Determination of Intermediate Biomarker Expression Levels by Quantitative Reverse Transcription-Polymerase Chain Reaction in Oral Mucosa of Cancer Patients Treated with Liarozole

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ABSTRACT

Liarozole is a 1-substituted imidazole derivative that inhibits cytochrome P450 activity and increases endogenous plasma concentrations of retinoid acid (RA). We have previously demonstrated that RA down-modulates transforming growth factor (TGF)–α and epidermal growth factor receptor (EGFR) levels in head and neck squamous cell carcinoma by decreasing the transcription rate of these two genes. Previous reports suggest that RA receptor (RAR)-β levels are down-modulated in head and neck cancer and are restored by RA therapy. Cellular RA-binding protein (CRABP)-II is up-regulated by RA and appears to modulate intracellular RA metabolism. In conjunction with a Phase I clinical trial, total intact RNA was extracted from oral cavity mucosa biopsied from 17 patients with advanced malignancies, before and after treatment with a 4-week course of liarozole. To analyze these limited quantities of total RNA (as little as 0.6 μg/sample), a quantitative reverse transcription-PCR assay was developed using delayed dropping of the 5′ β-actin primer to amplify the highly abundant β-actin gene as an internal control. We used this method to determine the expression levels of TGF-α, EGFR, RAR-β, and CRABP-II before and after treatment. There was a trend toward elevation of RAR-β levels in oral mucosa after liarozole therapy (P = 0.107), whereas TGF-α, EGFR, and CRABP-II were not modulated by systemic liarozole treatment. These results suggest that liarozole may up-regulate RAR-β in tissues from cancer patients and that expression levels of potential intermediate biomarkers may be determined in small tissue biopsies using a quantitative reverse transcription-PCR assay.

INTRODUCTION

Retinoids represent a group of compounds consisting of vitamin A (retinol), its natural metabolites, and a variety of synthetic analogues. Endogenous retinoids appear to modulate diverse biological functions such as cell growth, differentiation, and apoptosis by activating specific nuclear receptors (1–4). Evidence supports a major role for nuclear retinoid receptors in mediating the effects of retinoids on gene expression. SCCs3 and premalignant dysplasias demonstrate a specific down-modulation of RAR-β that can be restored by systemic retinoid therapy (5). CRABPs, particularly CRABP-II, participate in retinoid metabolism, storage, and transport and may mediate the effects of retinoids by regulating intracellular retinoid levels (6). Aberrant retinoid metabolism has been reported in patients with head and neck SCC (7) and may be associated with disease progression (8). In clinical trials, retinoid therapy eradicates oral leukoplakia, a premalignant oral cavity lesion (9, 10), and the administration of RA in the adjuvant setting effectively reduces the incidence of second primary tumors (11, 12).

Rubin Grandis and Tweardy (13) and Rubin Grandis et al. (14) reported previously that TGF-α and EGFR mRNA and protein are up-regulated both in histologically normal mucosa several centimeters away from the tumor and in tumors from patients with head and neck SCC when compared with levels in control normal mucosa from individuals without cancer, suggesting that this up-regulation is an early event in head and neck carcinogenesis. This up-regulation appears to be due to activated gene transcription (as opposed to increased DNA or prolongation of mRNA half-life) and can be down-modulated by RA to approach the transcription rate of normal mucosal epithelial cells (15). The potential utility of monitoring TGF-α and EGFR expression levels in chemoprevention trials is further supported by our demonstration of increased TGF-α and EGFR protein in premalignant dysplastic lesions compared with control normal mucosa (16).

The use of retinoids in the clinical setting has been limited by their toxicity, which includes skin, ocular, and muscle ab-
normalities; pseudo-tumor cerebri; hepatotoxicity; and hypertriglyceridemia (17). Liarozole is an inhibitor of RA metabolism that is thought to exert its biological effects through elevation of endogenous RA, with a reduced side effect profile compared with synthetic retinoids (18). The ability to predict response to therapy by assessing modulation of target gene expression levels would enable the clinician to determine the likely efficacy of treatment before definitive clinical outcome. To test the hypothesis that putative intermediate biomarker gene expression levels could be determined in surrogate tissue using a quantitative RT-PCR assay, we obtained oral mucosa biopsies before and immediately after a 4-week course of liarozole, in conjunction with preamplification, we preamplified the target genes with several cycles of PCR after the RT reaction before “dropping in” the sense β-actin primer to coamplify both genes for the remaining PCR cycles. The RT-PCR reaction volume was scaled down to 20 μl. A total of 0.01 μg of total RNA was used as a template in the reaction mixture with 1× RT-PCR buffer, 0.5 μl of 20 μM sense and antisense primers of the target gene, 0.5 μl of 20 μM β-actin antisense primer, 0.3 μl of [α-32P]dCTP (6000 Ci/mmol; 20 mCi/ml; New England Nuclear, Boston MA), and 0.5 μl of SuperscriptII RT/Taq mix. The RT reaction was performed for 30 min at 50°C and ended by incubating the reaction at 94°C for 2 min. PCR conditions for TGF-α and CRABP-II were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR conditions for EGFR and RAR-β were 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. After preamplification of the target gene (TGF-α, 6 cycles; EGFR and CRABP-II, 8 cycles; RAR-β, 14 cycles), 5 μl of a solution containing 1× RT-PCR buffer and 0.5 μl of 20 μM β-actin sense primer were added to each reaction, followed by 18 additional cycles.

Quantitation of PCR Products. Ten μl of each PCR product were electrophoresed on a 7.5% polyacrylamide gel containing 0.5× TBE [0.5× TBE = 0.045 M Tris-borate and 0.001 M EDTA (pH 8.0)] and 2.5% glycerol with 0.5× TBE as running buffer using the Mighty Small II SE250 vertical gel electrophoresis apparatus ( Hoefer-Amersham Life Science, Arlington Heights, IL). After electrophoresis, the gel was dried in a gel dryer (model 1583; Bio-Rad) on 3MM Whatman paper and exposed for 10 min to1 h. The absorbed

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-α sense</td>
<td>GCC-CTT-GTT-GCC-TGG-GGA-TAT</td>
<td>240</td>
</tr>
<tr>
<td>TGF-α antisense</td>
<td>AGG-AGG-TCC-GCA-TGC-TCA-CAG</td>
<td>644</td>
</tr>
<tr>
<td>EGFR sense</td>
<td>AAC-ACC-CTG-GTG-TGG-AAG-TAC</td>
<td>606</td>
</tr>
<tr>
<td>EGFR antisense</td>
<td>AGC-AGC-TCT-GAG-GAA-CTC-GCT-CCA</td>
<td>413</td>
</tr>
<tr>
<td>RAR-β sense</td>
<td>AGG-CGG-CCT-TCA-GCA-GGG-TAA-TTT</td>
<td>202</td>
</tr>
<tr>
<td>RAR-β antisense</td>
<td>CTC-TGC-GAC-GTA-GCC-CCT-GGG</td>
<td>0.66 –25.98 mg</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of preamplification cycles</th>
<th>No. of amplification cycles in combination with β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-α</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>EGFR</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>CRABP-II</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>RAR-β</td>
<td>14</td>
<td>18</td>
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</tbody>
</table>
radioactive signals on the phosphor screen (derived from the incorporated $[\alpha-32P]dCTP$ in the PCR products, which were in proportion to the density of each electrophoresed band) were scanned using the computerized PhosphorImager and analyzed by ImageQuant software (Molecular Dynamics). The ratio of the target gene:β-actin was used to designate gene expression levels. Designated gene expression levels in the mucosa before liarozole treatment were compared with expression levels after treatment.

**Statistics.** A paired $t$ test was used to compare total RNA in pre- and posttreatment biopsies. Treatment effects were calculated as the difference between pretreatment and posttreatment values. An exact signed rank test was then conducted to test for significant differences in any of the four intermediate biomarkers. The effect of liarozole dose on treatment effects was compared with expression levels after treatment.

**RESULTS**

**Tissues.** Pretreatment samples only were available for 11 patients. Seventeen patients had both pre- and posttreatment samples available for analysis. Three patients had partially degraded RNA in one of their paired samples, which prevented complete analysis of all four target genes (we were unable to determine EGFR, CRABP-II, and RAR-β in patient 1; TGF-α, EGFR, and CRABP-II in patient 17; and CRABP-II in patient 16). There was no difference in the total amount of RNA isolated from pre- and posttreatment biopsies ($P = 0.591$; data not shown).

**Quantitative RT-PCR Conditions.** A series of preliminary experiments were performed to establish and optimize the quantitative conditions by using total RNA from unpaired tissue samples. These studies included determination of the exponential phase for each PCR amplification, optimization of the primer dropping time point(s), and examination of the quantifiable range correlated with total RNA input. For the exponential phase, using different amounts of total RNA as a template, PCR amplification curves for each target gene and β-actin were generated. When 0.1 μg of total RNA was used in the one-step RT-PCR system, gene amplification remained in the exponential phase if the cycle number was less than 30 for TGF-α, EGFR, and CRABP-II; less than 35 for RAR-β; and less than 20 for β-actin (data not shown). These cycle numbers resulted in a linear relationship between the amount of mRNA input and PCR product. Using lower amounts of starting RNA widened the range of the exponential phase. Therefore, by using less than 0.1 μg of total RNA as a template to amplify TGF-α and less than 30 PCR cycles, we could insure that gene amplification was in the linear phase, thus avoiding a "plateau" in detecting the potential modulation of gene expression levels. Using β-actin as an internal control, these studies determined that the amplification cycle number should be less than or equal to 20.

**Primer Dropping Time Point.** To remain in the linear phase, we systematically examined different PCR cycle numbers for each target gene, alone and in combination with β-actin. Using this method, the ideal primer dropping point was determined for each gene [TGF-α, 6 cycles; EGFR and CRABP-II, 8 cycles; RAR-β, 14 cycles (Table 2)]. All target genes were coamplified with β-actin for 18 cycles. This primer dropping method minimized the competition for PCR substrates between target and control genes so that both gene products could reach detectable and comparable levels (Ref. 19; Fig. 1).

**Range of Total RNA Input.** Titration experiments were performed to determine the range of total RNA required for accurate quantitation of each target gene expression level using two pairs of primers. As demonstrated in Fig. 2, target gene determinations were most accurate when the sample was less than 0.08 μg of total RNA. The RNA input range for all target genes studied was 0.01–0.08 μg. Using nonradioactive methods, PCR products were difficult to detect when the total RNA input was below 0.005 μg (Fig. 2). This experiment was repeated twice for each gene, with similar results. To increase the sensitivity of the detection method, we introduced $[\alpha-32P]dCTP$ into the RT-PCR reactions as part of the DNA synthesis substrate. Designated expression levels of the target and control genes were then determined using a PhosphorImager. The variability of the established quantitative RT-PCR method was also evaluated (Fig. 3). Because the ratio of target gene:β-actin was used to designate expression levels, we measured the radioactive
strength of the PCR products and calculated this ratio in each reaction using different amounts of template, and then we determined the variability range. The largest variation was found to be 18.3% greater than the average (0.651). This method allowed us to detect and measure the final products when the total RNA was diluted from 0.08 to 0.00125 μg. In this range, the radioactive signal strength was in proportion to the RNA template input. Using this technique, we were able to measure up to a 64-fold change in target gene expression levels. This experiment was repeated three times with a high degree of consistency in the results calculated.

**Quantitation Control.** The titration studies revealed that 0.01 μg of total RNA was a suitable template amount to determine modulation of target gene expression levels. This input dose provided the ability to determine up to an 8-fold increase or decrease in target gene expression levels. Therefore, this method would be less sensitive in detecting expression level differences of >8-fold. In subsequent experiments, we used RNA extracted from a representative patient tissue sample as a control for quantitation. RT-PCR reactions using 0.01 μg of this positive control RNA were run with every batch of quantitation reactions (Fig. 4). Target gene quantitations obtained using the paired patient samples were all performed in the quantifiable range by monitoring the quantitation control.

**Modulation of Gene Expression Levels.** Quantitative RT-PCR determinations of putative intermediate biomarker gene expression levels in oral mucosa from cancer patients treated with liarozole demonstrated no significant modulation (Figs. 5 and 6). Experiments were repeated twice with reproducible results. There was a trend toward elevation of RAR-β levels after therapy that did not achieve statistical significance (Fig. 7).

**DISCUSSION**

RA has engendered considerable interest because of its clinical activity in acute promyelocytic leukemia and its ability to eradicate premalignant squamous epithelial lesions as well as prevent the formation of second primary upper aerodigestive tract tumors. Trials using RA have reported toxic side effects in as many as one-third of treated patients, thus limiting its clinical utility (11). In addition, patients receiving RA for leukemia generally become refractory to the drug and require standard chemotherapy (20). Liarozole is a novel imidazole derivative that has demonstrated antitumor activity in vitro and been shown to increase endogenous RA levels in preclinical animal models (18, 21). Establishment of intermediate end points as cancer surrogates would facilitate the design of clinical trials.
that are quicker, smaller, and less costly than studies that use malignancy as the end point. Techniques that facilitate the determination of expression levels of intermediate biomarker genes in accessible tissues would greatly enhance our ability to predict response to therapy.

Rubin Grandis et al. (15) reported previously that TGF-α and EGFR gene transcription rates are down-modulated by RA in vitro. Others have shown that RAR-β levels are restored by RA therapy (5) and that CRABP-II modulates intracellular RA metabolism and is up-regulated by RA (22). In conjunction with a multi-institutional Phase I clinical trial, we biopsied oral mucosa before and after a 4-week course of liarozole therapy to determine the modulation of intermediate biomarker gene expression levels in surrogate tissue. To accomplish this, we developed a quantitative RT-PCR assay using a primer dropping method to measure the expression level of target genes relative to a control (e.g., housekeeping) gene (e.g., β-actin). Because β-actin is more abundant than the target gene(s) examined and because the total RNA obtained from biopsies was often quite low, it was necessary to devise standard curves for each gene (primer pair) to determine the linear expression level range relative to RNA input. Thus, the primers for each target gene required incubation in the PCR reaction for a precisely determined number of cycles before the addition of the β-actin primer pairs.

The results of a RT-PCR-based quantitation are effected by each step, including quantitation of starting material (total RNA), cDNA synthesis (reverse transcription), and PCR reactions. Several quantitative RT-PCR methods have been reported previously, including variations of the primer dropping technique used in this study (18). In the widely used competitive RT-PCR quantitation method, a known amount of synthesized DNA is used as an internal control for PCR amplification (23, 24). However, errors in RNA measurement and template loading and varying efficiencies in different RT reactions may interfere with the results. In some cases, a plasmid construct of a housekeeping gene, such as β-actin, has been used for competitive RT-PCR purposes (25). An extra set of PCR reactions using this control aids in calibration of the cDNA used in subsequent PCR quantitation of the target gene. This method minimizes the effects caused by errors in total RNA quantitation and unequal synthesis of cDNA, but inaccuracies in the addition of the PCR template can still affect the results. We used endogenous β-actin mRNA as an internal control in our PCR quantitation. This method avoided errors by performing sample manipulation, RT, and PCR reactions in the presence of the control. A primer dropping technique controls for differential amplification of different genes, uses two pairs of primers, and minimizes competition for the substrate (19). Quantitative results can best be achieved with one PCR reaction using this assay instead of a series of reactions using different combinations of template and competitor (control). This one-step method is particularly

![Fig. 5 Pretreatment and posttreatment expression levels of TGF-α (A), EGFR (B), CRABP-II (C) and RAR-β (D). The ratio of the RT-PCR target gene product:control (β-actin) mRNA was used to determine gene expression levels.](image)
useful in translational studies in which the amount of patient sample may be extremely limited.

Our results suggest that expression levels of TGF-α, EGFR, RAR-β, and CRABP-II were not significantly altered in surrogate mucosa, although there was a trend toward elevation of RAR-β after liarozole treatment. There are several possible explanations for the lack of modulation of target gene expression by liarozole, including: (a) lack of efficacy of the compound, specifically with respect to increasing endogenous RA levels (a variable that was not examined in this study); (b) inappropriate selection of the target genes; (c) use of surrogate tissue (in contrast to tumor tissue); and (d) inability to detect

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**Fig. 6** Modulation of intermediate biomarker expression levels according to the liarozole treatment doses (75, 150, and 300 mg). The exact permutation distribution of the patient samples was determined by comparison of gene expression levels before and after liarozole treatment.

**Fig. 7** Mean RAR-β expression levels of the pretreatment and posttreatment sample groups. The expression levels are designated as the ratio of the amount of RAR-β RT-PCR product: β-actin RT-PCR product. A, scatter plot of relative pretreatment RAR-β levels compared with relative posttreatment levels, demonstrating a trend toward an increase in RAR-β expression with liarozole treatment. B, bar graph demonstrating a trend toward increased RAR-β expression with liarozole therapy.
statistical significance due to the relatively small number of patients (e.g., with RAR-β and possibly CRABP-II). The establishment of intermediate end points in cancer treatment requires that the method of determination be amenable to small biopsy samples. In addition, tumors are often not readily accessible for biopsy, thus restricting the analysis to surrogate tissue. Methods that are suitable for small quantities of RNA should be considered and devised for use in such studies.

REFERENCES

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