

SUPPRESSION OF RETINOIC ACID RECEPTOR- β IN PREMALIGNANT ORAL LESIONS AND ITS UP-REGULATION BY ISOTRETINOIN

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Abstract Background. Retinoids are effective in the treatment and prevention of certain human cancers. Most of their actions are thought to result from changes in gene expression mediated by nuclear retinoic acid receptors and retinoid X receptors. We conducted a study to determine whether the expression of these receptors was altered in premalignant oral lesions and, if so, whether their expression could be restored by treatment with isotretinoin.

Methods. We performed *in situ* hybridization of retinoic acid receptors and retinoid X receptors using antisense riboprobes in specimens of oral mucosa from 7 normal subjects and specimens of premalignant oral lesions from 52 patients before treatment with isotretinoin and from 39 of the 52 patients after three months of treatment.

Results. All the normal specimens expressed retinoic acid receptor- β messenger RNA (mRNA). In contrast, retinoic acid receptor- β mRNA was detected in only 21 of the 52 premalignant oral lesions ($P = 0.003$). Thirty-five of

the 39 specimens available for evaluation after treatment expressed retinoic acid receptor- β mRNA ($P < 0.001$). All normal and premalignant specimens expressed similar levels of mRNA for retinoic acid receptor- α and retinoic acid receptor- γ and the three types of retinoid X receptors, α , β , and γ . The levels of retinoic acid receptor- β mRNA increased in the specimens from 18 of the 22 patients who had responses to isotretinoin and in 8 of the 17 specimens from the patients without responses ($P = 0.04$).

Conclusions. The expression of retinoic acid receptor- β mRNA is selectively lost in premalignant oral lesions and can be restored by treatment with isotretinoin. Restoration of the expression of retinoic acid receptor- β mRNA is associated with a clinical response. Retinoic acid receptor- β may have a role in mediating the response to retinoids and may be a useful intermediate biologic marker in trials of these agents for the prevention of oral carcinogenesis. (N Engl J Med 1995;332:1405-10.)

RETINOIDS, including vitamin A and its analogues, regulate the morphogenesis, development, and growth and differentiation of cells.¹⁻³ Retinoids can also halt the progression of disease in premalignant lesions of the oral cavity, cervix, and skin and can prevent the development of second primary tumors associated with head and neck and lung cancer.⁴⁻¹⁴ Retinoids exert most of their effects by modulating gene expression.¹⁵ The effects of retinoids on gene expression depend on two types of nuclear retinoid receptors, retinoic acid receptors and retinoid X receptors, which act as transcription factors.^{3,16,17} Both types belong to the superfamily of steroid hormone receptors.¹⁶ The α , β , and γ subtypes of retinoic acid receptors and retinoid X receptors have distinct and conserved amino- and carboxy-terminal domains.^{3,16,17} Heterodimers of the retinoic acid receptors and the retinoid X receptors bind to a specific DNA sequence, the retinoic acid response element. This element is located in the promoter region of genes, including the retinoic acid receptor- β_2 gene,¹⁸ that retinoids regulate. Each receptor subtype has a specific pattern of expression during embryonal development and a different distribution in adult tissues. Each subtype is therefore thought to regulate the expression of a distinct set of genes.^{2,3}

The association between vitamin A deficiency and the development of cancer⁶ suggests that retinoid-dependent signaling pathways have a role in the suppression of carcinogenesis. Changes in the expression of specific

nuclear retinoid receptors may abrogate these pathways. The patterns of expression of retinoid receptors in normal, premalignant, and malignant tissue may therefore provide clues to the roles of these receptors in the development of cancer and in the response of premalignant lesions to retinoid treatment.

Most reports have described the expression of receptors in cultured normal or malignant cell lines and in embryos.^{2,3,15-19} There are only a few reports on the expression of retinoic acid receptors in tumor specimens,²⁰⁻²² and no reports on their expression in premalignant oral lesions *in vivo*.

Our group has demonstrated that 13-*cis*-retinoic acid (isotretinoin) prevents the development of cancer in patients with premalignant oral lesions (e.g., leukoplakia)^{4,7,11} and inhibits the development of second primary tumors in patients with previous head and neck cancer.^{8,12,13}

The current study was undertaken to determine whether the expression of retinoic acid receptors in normal tissue and in premalignant oral lesions differs and whether isotretinoin, which is active clinically,^{4-8,11-13} alters the expression of these receptors *in vivo*.

METHODS

Characteristics of the Patients

The specimens used in this study were from 7 normal subjects and 52 patients with histologically confirmed premalignant oral lesions. The patients consisted of 27 men and 25 women, many of whom used tobacco or alcohol or both at the time of the study (Table 1). On biopsy, the lesions of 20 of the patients showed hyperplasia, 24 mild dysplasia, 6 moderate dysplasia, and 2 severe dysplasia.

Tissue Specimens

Punch-biopsy specimens (4 mm in diameter) were obtained from all 52 patients before they underwent three months of therapy with isotretinoin at a dose of 1.5 mg per kilogram of body weight per day.¹¹

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Post-treatment specimens were available from 39 of these patients. (The post-treatment specimens from the other 13 patients had been totally consumed in previous laboratory studies.) All biopsies had been performed during an earlier, nonrandomized induction-phase clinical protocol.¹¹ The specimens were obtained from the premalignant oral lesions before treatment, from the residual lesions after treatment in the patients with partial or no responses, or from the sites of the original lesions, as identified from pretreatment photographs, in the patients with complete responses. In each of the two patients with biopsy specimens from more than one lesion, the specimen selected for analysis came from the largest lesion, so that only one specimen per patient was analyzed before and after treatment. In addition, punch-biopsy specimens of normal buccal mucosa were obtained from seven volunteers who did not smoke.

The specimens were fixed in 10 percent neutral formalin and embedded in paraffin. The pathology department provided blocks of the tissue specimens, which were cut into 4- μ m sections and collected on glass slides coated with poly-L-lysine. The sections were coded, and the receptor analyses were performed in a blinded fashion.

In Situ Hybridization

We used a previously described method of nonradioactive in situ hybridization, without any modifications, to analyze nuclear retinoid receptors in the formalin-fixed, paraffin-embedded histologic sections.^{22,23} The quality and specificity of the digoxigenin-labeled probes were determined with Northern blotting, and the specificity of the binding of the antisense riboprobes was verified by using sense probes as controls.²³

All sections to be analyzed for the expression of a particular receptor were stained on the same day with the same reagents to ensure reliable comparisons. The stained sections were reviewed under a Nikon microscope by three researchers, including two pathologists, who did not know the treatment status of the patients from whom the specimens had been obtained. The data in Table 2 are based on evaluation of the tissue sections for the presence or absence of staining. The data in Table 3 are based on staining-intensity scores ranging from 0 to 3 (0 indicates no staining, 1 weak staining, 2 strong staining, and 3 very strong staining).

Statistical Analysis

Frequency and summary data are given whenever appropriate. The chi-square test and Fisher's exact test were used to assess the association between two binary variables, such as the association between the clinical response and the modulation of nuclear retinoid receptors. McNemar's test was used to compare receptor expression

Table 2. Positive Expression of Retinoic Acid Receptor- β mRNA in Premalignant Oral Lesions before and after Treatment with Isotretinoin.

SITE OF ORAL LESION	EXPRESSION OF RETINOIC ACID RECEPTOR- β mRNA	
	BEFORE TREATMENT	AFTER TREATMENT
	<i>no. of patients/total (%)</i>	
Buccal mucosa	7/21 (33)	13/16 (81)
Tongue	12/16 (75)*	10/11 (91)
Lip	0/3	3/3 (100)
Soft palate	0/3	3/3 (100)
Hard palate	0/2	1/1 (100)
Gingiva	2/7 (29)	5/5 (100)
Total	21/52 (40)	35/39 (90)†

*The base-line level of retinoic acid receptor- β mRNA in the tongue was significantly higher than that in other sites ($P=0.002$ by Fisher's exact test).

† $P<0.001$ by McNemar's test.

before and after treatment.²⁴ The two-sample t-test was used to compare age and receptor expression. Two-sided P values were determined in all analyses.

RESULTS

Serial sections of two specimens of normal buccal mucosa hybridized with antisense riboprobes for messenger RNA (mRNA) for retinoic acid receptor- α , retinoic acid receptor- β , retinoic acid receptor- γ , and retinoid X receptor- α (Fig. 1A, 1B, 1C, and 1D). Similar results were observed with mRNA for retinoid X receptor- β and retinoid X receptor- γ (data not shown). These receptors were expressed in all the specimens of normal oral mucosa.

Loss of Retinoic Acid Receptor- β mRNA in Premalignant Oral Lesions

Figure 2 shows consecutive sections of a specimen from a premalignant oral lesion. The expression of mRNA for retinoic acid receptor- α , retinoic acid receptor- γ , and retinoid X receptor- α was similar to that found in normal tissue (Panels A, C, and D). The results were similar for retinoid X receptor- β and retinoid X receptor- γ mRNA (data not shown). In contrast, retinoic acid receptor- β mRNA was not detected in this lesion (Fig. 2B).

Retinoic acid receptor- β mRNA was detected in 21 of the 52 premalignant specimens (40 percent) (Table 2), whereas it was found in all 7 of the normal specimens ($P=0.003$ by Fisher's exact test). The expression of mRNA for the other retinoid receptors in the premalignant lesions ranged from 70 percent for retinoid X receptor- β to 100 percent for retinoid X receptor- α . None of these results differed significantly from those in the normal tissue (data not shown). There was no association between the expression of retinoic acid receptor- β mRNA and age ($P=0.96$ by the two-sample t-test), smoking status ($P=0.49$ by the chi-square test), or use of alcohol ($P=0.39$ by the chi-square test).

Because cell strains and cell lines derived from normal and premalignant tissue from different regions of

Table 1. Base-Line Characteristics of 52 Patients with Premalignant Oral Lesions.

CHARACTERISTIC	PATIENTS WITH PREMALIGNANT LESIONS
Age — yr	
Median	58
Range	24–83
Sex — no. of patients (%)	
Female	25 (48)
Male	27 (52)
Histologic type — no. of patients (%)	
Hyperplasia	20 (38)
Mild dysplasia	24 (46)
Moderate dysplasia	6 (12)
Severe dysplasia	2 (4)
Tobacco use — no. of patients (%)	
Current	28 (54)
Former	11 (21)
Never	13 (25)
Alcohol consumption — no. of patients (%)	
Current	38 (73)
Former	1 (2)
Never	13 (25)

Table 3. Relation between Increased Expression of Retinoic Acid Receptor- β mRNA and Clinical Response after Treatment with Isotretinoin.

CLINICAL RESPONSE (NO. OF PATIENTS)*	RETINOIC ACID RECEPTOR- β mRNA LEVEL AFTER TREATMENT†		
	INCREASED	NO CHANGE	DECREASED
	no. of patients (%)		
Response			
Complete (n = 4)	4	0	0
Partial (n = 18)	14	3	1
Total (n = 22)	18 (82)‡	3 (14)	1 (4)
No response (n = 17)	8 (47)	7 (41)	2 (12)

*Complete response was defined as the total disappearance of the lesion, and partial response as a decrease in the size of a lesion by at least 50 percent. Detailed information on the clinical responses is reported elsewhere.¹¹

†Increase indicates a higher score for staining after treatment than before treatment (e.g., an increase from 0 to 1 or from 1 to 2), no change indicates that the score did not change, and decrease indicates a lower score after treatment than before treatment.

‡P = 0.04 by Fisher's two-sided exact test.

the oral cavity express different levels of retinoic acid receptor- β mRNA,^{25,26} we analyzed our data according to the location of the tissue in the oral cavity (Table 2). From 67 to 100 percent of premalignant specimens from buccal mucosa and other regions of the oral cavity showed loss of retinoic acid receptor- β mRNA expression, as compared with only 25 percent of premalignant specimens from the tongue (P = 0.002 by Fisher's exact test).

Increased Retinoic Acid Receptor- β mRNA after Treatment with Isotretinoin

To determine whether treatment with retinoids modulates the expression of retinoid-receptor mRNA in vivo, we analyzed specimens from 39 of the 52 patients after three months of treatment with isotretinoin. Figure 2B shows selective loss of retinoic acid receptor- β mRNA in a biopsy specimen obtained before treatment. After treatment, the same premalignant lesion contained abundant mRNA for the receptor (Fig. 2F). Ninety percent of the 39 specimens from patients who were treated with isotretinoin expressed retinoic acid receptor- β mRNA, as compared with 40 percent of the pretreatment specimens (P < 0.001 by McNemar's test) (Table 2). The expression of retinoic acid receptor- β mRNA increased in specimens from all regions of the oral cavity (Table 2). Only 3 of the 39 specimens showed decreased levels of retinoic acid receptor- β mRNA after treatment.

Relation between Retinoic Acid Receptor- β mRNA and Clinical Response to Treatment

Of the 39 patients with premalignant oral lesions who were studied after treatment with isotretinoin, 22 (56 percent) had clinical responses to treatment (Table 3). There were 4 complete responses and 18 partial responses. The specimens from all 18 patients with partial responses (obtained from residual lesions) revealed histologic abnormalities. The specimens from the four patients with complete responses (obtained from the

sites of the original lesions) showed dysplasia in two and hyperplasia in two. Eighteen of the 22 patients with clinical responses (82 percent) had increased levels of retinoic acid receptor- β mRNA, as compared with 8 of the 17 patients without responses (47 percent). Eighteen of the 26 patients (69 percent) with up-regulated expression of retinoic acid receptor- β mRNA after treatment had clinical responses, as compared with only 4 of the 13 patients (31 percent) without retinoic acid receptor- β up-regulation (P = 0.04 by Fisher's two-sided exact test).

There was no association between the pretreatment expression of retinoic acid receptor- β mRNA and the clinical response to isotretinoin. Fifty-eight percent of the patients with no retinoic acid receptor- β mRNA at base line (14 of 24) had clinical responses, as compared with 53 percent of the patients with retinoic acid receptor- β mRNA at base line (8 of 15) (P = 0.76 by the chi-square test). Furthermore, all 14 patients without retinoic acid receptor- β mRNA at base line who had complete or partial responses had an up-regulation of retinoic acid receptor- β mRNA. All four patients with complete responses also had an up-regulation of retinoic acid receptor- β mRNA.

DISCUSSION

Encouraging results with retinoids in clinical prevention and therapy trials^{4,14} have stimulated efforts to understand how these agents act at the cellular and molecular levels. In this study, we analyzed the expression of mRNA for the six known retinoid-receptor subtypes in normal and premalignant oral tissue before and after treatment with isotretinoin. Because there are no

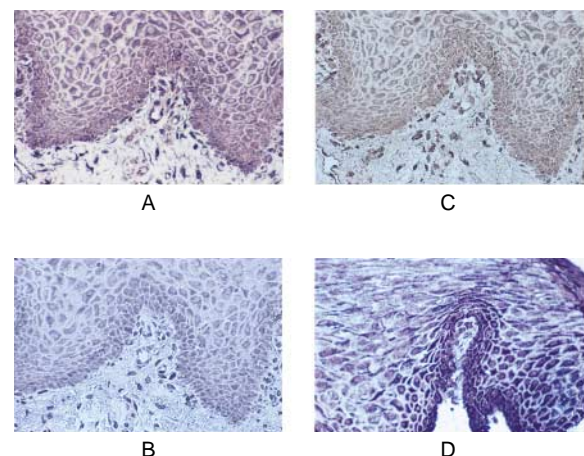


Figure 1. Expression of mRNA for Retinoic Acid Receptor- α (Panel A), Retinoic Acid Receptor- β (Panel B), Retinoic Acid Receptor- γ (Panel C), and Retinoid X Receptor- α (Panel D) in Consecutive Sections of Biopsy Specimens from Normal Buccal Mucosa.

The receptors were detected by nonradioactive in situ hybridization and light microscopy. Tissue sections were hybridized with digoxigenin-labeled antisense RNA probes, and the bound probes were detected with an antidigoxigenin antibody-alkaline phosphatase conjugate and a chromogenic substrate.

useful antibodies for the detection of nuclear retinoid receptors in histologic specimens, our analysis was limited to the detection of retinoid-receptor mRNA by in situ hybridization.

We found a selective loss of retinoic acid receptor- β mRNA in premalignant oral lesions. Because we were able to analyze only mRNA, we cannot exclude the possibility that in some specimens in which retinoic acid receptor- β mRNA was detected, the level of protein was suppressed at the post-transcriptional stage. Retinoic acid receptor- β mRNA was detected in all the samples of normal tissue but in only 40 percent of the samples of premalignant tissue. This finding is consistent with in vitro data^{25,26} and with our findings in specimens from patients with head and neck cancer.²² These studies suggest that loss of retinoic

acid receptor- β mRNA is an early event in oral carcinogenesis.

The mechanisms underlying the loss of retinoic acid receptor- β mRNA in premalignant oral lesions are not understood. We can speculate that no changes have occurred in the retinoic acid receptor- β gene. No gene deletions or rearrangements were found in cell lines from patients with head and neck cancer that failed to express retinoic acid receptor- β mRNA,²⁶ and in the present study its expression was up-regulated by treatment with isotretinoin.

The expression of retinoic acid receptor- β mRNA may depend on the intracellular level of retinoids. Studies in rats have demonstrated that the expression of retinoic acid receptor- β mRNA is selectively reduced in several organs during vitamin A deficiency and is enhanced by retinoic acid.^{27,28} It is unlikely that our patients had vitamin A deficiency, but we cannot exclude the possibility that the premalignant tissue was deficient in vitamin A because of a reduced uptake of vitamin A from plasma or an abnormally elevated rate of catabolism of intracellular retinoic acid.

Other possible causes of a reduction in the expression of retinoic acid receptor- β mRNA include overexpression of retinoic acid receptor- γ_1 mRNA, which could antagonize the transactivation of the retinoic acid response element for the retinoic acid receptor- β gene,^{17,29} and decreased levels of coactivators (e.g., cellular E1A-like proteins³⁰ and estrogen receptor-associated proteins³¹) essential for transactivation through the retinoic acid response element. The loss of expression of retinoic acid receptor- β mRNA by HeLa cells was not caused by mutations in the retinoic acid response element or other proximal regulatory elements of the retinoic acid receptor- β promoter.³²

Regardless of the mechanism, aberrations in the level and function of retinoic acid receptor- β mRNA may promote carcinogenesis. This hypothesis is supported by the finding that a number of lung-cancer cell lines fail to express retinoic acid receptor- β mRNA.^{20,21,33,34} Most of the neoplastic cells and tissues that express little or no retinoic acid receptor- β mRNA still express mRNA for retinoic acid receptor- α , retinoic acid receptor- γ , and at least one of the retinoid X receptors, which may mediate the transactivation of retinoid-responsive genes. This observation raises the possibility that retinoic acid receptor- β regulates specific genes that are important for the suppression of carcinogenesis. It is relevant that transfection of the retinoic acid receptor- β gene decreases the tumorigenicity of human lung-cancer cells.³⁵ Moreover, transient transfection with this gene results in retinoic acid-dependent suppression of cell proliferation.³⁶ The in vivo data presented here suggest that loss of the expression of retinoic acid receptor- β mRNA may have an important role in the expansion of premalignant clones.

We also found that a significant proportion of the patients had increased expression of retinoic acid receptor- β mRNA in oral premalignant tissue after treatment with isotretinoin. This finding is important,

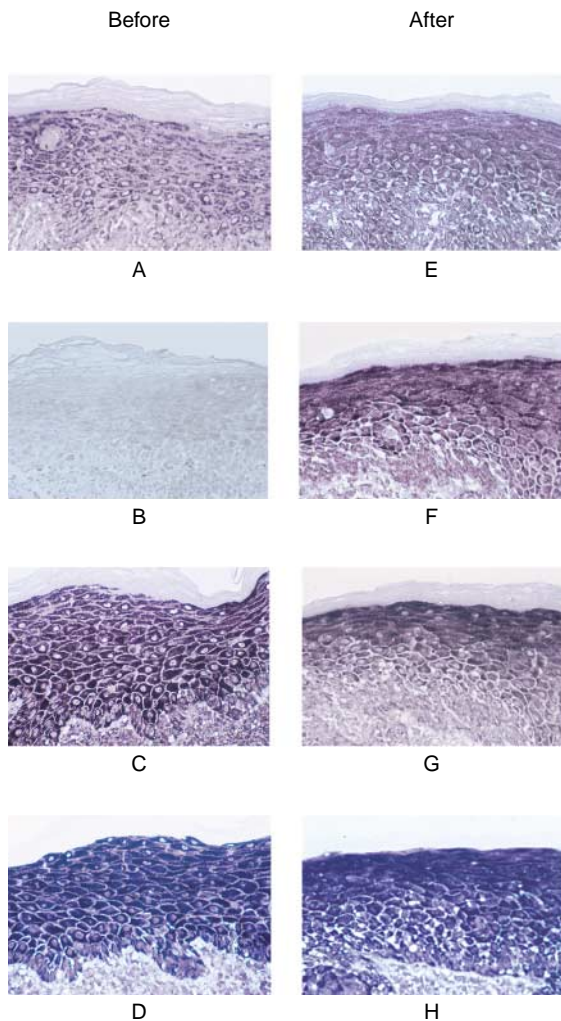


Figure 2. Expression of mRNA for Retinoic Acid Receptor- α , Retinoic Acid Receptor- β , Retinoic Acid Receptor- γ , and Retinoid X Receptor- α in Consecutive Sections of Biopsy Specimens from a Patient with a Premalignant Oral Lesion.

The expression of mRNA for retinoic acid receptor- α , retinoic acid receptor- β , retinoic acid receptor- γ , and retinoid X receptor- α is shown at base line (Panels A, B, C, and D, respectively) and after three months of treatment with isotretinoin (Panels E, F, G, and H, respectively).

even though our study was not randomized; the incidence of an increase in retinoic acid receptor- β mRNA had a random-chance probability of <0.001 (Table 2).

The ability of retinoic acid to induce the expression of retinoic acid receptor- β mRNA in cultured cell lines has been well documented,^{18,37} and in vivo induction has been reported in vitamin A-deficient rats^{38,39} and fetal mice.⁴⁰ Of the six subtypes of retinoid receptors, retinoic acid receptor- β appears to be the most closely regulated by retinoids. Retinoic acid can induce the expression of retinoic acid receptor- β mRNA in certain normal human cells (e.g., oral keratinocytes,²⁵ tracheobronchial epithelial cells,³⁴ and senescent mammary epithelial cells⁴¹) and in nontumorigenic HeLa-cell hybrids, but not in the malignant counterparts of these cells.³² These differences suggest that transformed cells have an aberrant response to retinoic acid.

Another important finding of this study is the significant association between the increased expression of retinoic acid receptor- β mRNA and clinical responses of premalignant oral lesions to isotretinoin. The importance of this association can be seen both in the high percentage of patients with clinical responses and increased expression of retinoic acid receptor- β mRNA (82 percent) and in the percentages of patients with increased and unchanged expression of retinoic acid receptor- β mRNA among those with clinical responses (69 and 31 percent, respectively; $P=0.04$). It is possible that retinoic acid receptor- β contributes to the suppression of the premalignant phenotype and is thus causally linked to the clinical outcome in chemoprevention trials of retinoids.

Our results suggest that retinoic acid receptor- β may be a useful intermediate marker in trials of retinoids for the prevention of oral carcinogenesis. However, we must be cautious in this recommendation, because 47 percent of the patients without clinical responses had increased expression of retinoic acid receptor- β mRNA. Our trial was brief (three months), and these patients might have had clinical responses in a longer trial. As with any potential intermediate marker (including the response of premalignant lesions), the use of retinoic acid receptor- β mRNA as a marker will have to be validated by comparison with the incidence of cancer (currently the only definitive end point for cancer prevention) in long-term clinical trials.⁴²

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