alpha\). RAR\(\gamma\) could not be detected in skin treated with TPA or mezerein after acute exposure. A previous report (32) had indicated that TPA could transiently down-modulate both RAR\(\alpha\) and RAR\(\gamma\) mRNA within 3.5 h of application to mouse skin. The marked effect of mezerein relative to TPA in down-modulating RAR\(\alpha\) after 48 h could reflect differences in the extent of down-modulation or the rate of regeneration of this protein after each treatment. Chronic treatment (15 weeks) of mouse skin with TPA caused a greater, but not complete, down-regulation of RAR\(\alpha\) protein compared to normal skin, whereas RAR\(\alpha\) was not detected in mezerein-treated skin after 15 weeks (data not shown). This suggests that the promoting agent alone may influence the complement of retinoid receptors expressed during skin carcinogenesis.

Transret (v-ral)\(^{\text{Re}}\) Keratinocytes with Constructs Containing \(\beta\)-RARE-LUC. \(\beta\)-RARE-tk-LUC (29) was a gift from Dr. Keiko Ozato (NIH). Primary keratinocytes were grown in 60-mm tissue culture dishes for 3 days until confluent and were transduced with the v-ral\(^{\text{Re}}\) retrovirus. Three days after infection, the medium was changed to 2 ml of serum-free Eagle’s minimal essential medium, and cells were cotransfected using Lipofectamine reagent (Life Technologies, Inc., Bethesda, MD) and electrophoretically separated as described previously (11). After transfer to NYTRAN, blots were probed with \(3^{\text{P}}\)-labeled mouse RAR\(\gamma\)-1 riboprobe and reprobed with a CDNA probe for mouse glyceraldehyde-3-phosphate dehydrogenase under conditions described previously (11).

Transret Mutation of Primary and v-ral\(^{\text{Re}}\) Keratinocytes with Constructs Containing \(\beta\)-RARE-LUC and \(\beta\)-RARE-tk-LUC (29) was a gift from Dr. Keiko Ozato (NIH). Primary keratinocytes were grown in 60-mm tissue culture dishes for 3 days until confluent and were transduced with the v-ral\(^{\text{Re}}\) retrovirus. Three days after infection, the medium was changed to 2 ml of serum-free Eagle’s minimal essential medium, and cells were cotransfected using Lipofectamine reagent (Life Technologies, Inc., Bethesda, MD) and electrophoretically separated as described (30). Six h after transfection, the latter was used to incubate for an additional 16 h in 8% serum containing fresh medium.

Subsequently, the cells were treated with 3 \(\times\) \(10^{-8}\) M RA or solvent DMSO for 24 h. Cells were lysed with reporter lysis buffer (Promega Corp., Madison, WI), and luciferase activity was determined in 1 \(\mu\)g of protein from each sample using the luciferase assay system from Promega in a monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA). \(\beta\)-Galactosidase activity was quantitated by the Fluoroprobe laZ/Galactosidase quantitation kit (Molecular Probes, Inc., Eugene, OR).

\(3^{\text{H}}\)H-Thymidine Incorporation. To assay the effect of expression of exogenous RARs on SP-1 cell proliferation, LRARSN-SP-1 and LXSN-SP-1 cells were plated (5 \(\times\) \(10^{3}\)) in 12-well Falcon tissue culture trays. Cells were treated with varying concentrations of RA (3 \(\times\) \(10^{-10}\) to 3 \(\times\) \(10^{-4}\) M) or DMSO for the time indicated in the figures and pulsed for 2 h with 0.5 \(\mu\)Ci of \(3^{\text{H}}\)H-thymidine (Amersham). The amount of label incorporated into DNA was determined as described (31). Triplicate wells were used to determine cell number, and the radioactivity associated with individual samples was reported as cpm/cell number.

Decreased Cell Proliferation and Altered Cell Cycle Distribution of SP-1 Cells Transduced with RAR\(\alpha\). The foregoing studies suggested that mezerein-promoted tumors have a constitutive or mezerein-induced deficiency in the RAR\(\alpha\) protein. Tumors induced

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by the mezerein protocol generally have a higher tumor cell labeling index than TPA-promoted tumors (10). To determine if changes in RARα might contribute to the regulation of proliferation in benign tumor cells, SP-1 cells were transduced with the RARα gene using a defective retroviral vector (Fig. 3A). The benign neoplastic SP-1 cells contain an activated ras gene and express very low levels of RARα protein compared to normal keratinocytes (compare Figs. 3A and 4A).

Introduction of the LRARSN expression vector substantially increases the RARα levels in SP-1 cells (Fig. 3A). The effect of RA on cell growth of transduced and control SP-1 cells was tested at doses between 3 × 10⁻⁶ and 3 × 10⁻⁹ M. Cell proliferation was inhibited by RA treatment for 42 h in LRARSN-SP-1 cells in a dose-dependent manner (Fig. 3B). LRARSN-SP-1-transduced cells treated with 3 × 10⁻⁶ M RA showed a 44% inhibition of [³H]thymidine incorporation, whereas this dose of RA had no effect in LXSN-SP-1 control cells. At 3 × 10⁻⁶ M RA, DNA synthesis was inhibited in both cell types, but LRARSN-SP-1 cells were consistently more sensitive to the inhibitory effects of RA.

The decrease in [³H]thymidine uptake in RA-treated LRARSN-SP-1 cells was accompanied by an early redistribution of cells in stages of the cell cycle (Fig. 3C). After 24 h exposure to 3 × 10⁻⁶ M RA, LRARSN-SP-1 cells had a 40% reduction in S-phase cells compared to DM50-treated and vector-transduced controls. An increase in the G0-G1 population was also detected at this time.

Decreased Levels of RARα and RARγ Proteins Are Associated with Expression of the v-ras⁶⁷⁸ Oncogene in Keratinocytes. To determine if the reduced level of endogenous RARα and/or γ proteins in skin tumors and SP-1 cells was related to ras activation, normal cultured mouse keratinocytes were transduced with v-ras⁶⁷⁸ by retroviral infection. Introduction of v-ras⁶⁷⁸ into normal keratinocytes resulted in 60–70% reduction in RARγ and RARα proteins, respectively (Fig. 4A). The magnitude of reduction in RAR protein levels was smaller when lower titers of the v-ras⁶⁷⁸ retrovirus were used for infection (data not shown). Thus, the expression of a mutant ras⁶⁷⁸ p21 protein in primary keratinocytes is sufficient to suppress the level of RARs.

Transactivation of β-RARE-­tk-LUC by RA Is Reduced in v-ras⁶⁷⁸ Keratinocytes. To determine whether the functional response to RA is reduced by ras activation coordinately with the protein levels of RARs, keratinocytes were transfected with DNA constructs containing a reporter gene, LUC, under the control of a β-RARE.
Fig. 3. A. LRARSN-SP-1 cells express elevated levels of RARα protein and respond to RA. SP-1 cells were transduced with the LXSN or LRARSN retroviral vectors as described in "Materials and Methods." Total cell lysates (30 μg) were examined by immunoblot analysis using antibodies specific for RARα as described (26). B. LRARSN-SP-1 and LXSN-SP-1 cells were plated at equal cell density. Twenty-four h following plating, cells were treated for 42 h with varying concentrations of RA or DMSO and pulsed for 2 h with 0.5 μCi [3H]thymidine as described. The radioactivity associated with individual samples was reported as cpm/cell number. [3H]Thymidine incorporation is shown as a percentage of DMSO-treated cells for each group. The values are averages of triplicate measurements; bars, SE. Similar results were produced in three independent experiments. C. 24 h following plating, LRARSN-SP-1 and LXSN-SP-1 cells were treated for 24 h with 3 X 10^-6 M RA or DMSO. Cell cycle analysis was performed as described in "Materials and Methods." Results are representative of two independent experiments.

Transactivation of the reporter gene was determined after 24 h treatment with RA in control and v-ras^Ht keratinocytes. RA caused an 18-fold induction of β-RARE-dependent luciferase activity in control cells and only a 3-fold increase in reporter gene activity in v-ras^Ht keratinocytes (Fig. 4B). Thus, reduction of RAR protein levels in keratinocytes expressing an oncogenic v-ras^Ht gene is coordinated with functional loss of receptor-dependent RA responsiveness.

Down-Regulation of PKC by Bryostatin Partially Restores Normal Levels of RARα Proteins in v-ras^Ht Keratinocytes. Certain phenotypic changes in v-ras^Ht keratinocytes are mediated by activation of PKCα (19), and these can be reversed by bryostatin, a potent ligand for PKC that causes rapid down-modulation in a dose-dependent and isoform-specific manner and thus antagonizes PKC activity (33). In Fig. 5, RARα is reduced by 50% in v-ras^Ht keratinocytes compared to control cells. Bryostatin at 60 nM completely down-regulated PKCα protein in control and v-ras^Ht keratinocytes and restored RARα protein levels up to 82% of control values in v-ras^Ht keratinocytes (Fig. 5). At this dose, bryostatin is relatively specific for down-modulating PKCα (33). Bryostatin did not alter RARα proteins in control cells. These results suggest that the reduction of RARα protein levels in v-ras^Ht keratinocytes is at least partly mediated by PKC and specifically by the α isoform.

TPA Treatment Down-Regulates RARα Proteins in Keratinocytes. To further confirm that PKC activation contributes to the down-regulation of RAR proteins, keratinocytes were treated with 100 nM TPA, and the endogenous levels of RAR proteins were quantitated.
LOSS OF RARs AND ACTIVATION OF ras

Reduction of RARs in tumors may in part be a consequence of ras gene activation. However, it now seems clear that the promoting agent can also influence the extent of RAR loss. Previous reports have demonstrated that RARs are essential for epidermal differentiation (34–37). Overexpression of a dominant-negative RAR in the skin of transgenic mice blocked the formation of mature suprabasal squamous cells (37). The reduction or loss of RARs could be associated with less differentiated squamous states, particularly the

Fig. 4. Decreased levels of RARα (■) and RARγ (□) proteins and decreased RA responsiveness are associated with the expression of the v-rasHa gene in keratinocytes. A, primary mouse keratinocytes were infected with the v-rasHa retrovirus at a MOI of 1 at day 4 of culture. Mock-infected cells were treated identically. Control and v-rasHa keratinocytes were grown in 0.05 mM Ca2+ standard medium for 1 week. Total cell lysates were prepared, and 30 µg from each sample were examined by immunoblot analysis using antibodies specific for RARα, RARγ, and p21^ras^ as described in “Materials and Methods.” Equal protein loading and transfer were verified by Ponceau staining of membranes. Intensity of the RARα and RARγ signals in the v-rasHa group relative to the control (%) was determined by densitometry. Reproducible reductions were obtained, and the extent of reduction mixed with the v-rasHa titer used for infections (MOI of 1 versus MOI of 0.25; data not shown). B, control and v-rasHa keratinocytes transfected with B-RARE-tk-LUC were treated with DMSO or 3 X 10^-8 M RA. After 24 h of treatment, LUC activity was measured as indicated in “Materials and Methods.” Relative LUC activity is shown, calculated as the ratio of LUC activity in RA-treated and in DMSO-treated cells of each group. β-Galactosidase activity was used to normalize for transfection efficiency (see “Materials and Methods”). The values are averages of triplicate measurements; bars, SE.

DISCUSSION

These data indicate that the loss of RARs, particularly RARα, in mouse skin and mouse skin tumors is associated with tumor promotion protocols designed to yield tumors at a high risk for malignant

Fig. 5. Down-regulation of PKC by bryostatin (Bryo) partially restores the normal level of RARα proteins in v-rasHa keratinocytes. Cells were cultured in 0.05 mM Ca2+ and subsequently transduced with v-rasHa on day 3 of culture. Twenty-four h later, control and v-rasHa keratinocytes were treated with 60 nM bryostatin for 48 h to down-regulate PKC. Cells were harvested for analysis of RARα, PKCa, and p21^ras^ by immunoblotting. Keratin 14 (K-14) immunostaining was used to verify equal loading. The intensity of the RARα signal was determined by densitometry, and the relative RARα protein values were normalized to mock-transduced control and untreated keratinocytes (100%). A: column 1, keratinocytes; column 2, bryostatin-treated keratinocytes; column 3, v-rasHa-keratinocytes; column 4, bryostatin-treated v-rasHa-keratinocytes. Similar results were obtained in two independent experiments. B, solvent-treated, +, bryostatin-treated.

in whole-cell lysates over a 24-h period (Fig. 6). TPA treatment down-regulated RARα protein levels by 12, 26, 46, 64, 58, and 33% at 2, 4, 8, 12, 18, and 24 h after treatment, respectively (Fig. 6A and B). This response profile suggests that PKC activation contributes to RARα reduction, and that recovery may occur as PKC is down-regulated. TPA and mezequin (data not shown) also caused almost complete RARα and RARγ down-modulation at 12 h when nuclear extracts were analyzed by immunoblotting (Fig. 6C).
dysplasia seen in progressing papillomas. This may explain the constitutively low level of RARs in mezerein-promoted papillomas seen at 50 weeks, when mezerein promotion was stopped 30 weeks earlier. Such tumors are generally more dysplastic than TPA-promoted tumors (9).

Mouse skin tumor progression is the result of multiple genetic and phenotypic changes (38). Presently, we cannot determine whether the loss of RARs contributes directly to premalignant progression of papillomas or indirectly by modulating other associated factors such as integrin changes, loss of TGF-β, or changes in the proliferative compartment (9, 10, 39). The reconstitution of RARα-deficient papilloma cells with recombinant RARα sensitizes the cells to growth inhibition by RA, suggesting that changes in RARs could influence tumor growth directly. In adult human skin, relative retinoid receptor protein levels were found to reflect relative transcript levels (40). The loss of RARγ proteins in tumors shown previously to express RARγ transcripts by in situ hybridization (11), and here by Northern blot analysis, could reflect a posttranscriptional regulation of RARγ expression in skin tumors, as shown previously for TGF-β1 (10). Further studies will be needed to clarify this observation.

The potential genetic or biochemical differences that distinguish tumors with a high or low risk for malignant conversion remain obscure, although the phenotypic distinction can be detected early (9, 10). Alterations in the AP-1 family of transcription factors may contribute to the high risk phenotype, since overexpression of v-fos can accelerate premalignant progression in benign tumor cells with a ras oncogene (41). Furthermore, premalignant progression is completely inhibited in skin tumors produced on c-fos null mice (42). An increase in AP-1 activity has been associated with malignant conversion in epidermal tumors and cell lines (43–45). The RAR and AP-1 families of transcriptional factors antagonize each other at the level of DNA binding and transactivation (46–50). Therefore, one possible mechanism whereby the loss of RAR proteins in tumors during promotion or premalignant progression could contribute to cancer risk is by a consequent increase in AP-1 activity. If AP-1 activity enhances proliferation of tumor cells, tumors that have lost RARs may arise earlier and grow faster. These are characteristics of high-risk papillomas in skin carcinogenesis (51). Such a possibility is supported by the growth-inhibitory activity of a transduced RARα gene in SP-1 papilloma cells treated with RA. However, we cannot discount the possibility that differential effects of mezerein and TPA on RARs in surrounding normal skin cells contribute to the rapid emergence of high-risk tumors. In vitro studies have suggested that the influence of promoting agents on normal skin keratinocytes is fundamental to their promoting activity (52). The rapid depletion of RARα and γ by mezerein could accelerate the clonal expansion of initiated cells to form clinically visible tumors through combined effects on normal and initiated cells.

Mutation of the c-rasH10 gene is commonly detected in tumors induced by certain initiating agents in mouse skin carcinogenesis (14, 53). The transduction of primary keratinocytes with the v-rasH10 oncogene reduced both RARα and RARγ proteins and inhibited transactivation of β-RARE-ik-LUC by RA. Thus, at an early stage of tumorigenesis, probably initiation, incipient tumor cells are likely to be less responsive to gene activation by retinoids. Reduction to homozygosity of the mutant ras allele is well documented during premalignant progression of chemically induced skin tumors, and this could contribute to further loss of RARs and retinoid responses at the time of malignant conversion (11, 54). Certain of the alterations in v-rasH10 keratinocytes have been attributed to the PKC signal transduction pathway (19, 20), and TPA treatment of cultured keratinocytes and mouse skin attenuated the levels of RARα and RARγ proteins. Previously, the application of TPA to mouse skin was shown to decrease the binding of [3H]RA to RARs and transiently reduce the steady-state level of mRNA for RARα and γ (32). This suggests that PKC activation could regulate expression of retinoid receptors in skin. However, in T lymphocytes, PKC activators enhance transactivation by RA (55), and a synergy was observed between phorbol esters and RA in inducing brain PKC activation (56). It is documented that retinoids can antagonize phorbol ester-induced ornithine decarboxylase activity when applied topically prior to TPA, but retinoids are less effective when applied after TPA (57). Furthermore, retinoid responses are altered markedly in skin that has been previously exposed to TPA or mezerein (58). Our study suggests this could be a result of phorbol ester or mezerein down-modulating RARs. Since mezerein appears to be more potent in this activity, modulation of RARs could be less responsive to gene activation by retinoids. Reduction to homozygosity of the mutant ras allele is well documented during premalignant progression of chemically induced skin tumors, and this could contribute to further loss of RARs and retinoid responses at the time of malignant conversion (11, 54). Certain of the alterations in v-rasH10 keratinocytes have been attributed to the PKC signal transduction pathway (19, 20), and TPA treatment of cultured keratinocytes and mouse skin attenuated the levels of RARα and RARγ proteins. Previously, the application of TPA to mouse skin was shown to decrease the binding of [3H]RA to RARs and transiently reduce the steady-state level of mRNA for RARα and γ (32). This suggests that PKC activation could regulate expression of retinoid receptors in skin. However, in T lymphocytes, PKC activators enhance transactivation by RA (55), and a synergy was observed between phorbol esters and RA in inducing brain PKC activation (56). It is documented that retinoids can antagonize phorbol ester-induced ornithine decarboxylase activity when applied topically prior to TPA, but retinoids are less effective when applied after TPA (57). Furthermore, retinoid responses are altered markedly in skin that has been previously exposed to TPA or mezerein (58). Our study suggests this could be a result of phorbol ester or mezerein down-modulating RARs. Since mezerein appears to be more potent in this activity, modulation of RARs could
contribute to the unique properties of mezezin as a tumor promoter (59–61). Previous studies have suggested that a unique relationship exists between mezezin-promoted tumors and retinoid status of the host, because pharmacological doses of dietary RA can inhibit papilloma formation promoted by mezezin but not by TPA (12, 52). In contrast, carcinoma incidence is inhibited by high dietary RA in both promotion protocols. Although the mechanisms underlying this difference are unknown, the results suggest that the unique promoting action of mezezin on skin and skin tumor cells may in part relate to its effects on retinoic acid receptors. Furthermore, the efficacy of retinoids in the therapy or chemoprevention of human tumors may depend on the specific complement of retinoid receptors in the target tissue and modification of these receptors by the specific pathways involved in the carcinogenic process for that site.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the advice and assistance of Drs. Andrzej A. Dlugosz and Mitchell F. Denning and the editorial assistance of Margaret Taylor.

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Loss of RARα and Activation of Retinoic Acid Receptors


Loss of Retinoic Acid Receptors in Mouse Skin and Skin Tumors Is Associated with Activation of the $\text{ras}^{\text{Ha}}$ Oncogene and High Risk for Premalignant Progression

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