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Advances in Brief

Progressive Decreases in Nuclear Retinoid Receptors during Skin Squamous Carcinogenesis[1]

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Abstract

Retinoids are essential for normal skin growth, differentiation, and apoptosis and are active pharmacologically in the prevention and treatment of skin cancers and other lesions. Retinoid effects are mediated mainly by retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which act as transcription factors to alter gene expression. Using *in situ* hybridization, we analyzed the expression of RARs and RXRs in normal sun-exposed skin ($n = 85$), squamous cell carcinoma (SCC; $n = 28$), and actinic keratosis [AK (a precursor to SCC); $n = 38$]. The expressions of five receptors (RAR-α and -γ and RXR-α, -β, and -γ) were moderate to very strong in normal skin, with higher expressions in spinous and granular layers than in the basal layer. RAR-β expression was weak or absent in normal and lesion samples. All five receptors expressed in the skin were suppressed progressively from normal skin to premalignant skin (AK) to invasive skin SCC. Specific receptor decreases in lesions relative to normal skin ranged from 75% (RXR-β) to 96% (RAR-α) in SCC and from 37% (RAR-γ) to 68% (RXR-β) in AK. The degree of suppression of RXR-α and RAR-γ, the two predominant retinoid receptors in skin, was relatively less for RXR-α (58% *versus* 86%; $P = 0.015$) and relatively greater for RAR-γ (37% *versus* 89%; $P = 0.0001$) between AK and SCC, suggesting that suppression of RXR-α may be an earlier event and expression of RAR-γ may be a later event of multistep squamous skin carcinogenesis. Our results indicate that suppressed expression of retinoid receptors occurs early (in AK) and is associated with progression of squamous skin carcinogenesis to SCC.

Introduction

Non-melanoma skin cancer is the single most commonly diagnosed cancer in the Caucasian population, with an estimated 1,000,000 new cases reported in the United States each year (1). Of the two most common forms of skin cancer (SCC[4] and BCC), SCC is clinically more aggressive, accounting for most of the non-melanoma skin cancer deaths (2), and has been increasing in incidence at a rate of 4–8% per year since the 1960s, with particularly increased rates (up to 10% annually) in recent years (3, 4).

Although most early skin SCCs and BCCs are controlled successfully with conservative local therapy, they still are responsible for profound quality of life consequences, such as substantial pain, cosmetic morbidity, and medical costs (4). Therefore, new strategies to prevent skin cancer are necessary and are being developed. One of the best studied of these new approaches is retinoid chemoprevention, which has established clinical activity in several sites of epithelial carcinogenesis, including the skin (5–8).

Retinoids exert their effects primarily through two subfamilies of the steroid/thyroid hormone receptor superfamily, the RARs and RXRs. There are three types each of RARs (RAR-α, -β, and -γ) and RXRs (RXR-α, -β, and -γ), and all are nuclear, ligand-dependent, DNA-binding transcriptional transactivator proteins (9).

The various RAR and RXR subtypes are encoded by different genes, are highly conserved in evolution, and display distinct spatiotemporal expression patterns in early and adult stages of development, suggesting that each receptor has distinct physiological functions. RAR-α is expressed in most tissues. RAR-β has a more restricted distribution pattern, which does not include the skin. RAR-γ is expressed mainly in the skin, accounting for 87% of the RAR protein in that tissue. RXR-α is the skin's predominant RXR, accounting for 90% of the RXR protein in the skin (10). Therefore, it is highly likely that the RAR-γ/RXR-α heterodimer is the principle transducer of the retinoid signal in human skin (11).

We designed our present analysis of retinoid receptor status in premalignant and malignant skin lesions (relative to that in adjacent normal skin) to assess possible relationships among the levels of these receptors and cancer development and progression. This information is important for improving our ability to predict responsiveness to retinoid treatment. To our knowledge, this is the first systematic comparative analysis of all six retinoid receptor expressions in human skin SCCs and BCCs, AK, and normal skin. Isolated findings of retinoid receptor expressions in human skin (10, 12) and a decrease in RAR-γ expression in skin SCC (13) have been reported.

Materials and Methods

In general, the specimens were obtained from sun-exposed areas of the skin from Caucasian patients (age range, 46–92 years). The specimens of human AK and SCC and adjacent normal skin were removed surgically or by shave biopsy, routinely fixed in 10% neutral formalin, and embedded in paraffin. The paraffin blocks were then cut into 4-μm-thick sections, which were collected in a bath containing diethylpyrocarbonate-treated water to prevent RNase contamination. For the same reason, the glass microscope slides used had been cleaned and baked at 180°C for 4 h and then coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO; Ref. 14).

We used a nonradioactive *in situ* hybridization method based on digoxygenin-labeled antisense riboprobes prepared for each of the six receptors, as described previously (14, 15). The quality and specificity of the digoxigenin-labeled probes were determined by Northern blot analysis (14). The specificity of the binding of antisense riboprobes was verified by negative control sections to which sense probes were hybridized or no probe was hybridized. These controls were found to be negative. The stained sections were reviewed with a Nikon microscope. Lesion or normal skin staining within each section was

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[4] The abbreviations used are: SCC, squamous cell carcinoma; BCC, basal cell carcinoma; RAR, retinoic acid receptor; RXR, retinoid X receptor; AK, actinic keratosis; RA, retinoic acid.

scored from 0–4 as follows: (*a*) 0, no staining; (*b*) 1, weak staining; (*c*) 2, moderate staining; (*d*) 3, strong staining; and (*e*) 4, very strong staining.

Results

We analyzed retinoid receptor expressions in 85 normal skin samples adjacent to our study lesions. RAR-α and -γ and RXR-α, -β, and -γ expressions ranged from moderate to very strong in normal skin. However, the patterns of receptor expression were not consistent throughout the epidermal layers, with generally higher expressions in the spinous and granular layers than in the basal layer. RAR-β expression ranged from weak to absent in normal skin and in the lesion samples as well.

We then analyzed receptor expression in 38 AK and 28 SCC specimens by *in situ* hybridization (Figs. 1 and 2). The staining category (scores, 0–4) of each of 38 AK specimens and 28 SCC specimens is summarized in Table 1, and the number of cases in which the staining in AK or SCC was lower than that in the corresponding adjacent normal specimen was determined and is presented in Table 2.

The expressions of the nuclear retinoid receptors were suppressed in AK (premalignant skin) and SCC, compared with adjacent normal skin. All five skin retinoid receptors were profoundly suppressed in SCC as compared with normal skin (Tables 1 and 2; Fig. 1). The skin receptors also were substantially suppressed in AK lesions (only RAR-γ suppression occurred in $<$50% of samples) compared with normal skin (Table 2; Fig. 2). Statistically significant greater retinoid receptor suppression occurred in SCC than in AK, with the greatest difference occurring in RAR-γ suppression (Table 2). In SCC, specific receptors were decreased in 75% (RXR-β) to 96% (RAR-γ) of samples (Table 2). No increases in receptor expression appeared in the SCC samples. Receptor expression levels varied according to the extent of differentiation. In general, specimens of poorly differentiated SCC had much lower receptor expression than did specimens of highly differentiated SCC. This pattern held true even within less differentiated and more differentiated areas within the same SCC specimen. Greater expression of receptors occurred near positive cytokeratin CK-1 staining than near CK-1-negative staining (data not shown). Our results in advanced SCC lesions (*e.g.*, those having nerve, muscle, or lymph node involvement) paralleled those in early SCC lesions (data not shown).

Discussion

Our present study involved a systematic analysis of the differential expression of nuclear retinoid receptors in human skin carcinogenesis. Our major finding is that suppression of nuclear retinoid receptors occurs progressively during multistep skin squamous carcinogenesis. Normal skin had moderate to very strong expression of five retinoid receptors, all except RAR-β, which was weak to absent in normal samples and all lesion samples. Receptor expression loss began at an early stage of skin carcinogenesis, with suppression in 37–68% of AK lesions. Specific receptor decreases in SCC lesions (*versus* those in normal tissue) ranged from 75% (RXR-α) to 96% (RAR-α). We found that expression of the two predominant skin receptors, RAR-γ and RXR-α, was reduced in 89% and 86% of SCC lesions, respec-

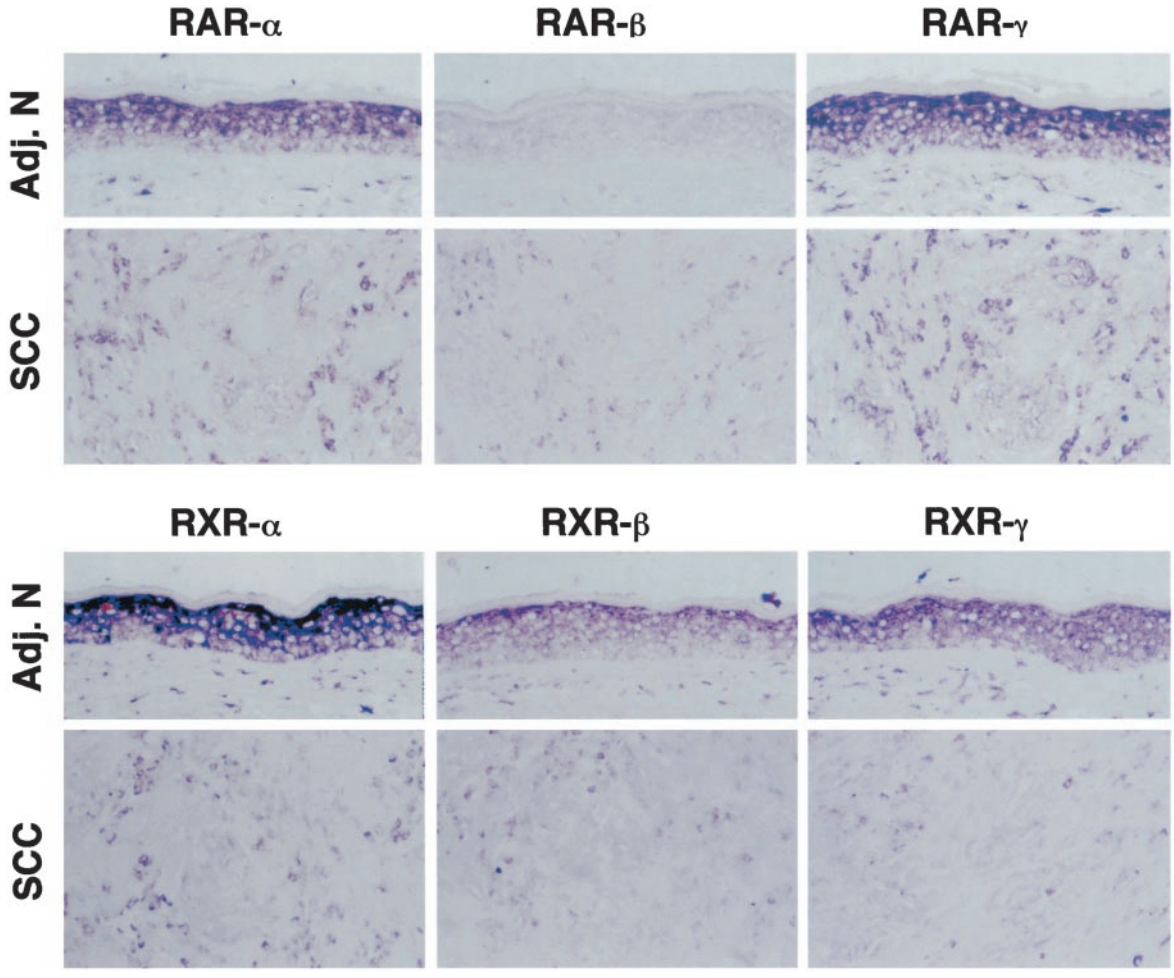

Fig. 1. Expression of RAR and RXR mRNAs in SCC, as shown by *in situ* hybridization.

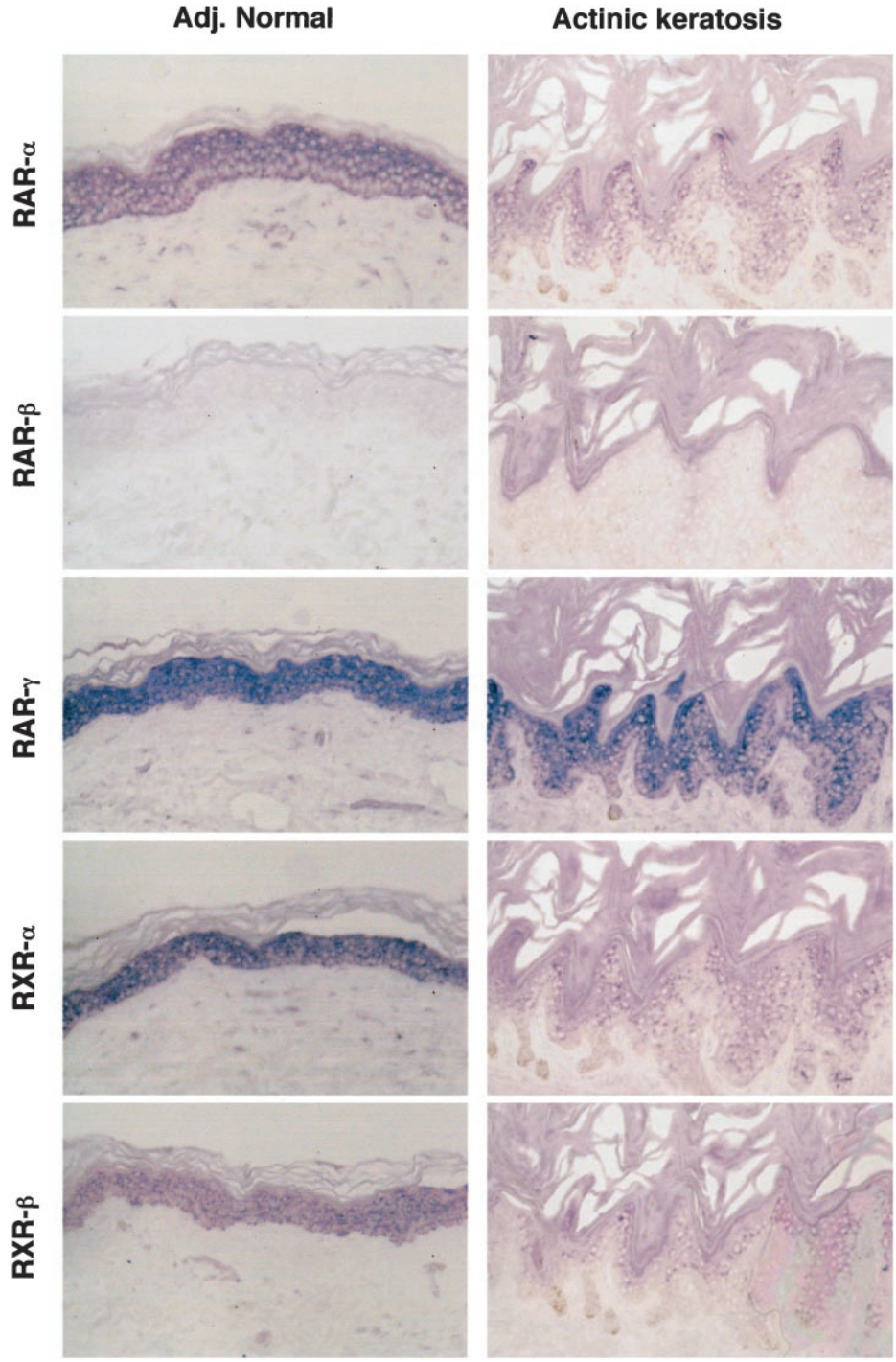

Fig. 2. Expression of RAR and RXR mRNAs in AK and adjacent skin, as shown by *in situ* hybridization.

tively. RXR-α suppression occurred in 58% of AKs compared with 86% of SCCs ($P = 0.015$, χ^2 test), indicating that this is an earlier event during carcinogenesis, whereas RAR-γ suppression occurred in 37% of AKs compared with 89% of SCCs ($P = 0.0001$, χ^2), indicating that this is a later event. All these data support our conclusion that a progressive decrease in the expressions of RAR-α, RAR-γ, RXR-α, RXR-β, and RXR-γ occurs in carcinogenic progression from normal skin to AK to SCC.

Retinoids and their nuclear receptors are critically important to skin physiology. Several groups have previously described the expressions of RARs and RXRs in normal human skin (10–13). RAR-γ is highly expressed, followed by RAR-α whereas RAR-β is expressed at low levels or is not expressed at all (10, 16). RXR-α predominates, and RXR-β can be detected; but neither RXR-γ message or protein has been detected previously in normal skin (10). Except for those regarding RXR-γ expression, our results are consistent with these previously reported findings on RAR-γ and RXR-α expression patterns in normal skin. Our samples of normal skin (which were from skin adjacent to SCCs) did express the RXR-γ transcript. This difference between our report and previous reports probably reflects the greater sensitivity of *in situ* hybridization (our method) as compared with Northern blotting (the method used in previous studies). *In situ* hybridization is very sensitive because it can detect mRNA in individual cells.

Table 1 *Analysis of nuclear retinoid receptor expression in skin tissues by in situ hybridization, presented as number of cases in each of five staining categories (0–4)*

	Normal skin adjacent to AK (n = 38)					AK (n = 38)					Normal skin adjacent to SCC (n = 28)					SCC (n = 28)				
Receptor	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
RAR-α	0	6	14	15	3	1	20	10	6	1	0	4	10	11	3	10	13	3	2	0
RAR-β	38	0	0	0	0	38	0	0	0	0	28	0	0	0	0	28	0	0	0	0
RAR-γ	1	11	16	10	0	4	17	12	5	0	0	9	9	6	4	10	9	7	2	0
RXR-α	1	5	20	11	1	8	13	14	3	0	0	5	10	11	2	6	12	7	3	0
RXR-β	5	6	23	4	0	10	16	11	1	0	4	8	12	4	0	9	14	4	1	0
RXR-γ	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	4	9	13	2	0	10	12	5	1	0

[a] ND, not determined.

We are aware of only one isolated previous report of nuclear retinoid receptor expression in SCC, which showed suppressed RAR-γ (13). Altered expression levels or aberrant function of retinoid receptors, however, has been associated with several other epithelial neoplasms, including head and neck cancer (RAR-β; Ref. 15), breast cancer (RAR-α and -β; Ref. 17), esophageal cancer (RAR-β; Ref. 18), non-small cell lung cancer (RAR-β, -γ, and RXR-γ; Ref. 19), prostate cancer (RAR-β and RXR-β; Ref. 20), and dysplastic nevi (RXR-β; Ref. 21). The expression of RAR-α, RAR-γ, and RXR-α mRNA in mouse skin was found to decrease a few hours after exposure of the skin to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (22), and RAR-α and -γ were progressively lost during mouse skin carcinogenesis (23). The profound loss we report here in human skin SCC of expression of all retinoid receptors found in normal skin has not been reported for any other cancer, including non-cutaneous SCCs. Consistent with aberrant RA signaling in skin SCC, we recently found significant suppression of the RA-regulated gene *TIG-3* in skin SCC (24). Because retinoid receptors have been implicated in differentiation of normal keratinocytes *in vitro* (25) and skin epidermis *in vivo* (26), it is plausible that decreased expression of these receptors may be the cause of dysregulation of differentiation and may contribute to early stages of skin carcinogenesis.

The mechanism underlying the suppression of nuclear retinoid receptor expression in the development and progression of human squamous carcinogenesis of the skin remains unknown. Recent studies, however, have shown that exposure of human skin to low levels of UV irradiation results in a reduction in RAR-γ and RXR-α mRNA and protein, which can be prevented by RA treatment (27). Subsequently, it was shown that UV irradiation inhibits the synthesis of RAR-γ and RXR-α protein and that these receptors are substrates for ubiquitination and proteasome-mediated degradation (28).

Our present results suggest the following hypotheses: (*a*) the loss of receptor expression may result in diminished ability of epidermal cells to respond to physiological levels of RA, and this may contribute to SCC development; and (*b*) pharmacological RA levels may restore the ability of skin SCC cells to use RA by saturating the remaining signaling machinery and thus up-regulating the skin RARs. These hypotheses are consistent with the positive results of a recent definitive Phase III retinol trial (7). We are assessing these hypotheses in an ongoing National Cancer Institute skin Phase III trial underway at The University of Texas M. D. Anderson Cancer Center.

Table 2 *Decreased expression of nuclear retinoid receptors in premalignant and malignant squamous skin lesions (compared with paired adjacent normal skin)*

	No. of cases with reduced receptor expression/total (%)		P^a	
Receptor	AK	SCC	Fisher's exact test	χ^2 test
RAR-α	24/38 (63)	27/28 (96)	0.0011	0.0014
RAR-β	0/38 (0)	0/28 (0)		
RAR-γ	14/38 (37)	25/28 (89)	0.00001	0.0001
RXR-α	22/38 (58)	24/28 (86)	0.014	0.015
RXR-β	26/38 (68)	21/28 (75)	0.38	0.56
RXR-γ^b	ND[c]	22/28 (79)	ND	

[a] Comparing AK with SCC.
[b] RXR-γ was not analyzed in AK.
[c] ND, not determined.

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