Accumulation of p53 Protein and Retinoic Acid Receptor $\beta$ in Retinoid Chemoprevention


Departments of Thoracic/Head and Neck Medical Oncology [D. M. S., J. S. L., W. K. H.], Clinical Cancer Prevention [X-C. X., S. M. L.], Biomathematics [J. J. L.], Pathology [J. G. B., J. Y. R.], Dental Oncology [W. W. M.], Clinical Investigation [W. N. H.], and Tumor Biology [R. L.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Although retinoids have proven to be effective as chemopreventive agents in reversing premalignant oral lesions and preventing second primary tumors, their mechanisms of chemopreventive efficacy in clinical settings have not been established. To better define this mechanism, we studied p53 protein and retinoic acid receptor $\beta$ (RAR-$\beta$) expression in 52 baseline biopsy samples taken from premalignant oral lesions. We then studied p53 expression in 39 matched samples and RAR-$\beta$ expression in 38 matched samples before and after treating them with isotretinoin. The study results were then compared with clinical responses. To detect p53 protein expression, 4-$\mu$m sections of formalin-fixed, paraffin-embedded tissue specimens were used for immunohistochemical analysis with a monoclonal anti-p53 antibody, and levels of p53 expression were recorded with a labeling index (LI). Expression of RAR-$\beta$ mRNA was determined using nonradioactive in situ hybridization, and the staining intensity of RAR-$\beta$ mRNA was semiquantitated using scores from 0 (no expression) to 3+ (highest expression). p53 protein was detected in 85% of all lesions. High p53 protein expression (LI $\geq$ 0.2) was detected in 25% of the lesions at baseline and in 18% of the lesions after isotretinoin therapy. The clinical response was 65% for lesions having low p53 expression and either no change or down-regulation of RAR-$\beta$ or with high p53 expression at baseline and up-regulation of RAR-$\beta$ after isotretinoin therapy achieved a 70% rate of major response. The patients with low p53 protein expression and either no change or down-regulation of RAR-$\beta$ or high p53 expression, compared with 22% of patients with high p53 expression (P = 0.003). We correlated baseline p53 protein expression with RAR-$\beta$ modulation and clinical responses to isotretinoin therapy. The patients with low p53 protein expression at baseline and up-regulation of RAR-$\beta$ after isotretinoin therapy had a response rate of 50%. The patients with high p53 protein expression and either no change or down-regulation of RAR-$\beta$ had a response rate of only 14% to isotretinoin therapy. The basic mechanisms underlying the association between clinical responses and these two biomarkers need to be explored.

INTRODUCTION

Chemoprevention, the use of specific natural or synthetic agents to reverse, suppress, or block the carcinogenesis pathway before invasion occurs (1), is one of the leading new investigative approaches for controlling epithelial tumors, including those in head and neck cancer. Retinoids have effectively reversed premalignant oral lesions and prevented second primary cancers in the aerodigestive tract (2–8), but the mechanisms of their clinical efficacy have not been well characterized.

Previous studies have suggested that p53 alterations occur during the early stages of head and neck tumorigenesis (9–11) and are associated with genetic instability, efficacy of cancer therapy, and head and neck cancer prognoses (12–14). Furthermore, p53 protein levels were directly associated with histological grades and inversely related to retinoid responses (15).

Retinoid effectiveness may result from the modulation of gene expression, which is mediated by two classes of nuclear retinoid receptors, RARs and RXRs, that are members of the steroid hormone receptor superfamily (16–19). Three subtypes of both RARs (RAR-$\alpha$, RAR-$\beta$, and RAR-$\gamma$) and RXRs (RXR-$\alpha$, RXR-$\beta$, and RXR-$\gamma$) have been identified, each having distinct evolutionarily conserved sequences. The RXRs usually bind all-trans-retinoic acid and 9cRA, whereas the RARs bind only 9cRA (20, 21). RAR-RXR heterodimers bind to a specific DNA sequence that is a retinoic acid-response element in the promoter regions of genes regulated by retinoids (16–19).

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2 To whom requests for reprints should be addressed, at Department of Thoracic/Head and Neck Medical Oncology, Box 080, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030.

The abbreviations used are: RAR, retinoic acid receptor; LI, labeling index; RXR, retinoid X receptor; 9cRA, 9-cis-retinoic acid.
Each subtype exhibits specific, distinct patterns of expression and is thought to regulate the expression of specific genes (16–18).

To understand the mechanisms of action of retinoids at the tissue level, our group studied the expression of p53 and RAR-β (15, 22) using premalignant oral tissue samples from a previously conducted prospective chemoprevention trial (4). The study showed that p53 expression increased in direct association with the histological grade. We also found that p53 protein accumulation correlated inversely with the response to isotretinoin therapy, although isotretinoin did not modulate p53 protein expression (15). However, expression of RAR-β mRNA was selectively lost in premalignant oral lesions and up-regulated by isotretinoin therapy. Furthermore, up-regulation of RAR-β expression was associated with clinical responses (22). This study was undertaken to determine whether the expression of the p53 protein and RARs are related before and after isotretinoin therapy.

MATERIALS AND METHODS

**Tissue Specimens.** Specimens were obtained from a previously conducted chemoprevention trial (4) in which 38 patients received high-dose isotretinoin therapy (1.5 mg/kg/day) for 3 months. Tissue samples obtained at baseline and after the 3-month isotretinoin therapy were analyzed. Fifty-two baseline tissue samples were analyzed for expression of p53 and RAR-β. Thirty-eight posttreatment samples were analyzed for p53 expression, whereas 39 posttreatment samples were analyzed for RAR-β expression (fourteen samples analyzed for p53 expression and 13 samples analyzed for RAR-β expression after therapy were depleted in previous laboratory studies.) Normal oral epithelium samples were obtained from seven healthy, nonsmoking volunteers. Two pathologists performed histological evaluation of H&E-stained slides for immunostaining of p53 and in situ hybridization of RAR-β mRNA. All of the slides were coded and interpreted in a blind fashion.

**Immunohistochemistry for p53 Expression.** A monoclonal anti-p53 antibody, clone D07 (Biogenex Laboratories, San Ramon, CA), was applied to 4-μm sections of formalin-fixed, paraffin-embedded tissue specimens, and its binding was analyzed using immunohistochemistry (11). All tissue sections were analyzed after application of the antigen retrieval technique using 1% zinc sulfate in a microwave for 3 min (23). Levels of p53 expression in the entire epithelium were measured and recorded with a LI (the number of cells with unequivocal nuclear staining divided by the total number of cells counted) as described previously (15).

**In Situ Hybridization for RAR-β mRNA.** Nonradioactive in situ hybridization for expression of RAR-β mRNA was used with formalin-fixed, paraffin-embedded histological sections as described previously (22, 24, 25). The binding specificity of the antisense riboprobes was verified using sense probes as controls (24). The staining intensity scored ranged from 0 to 3+ (0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining).

**Statistical Analysis.** The χ² test and Fisher’s exact test were used to assess the association between binary variables, such as the association between clinical response and p53 expression or RAR-β mRNA modulation. McNemar’s test was used to determine p53 and RAR-β expression before and after treatment (26). Two-sided P values were determined in all analyses; P ≤ 0.05 was considered statistically significant.

**RESULTS**

**Characteristics of Patients.** Table 1 summarizes the clinical characteristics of 52 patients. Seventy-five percent of the patients were either current (54%) or former (21%) smokers. Sixty-nine percent of the patients had either mild (46%) or moderate (23%) dysplasia. Major clinical responders included 5 complete (10%) and 24 partial (46%) responders after 3 months of isotretinoin therapy.

**p53 Protein and RAR-β mRNA Expression before and after Isotretinoin Treatment.** Table 2 summarizes the results of p53 and RAR-β analyses before and after isotretinoin therapy. The p53 protein was not detected in the seven control samples taken from normal oral tissues. Nuclear p53 protein expression was detected in 44 of 52 (85%) lesions. We analyzed p53 expression according to the different oral sites as shown in Table 2. High p53 protein expression (LI ≥ 0.2) was detected in 13 of 52 (25%) lesions at baseline and in 7 of 38 (18%) lesions after isotretinoin therapy (P = 0.375). The group with high p53 expression had a 27% clinical response to isotretinoin therapy, whereas the group with low p53 expression had a 65% clinical response (P = 0.027). Expression of p53 also increased as tissues progressed from hyperplasia to mild or moderate dysplasia (data not shown).

**Table 1.** Clinical characteristics of 52 patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean yr. (range)</td>
<td>58 (24–83)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27 (52)</td>
</tr>
<tr>
<td>Female</td>
<td>25 (48)</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>16 (31)</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>24 (46)</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>12 (23)</td>
</tr>
<tr>
<td>Tobacco use</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>28 (54)</td>
</tr>
<tr>
<td>Former</td>
<td>11 (21)</td>
</tr>
<tr>
<td>Never</td>
<td>13 (25)</td>
</tr>
<tr>
<td>Alcohol use</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>38 (73)</td>
</tr>
<tr>
<td>Former</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Never</td>
<td>13 (25)</td>
</tr>
<tr>
<td>Clinical Response</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>5 (10)</td>
</tr>
<tr>
<td>Partial</td>
<td>24 (46)</td>
</tr>
<tr>
<td>No change (stable)</td>
<td>20 (38)</td>
</tr>
<tr>
<td>Progression</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

Fig. 1 shows a representative case of RAR-β mRNA expression (i.e., Fig. 1F shows high RAR-β expression) in dysplastic oral lesions using nonisotopic in situ hybridization. All seven normal control epithelium samples showed expression of RAR-β mRNA.

RAR-β mRNA expression was present with varying intensity in 21 (40%) of 52 baseline tissue samples before isotretinoin...
Table 2  p53 and RAR-β expression by isotretinoin therapy in premalignant oral lesions

<table>
<thead>
<tr>
<th>Site</th>
<th>p53 protein expression (LI &gt; 0.2; % of lesions)</th>
<th>RAR-β mRNA expression (SI' 1+, 2+, and 3+: % of lesions)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>4/20 (20)</td>
<td>1/15 (7)</td>
</tr>
<tr>
<td>Tongue</td>
<td>7/16 (44)</td>
<td>5/11 (45)</td>
</tr>
<tr>
<td>Lip</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Soft and hard palate</td>
<td>0/6 (0)</td>
<td>1/4 (25)</td>
</tr>
<tr>
<td>Gingiva</td>
<td>2/7 (29)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>13/52 (25)</td>
<td>7/38 (18)</td>
</tr>
</tbody>
</table>

Normal control         | 0/7 (0)          | 7/7 (100)       |                                                          |

*SI, staining intensity (0, no expression; 1+, minimal intensity; 2+, moderate intensity; 3+, strong intensity).

† McNemar’s test indicates that an increase in p53 expression after treatment is not statistically significant.

‡ RAR-β significantly increased after isotretinoin therapy (P < 0.001).

treatment; the expression was detected in 35 (90%) of 39 post-treatment samples (P < 0.001; Table 2).

Correlation between p53 Protein Expression and Expression of RAR-β mRNA. To determine whether baseline p53 protein expression correlates with RAR-β mRNA expression before isotretinoin therapy, RAR-β mRNA expression was analyzed by either low or high p53 expression status. The 38 patients were divided into 2 groups: low p53 expression (LI < 0.2) and high p53 expression (LI ≥ 0.2). Twenty-nine patients were in the low p53 expression group, and nine patients were in the high p53 expression group. Twenty-one of 29 patients (72%) having low p53 expression had no detectable RAR-β mRNA expression, whereas 2 of 9 patients (22%) having high p53 expression had no detectable RAR-β mRNA expression (Fisher’s exact test, P = 0.016). These results indicate that patients in the low p53 expression group had lost RAR-β mRNA expression in the tissue specimens.

Because p53 was not modulated, but RAR-β mRNA was up-regulated by isotretinoin therapy, we analyzed the data for a correlation between baseline p53 protein expression and RAR-β mRNA up-regulation. Of 29 patients with low p53 expression, 24 (83%) had up-regulation of RAR-β mRNA after isotretinoin therapy; only 22% (2 of 9) of the patients having high p53 expression had up-regulation of RAR-β mRNA (P = 0.003). Two patients who had high p53 expression achieved major clinical responses. These data suggest that patients with low p53 expression in their premalignant lesions at baseline are more likely to have up-regulation of RAR-β mRNA than are patients with high p53 expression.

We observed that tissue samples taken from lesions with low p53 levels at baseline were not likely to express RAR-β mRNA. To determine whether the greater likelihood of RAR-β up-regulation was caused by the expression status of p53 or RAR-β at baseline, up-regulation of RAR-β was analyzed according to the baseline p53 and RAR-β values (Table 3). The RAR-β up-regulation percentage in the low baseline p53 group was as follows: 90% in the patients having no detectable RAR-β expression (0); 60% in the patients having low baseline RAR-β
expression (1+), and 67% in the patients having intermediate baseline RAR- (2+) expression. These data indicate that lesions with low baseline p53 levels, regardless of baseline RAR-β status, had a higher frequency of RAR-β up-regulation after retinoid therapy than did lesions with high baseline p53 levels.

**p53 Protein, RAR-β Modulation, and Clinical Response.** We correlated baseline p53 protein and RAR-β modulation with clinical responses to retinoid chemoprevention. Of the 38 patients whose tissue samples and clinical responses were analyzed for p53 and RAR-β expression before and after retinoid therapy, 3 had a complete response, 18 had partial responses, 15 had stable disease, and 2 had progressive disease. Twenty-three patients had low p53 protein levels at baseline with up-regulation of RAR-β after retinoid therapy; 16 of them (70%) had major clinical responses. Eight patients had either low p53 protein levels at baseline with no change or down-regulation of RAR-β or high p53 levels with up-regulation of RAR-β; four of them (50%) had major responses. Seven patients had high p53 protein levels at baseline with either no change or down-regulation of RAR-β; one of them (14%) had a major clinical response. The differences between the three groups was statistically significant (P = 0.034; Fig. 2). All three complete clinical responders had low p53 protein levels at baseline and up-regulation of RAR-β after retinoid therapy (a representative case is shown in Fig. 1.).

**DISCUSSION**

The promising results of using retinoids in chemoprevention and therapy trials (1–6) have prompted our investigation into their mechanisms of action at the cellular and molecular levels. We previously reported that abnormally high p53 protein accumulation in premalignant oral lesions was associated with resistance to retinoid chemoprevention (15) and that RAR-β restoration was associated with the clinical response to this therapy (22).

The relationship between p53 and RAR-β, however, is undetermined. To understand the relationship between these two markers, we analyzed p53 protein and RAR-β mRNA expression before and after isotretinoin therapy. Before therapy, p53 protein was expressed in 85% of the lesions analyzed; we recorded a similar percentage of p53 expression (89%) in a previous, smaller study (15). Lesions in patients with high baseline p53 protein levels had a lower response rate to isotretinoin therapy than did lesions in patients with low p53 protein levels. The high levels of p53 protein may be associated with mutant p53, because most mutant forms have long half-lives (up to 6 h), probably due to stabilization of the protein (27). In contrast, wild-type p53 protein is thought to have a short half-life (6–20 min) and therefore may not be detected in tissues by immunohistochemical analysis. Cells with mutant forms of p53 usually do not undergo the apoptosis that plays an essential role in cell cycle checkpoints (28–30). Oncogenetically transformed fibroblasts derived from wild-type p53 or p53 knockout mice differ dramatically in their susceptibility to the induction of apoptosis by irradiation and chemotherapy agents (13, 31). Wild-type p53 cells are more radiosensitive and chemosensitive than p53 knockout mice cells and undergo apoptosis, which causes cell cycle arrest, whereas cells with a loss of p53 function caused by either mutations or deletions have lost the apoptotic pathway and are often resistant to irradiation and chemotherapy agents (13, 31). Our observation that the lesions with low p53 expression had higher response rates to isotretinoin therapy expands upon the recent findings on cytotoxic agents (31) and raises important questions about retinoid mechanisms.

Our previous report showed that RAR-β mRNA expression was lost in premalignant oral lesions (24) and induced by isotretinoin in association with clinical responses (22). This study indicates that lesions with low p53 expression tend to have low RAR-β mRNA expression as well. After isotretinoin therapy, RAR-β mRNA was up-regulated in most of the responding lesions. We tested whether RAR-β mRNA up-regulation was related to baseline RAR-β or p53 status. To answer this question, we evaluated the modulation of RAR-β according to the baseline p53 status and RAR-β expression. Baseline RAR-β status had no effect on RAR-β mRNA up-regulation in clinical responses in the low p53 expression group.

The subset of patients having the best clinical responses after the administration of isotretinoin was the group that had low baseline p53 expression and RAR-β mRNA up-regulation after isotretinoin therapy. The group with the worst clinical responses had high baseline p53 expression and no up-regula-
tion of RAR-β mRNA. This clinical observation may be explained in part by defective AF-2-specific cofactors studied in lung cancer cell lines. This study, using lung cancer cell lines and tumors, indicated that abnormal RAR-β mRNA expression (i.e., absent or weak expression of the RAR-β2 isoform) occurred even after retinoid treatment. Because RAR-β2 inactivation may contribute to lung carcinogenesis, Moghal and Neel (32) investigated the molecular mechanism of defective RAR-β2 expression. They found that novel RAR-thyroid hormone receptor AF-2-specific cofactors are necessary for high levels of transcription and are frequently inactivated in human lung cancers (32). RAR-β mRNA that was not up-regulated after retinoid therapy may have inactivated AF-2-specific cofactors in the target tissues. To further understand the mechanisms of resistance to retinoid therapy, the study with AF-2-specific cofactors needs to be addressed using biopsied samples derived from the patients who participate in the retinoid therapy.

Another speculative mechanism of the clinical association between p53 and RAR-β was identified in a different system. A recent study indicated that LG100153, a synthetic retinoid that binds only to RXRs, induced mRNA and protein expression of p21WAF1, an inhibitor of cyclin-dependent kinases that is inducible by p53. Antisense oligonucleotides directed against p21WAF1 specifically inhibited both the increase in the p21WAF1 protein level and the induction of apoptosis by LG100153 (33). For example, 9cRA, a RXR-selective retinoid, may induce the p33-independent pathway of p21WAF1 and lead to apoptosis, regardless of whether the target cell population contains wild-type or mutant forms of the p53 gene (33). These results suggest that p53-independent induction of p21WAF1 is required to induce apoptosis in these cells by a RXR-selective retinoid. Other extremely complex systems may link p53 function and retinoid action, because the large transforming (T) antigen of the SV40 DNA virus binds not only with p53 protein (34) but with other growth-regulatory proteins as well, such as p16, p17, and p27, and retinoblastoma proteins in cell cycle machinery (12, 34). However, candidate consensus sequences of the p53 binding site have not been described in the promoter elements of the RAR-β gene and need to be pursued further.

The success of chemoprevention depends on the identification of intermediate biomarkers (1). To this end, many biomarker candidates have recently been described, including p53 alterations, chromosomal changes, proliferating cell nuclear antigens, epidermal growth factor receptors, RARs, RXRs, and loss of heterozygosity in certain chromosomal regions (11, 15, 22, 34–39). Among these candidates, p53 protein alteration and RAR-β modulation seem to be the promising biomarkers in oral chemopreventive studies.

Detailed further in vitro and in vivo study will be required to figure out the complex relationship among aberrant p53 function, retinoid receptors, and retinoid sensitivity in head and neck premalignant lesions.

The study of biomarkers is equally important for clinical chemopreventive and therapeutic trials, including gene therapy trials. It is impossible to target therapy without knowing the expression of certain genes in target tumor tissues. Such translational biomarker studies therefore need to be integrated into future clinical trials.

REFERENCES


Accumulation of p53 protein and retinoic acid receptor beta in retinoid chemoprevention.

D M Shin, X C Xu, S M Lippman, et al.