Topical Delivery of 13-cis-Retinoic Acid by Inhalation Up-Regulates Expression of Rodent Lung but not Liver Retinoic Acid Receptors

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ABSTRACT

Chemopreventive retinoids may be more effective if delivered to the lung epithelium by inhalation. 13-cis-Retinoic acid (13-cis-RA) was comparable to all-trans-retinoic acid (RA) in inducing transglutaminase II (TGase II) in cultured human cells. Inhaled 13-cis-RA had a significant stimulatory activity on TGase II in rat lung (P < 0.001) but not in liver tissue (P < 0.544). Furthermore, inhaled 13-cis-RA at daily deposited doses of 1.9 mg/kg/day up-regulated the expression of lung retinoic acid receptors (RARs) α, β, and γ at day 1 (RARα by 3.4-fold, RARβ by 7.2-fold, and RARγ by 9.7-fold) and at day 17 (RARα by 4.2-fold, RARβ by 10.0-fold, and RARγ by 12.9-fold). At a lower aerosol concentration, daily deposited doses of 0.6 mg/kg/day were also effective at 28 days. Lung RARα was induced by 4.7-fold, RARβ by 8.0-fold, and RARγ by 8.1-fold. Adjustment of dose by exposure duration was also effective; thus, inhalation of an aerosol concentration of 62.2 μg/liter, for durations from 5 to 240 min daily for 14 days, induced all RARs from 30.6- to 74-fold at the shortest exposure time. None of the animals exposed to 13-cis-RA aerosols showed RAR induction in livers. By contrast, a diet containing pharmacological RA (30 μg/g of diet) failed to induce RARs in SENCAR mouse lung, although it induced liver RARs (RARα, 21.8-fold; RARβ, 13.5-fold; RARγ, 12.5-fold); it also failed to induce lung TGase II. A striking increase of RARα expression was evident in the nuclei of hepatocytes. Pharmacological dietary RA stimulated RARα, RARβ, and RARγ as early as day 1 by 2-, 4-, and 2.1-fold, respectively, without effect on lung RARs. Therefore, 13-cis-RA delivered to lung tissue of rats is a potent stimulant of lung but not liver RARs. Conversely, dietary RA stimulates liver but not lung RARs. These data support the concept that epithelial delivery of chemopreventive retinoids to lung tissue is a more efficacious way to attain up-regulation of TGase II and the retinoid receptors and possibly to achieve chemoprevention.

INTRODUCTION

Lung cancer is the leading cause of cancer death among men and women in the United States, as well as around the world (1, 2). Because conventional treatments for lung cancer have had limited success in improving survival outcome, alternative strategies to combat lung cancer have been introduced. Oral and i.v. delivery of retinoids, such as 13-cis-RA,3 have been investigated in both animal and human trials. However, retinoid availability to epithelial targets is relatively small when the retinoid is administered systemically, because of retinoid interaction with albumin, with another protein, or both (3). The group from Arizona reported that 99.9% of (radiolabeled) 13-cis-RA was present as albumin bound and that this interaction could not be reversed by competition with high concentrations of unlabeled retinoid (3, 4). 13-cis-RA has shown effectiveness as a chemopreventive agent of oral leukoplakia (5) and head and neck cancer (6), but with significant toxicity. For the purpose of increasing target tissue bioavailability and reducing general toxicity, inhalation of (13-cis-RA) has been proposed as an alternative chemopreventive approach (7). Ideally, this would allow delivery of appropriate concentrations of 13-cis-RA to the pulmonary epithelium, bypassing the marked enterohpatic clearance as well as near-universal interaction with albumin and permitting a higher final concentration of active retinoid at the target epithelium.

As a preclinical study, we exposed normal rats to inhaled concentrations of 13-cis-RA and looked at specific biomarkers to monitor effect. TGase II and the RARs were chosen as biomarkers because they are first order dependence genes (8), i.e., they have been shown to contain a RA-responsive element in their promoter.

MATERIALS AND METHODS

13-cis-RA Treatment of Breast Cancer MCF-7 Cells. MCF-7 cells were seeded at a density of 1.5 × 10^6 cells/ml medium (500 ml of DMEM + 56.2 ml of fetal bovine serum + 5.6 ml of antibiotic/antimycotic) in 6-cm-diameter dishes for 24 h and treated with either DMSO, RA, or 13-cis-RA at 10^{-6}

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2 Present address: Guilford Pharmaceuticals, Inc., 6611 Tributary Street, Baltimore, Maryland 21224.
3 The abbreviations used are: 13-cis-RA, 13-cis-retinoic acid; RA, all-trans-retinoic acid; TGase II, transglutaminase II; RAR, retinoic acid receptor; MMAD, mass median aerodynamic diameter; GSD, geometric SD.
m and grown to confluence (about 72 h). Cells were harvested, and TGase II activity was measured as described below.

**Inhalation Experiments A and B.** Male Sprague Dawley rats were received from Charles River Laboratory. They were quarantined and observed for a period of 7–8 days prior to inhalation exposure to evaluate their health. After an examination by a staff veterinarian, the animals were released for use in the study. All animals were considered healthy and acceptable for use in the study. All study animals were introduced into the inhalation exposure tubes for at least 5 days with increasing duration up to 120 min prior to the first actual inhalation exposure. The rats were approximately 17 weeks of age and ranged in body weight from 200 to 350 g on the first day of dose administration. For experiment B, the rats were treated in the same way as for A, except that they were 7–14 weeks of age and ranged in body weight from 200 to 350 g on the first day of exposure. The rats were allowed access to Certified Rodent Diet (P.M.I. Feeds, Inc.) ad libitum (except during dose administration). Fresh water from the Columbus municipal water supply was provided ad libitum during dose administration.

Within 24 h of the last exposure, animals were euthanized by pentobarbital overdose; their lungs were removed, flash-frozen in liquid nitrogen, and sent on dry ice to the National Cancer Institute for biomarker determination.

**13-cis-RA.** 13-cis-RA was received from Hande-Tech (Houston, TX) or Sigma Chemical Co. (St. Louis, MO) or Toronto Research (North York, Ontario, Canada). The shipment was received at room temperature and was stored at ~5°C prior to formulation.

**Formation of Nebulizer Solutions.** For rat experiment A, formulations of isotretinoin in 100% ethanol dosing solution were prepared at 1.4 mg/ml. Solutions were dispensed into Teflon-lined amber glass bottles with Teflon-lined lids and stored at 5°C prior to dose administration. Verification of the shipment (except during dose administration). Fresh water from the Columbus municipal water supply was provided ad libitum (except during dose administration).

**Calculations of Deposited Dose.** Deposited doses were calculated as follows:

\[
\text{Aerosol concentration (µg/liter)} \times (2.1 \times \text{BW(g)}^{0.75}) \text{ ml/min} \times \frac{1 \text{ liter}}{1000 \text{ ml}} \times \text{time (min)} \times \frac{1}{\text{BW (kg)}} \times f \quad (A)
\]

where \(2.1 \times \text{BW}^{0.75}\) is the Guyton formula for minute volumes in ml/min (10), BW is body weight in grams, and \(f\) is the deposition fraction.

Fractional depositions were assumed the same as 1.09- and 1.03-µm monodisperse aerosols (11) for mice and rats, respectively.

**Dietary RA Studies in SENCAR Mice.** Male SENCAR mice (\(n = 10\)) were divided into two experimental groups and were fed varying amounts of RA in the diet. The experimental groups were divided into two groups of five mice (Table 1), each including a low-dose and high-dose group that were fed either a physiological RA diet (3 µg/g of diet) or a pharmacological RA diet (30 µg/g of diet) for 75 weeks, respectively.

**Immunohistochemical Staining.** Liver tissue (approximately 300 mg) was fixed in 10% formalin and embedded in paraffin, and 5-µm sections were used for immunohistochemistry. Staining for RARα was similar to our previously described protocol (12). ABC kit, mouse/rabbit IgG and DAB substrate kit were used (Vector Laboratories Inc., Burlingame, CA).

### Table 1  TGase II activity of liver tissue from SENCAR mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein assay (µg/ml)</th>
<th>TGase II assay (DPM/µg protein pmol/µg of protein/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>36</td>
<td>9820, 0.125 ± 0.02</td>
</tr>
<tr>
<td>30</td>
<td>37</td>
<td>49,260, 0.630 ± 0.16</td>
</tr>
</tbody>
</table>

### Table 2  Liver and lung samples from SENCAR mice

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of specimens</th>
<th>Experimental period (days)</th>
<th>Dietary RA (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1</td>
<td>30</td>
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<td>28</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>28</td>
<td>30</td>
</tr>
</tbody>
</table>
Fig. 1  Stimulation of TGase II activity by retinoids. A, 13-cis-RA and RA stimulate TGase II activity in cultured human breast cancer MCF-7 cells. The average of TGase II activity analysis of three separate dishes ± SE for each treatment group is shown. In column 1, cells were treated with DMSO.
**Time Course of Dietary RA Effect on RARs.** SENCAR mice \( n = 30 \) were divided into six experimental groups. The experimental groups were as follows (Table 2): groups 1, 3, and 5 (five mice each) were fed a physiological RA diet \((3 \mu g/g \text{ of diet})\) for 1, 14, and 28 days; groups 2, 4, and 6 (five mice each) were fed a pharmacological RA diet \((30 \mu g/g \text{ of diet})\) for 1, 14, and 28 days.

**Antibodies.** Polyclonal rabbit anti-mouse antibodies against RARα, RARβ, and RARγ (Santa Cruz Biotechnology Inc., San Francisco, CA) were used. BM Chemiluminescence Western blotting kit (mouse/rabbit) was used (Roche Molecular Biochemicals, Indianapolis, IN) for the Western blots. Each antibody was tested with specificity with blocking peptides.

**Apparatus and Reagents for Western Blot Analysis.** X Cell II Mini-Cell & Blot module was used with 10% Tris-glycine gels and transfer buffer and Tris-glycine SDS sample buffer; Tris-glycine SDS was used as running buffer (Novex, Novel Experimental Technology Inc., San Francisco, CA).

**TGase II Assay.** Cultured cells were placed in 100 μl of scraping buffer \([2800 \mu l \text{ of buffer A (400 } \mu l \text{ of } 0.5 \text{ m sodium phosphate, } 500 \mu l \text{ of } 0.01 \text{ m EDTA, } 100 \mu l \text{ of } 1 \text{ m DTT, } 9 \mu l \text{ of PBS; total, } 10 \mu l \text{ } + 700 \mu l \text{ of buffer B (10 } \mu l \text{ of } 20 \text{ mg/ml phenylmethylsulfonyl fluoride, } 790 \mu l \text{ of PBS; total, } 800 \mu l \text{) for each dish. Cells were broken by a sonicator and kept in ice until used. TGase II assay was conducted as described previously (13).**

![Graph](https://via.placeholder.com/150)

*Fig. 1 Continued*

for 72 h (TGase II activity = 0.183 ± 0.005 pmol of putrescine/\( \mu g \) of protein/30 min); in column 2, cells were treated with RA \( (10^{-6} \text{ m}) \) for 72 h (TGase II activity = 1.359 ± 0.009 pmol/\( \mu g \) of protein/30 min). The difference between columns 1 and 2 is highly significant \((P < 0.001)\). In column 3, cells were treated with 13-cis-RA \( (10^{-6} \text{ m}) \) for 72 h (TGase II activity = 1.118 ± 0.016 pmol/\( \mu g \) of protein/30 min). The difference between columns 1 and 3 is highly significant \((P < 0.001)\), but there is no significant difference between columns 2 and 3 \((P < 0.07)\). B, 13-cis-RA by inhalation significantly increases TGase II activity (experiment A) of rat lung tissue. Four left lungs (one from each rat) were used; as for the lungs, mean values of 12 measurements (triplicates for each liver) are plotted for 72 h (TGase II activity = 0.037 ± 0.006 pmol/\( \mu g \) of protein/30 min). The difference between columns 1 and 2 is highly significant \((P < 0.001)\). In column 3, cells were treated with 13-cis-RA (TGase II activity = 0.080 ± 0.004 pmol/\( \mu g \) of protein/30 min); 2 (low dose), 39 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.0955 ± 0.004 pmol/\( \mu g \) of protein/30 min; \( P < 0.001 \) between 1 and 2); 3 (low-middle dose), 117 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.1150 ± 0.006 pmol/\( \mu g \) of protein/30 min; \( P < 0.001 \) between 1 and 3); 4 (middle dose), 351 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.1330 ± 0.009 pmol/\( \mu g \) of protein/30 min; \( P < 0.001 \) between 1 and 4); 5 (middle-high dose), 936 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.1020 ± 0.005 pmol/\( \mu g \) of protein/30 min; \( P < 0.001 \) between 1 and 5); 6 (high dose), 1872 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.1025 ± 0.004 pmol/\( \mu g \) of protein/30 min; \( P < 0.001 \) between 1 and 6). C, inhaled 13-cis-RA fails to significantly alter liver TGase II activity (experiment A). Rats inhaled 13-cis-RA aerosol (Table 3). Measurements were conducted on liver tissue. Methods were the same as for B. 1 (vehicle control), liver tissue from rats that inhaled vehicle (deposited dose = 0); TGase II activity = 0.260 ± 0.005 pmol/\( \mu g \) of protein/30 min; 2 (low dose), 39 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.289 ± 0.007 pmol/\( \mu g \) of protein/30 min; \( P < 0.285 \) between 1 and 2); 3 (low-middle dose), 117 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.273 ± 0.018 pmol/\( \mu g \) of protein/30 min; \( P < 0.619 \) between 1 and 3); 4 (middle dose), 351 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.269 ± 0.015 pmol/\( \mu g \) of protein/30 min; \( P < 0.993 \) between 1 and 4); 5 (middle-high dose), 936 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.313 ± 0.025 pmol/\( \mu g \) of protein/30 min; \( P < 0.065 \) between 1 and 4); 6 (high dose), 1872 \( \mu g/\text{kg} \) was the total deposited dose of 13-cis-RA (TGase II activity = 0.271 ± 0.015 pmol/\( \mu g \) of protein/30 min; \( P < 0.758 \) between 1 and 6). D, dietary RA significantly increases mouse liver TGase II activity. Mice were fed RA for 75 weeks at two levels, 3 and 30 \( \mu g/g \) of diet. Four different mice from each dietary RA group were used; as for the lungs, mean values of 12 measurements (triplicates for each liver) are plotted ± SE (Table 1). 1, TGase II activity from the livers of SENCAR mice fed a physiological RA diet \((3 \mu g/g)\) for 75 weeks (TGase II activity = 0.125 ± 0.02 pmol/\( \mu g \) of protein/30 min); 2, TGase II activity from the livers of SENCAR mice fed a pharmacological RA diet \((30 \mu g/g)\) for 75 weeks (TGase II activity = 0.630 ± 0.16 pmol/\( \mu g \) of protein/30 min; \( P < 0.003 \) between columns 1 and 2).
Retinoid Inhalation Up-Regulates Lung RARs

RESULTS

Samples were centrifuged at 14,000 × g approximately 2 volumes of scraping buffer for 2–3 min at 4°C. The supernatant was removed and kept in ice until used.

Table 3 Dose of inhaled 13-cis-RA in experiment A rats exposed to 13-cis-RA aerosols

<table>
<thead>
<tr>
<th>Daily exposure duration (min)</th>
<th>Calculated total daily deposited dose (μg/kg)</th>
<th>Calculated pulmonary daily deposited dose (μg/kg)</th>
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<tbody>
<tr>
<td>240</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
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</tr>
<tr>
<td>15</td>
<td>117</td>
<td>44</td>
</tr>
<tr>
<td>45</td>
<td>351</td>
<td>131</td>
</tr>
<tr>
<td>120</td>
<td>936</td>
<td>350</td>
</tr>
<tr>
<td>240</td>
<td>1872</td>
<td>700</td>
</tr>
</tbody>
</table>

Table 4 Dose of inhaled 13-cis-RA in experiment B rats exposed to 13-cis-RA aerosols

<table>
<thead>
<tr>
<th>Study duration (days)</th>
<th>Targeted 13-cis-RA aerosol concentration (μg/liter)</th>
<th>Calculated total daily deposited dose (μg/kg)</th>
<th>Calculated pulmonary daily deposited dose (μg/kg)</th>
</tr>
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<tbody>
<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>104</td>
<td>1892</td>
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<td>1892</td>
<td>708</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>31</td>
<td>564</td>
<td>211</td>
</tr>
</tbody>
</table>

Four male rats for each exposure duration were exposed to 13-cis-RA aerosol at concentrations of −62.2 μg/liter [MMAD (GSD), 1.5 μm (~2.0)] daily for 14 days and were sacrificed on day 15.

Three vehicle control animals were exposed for 240 min daily. The vehicle was 100% ethanol.

For liver tissue, approximately 100–400 mg were used. Tissue was diced into small pieces and homogenized in approximately 2 volumes of scraping buffer for 2–3 min at 4°C. Samples were centrifuged at 14,000 × g for 30 min at 4°C. The supernatant was removed and kept in ice until used.

Protein concentration determination was conducted by the Bradford method (14).

RESULTS

Demonstration that 13-cis-RA Stimulates TGase II Activity and Comparison with RA in Cultured Human Breast Cancer MCF-7 Cells. Prior to using 13-cis-RA by the inhalation route, we tested its ability to up-regulate the expression of the retinoid responsive TGase II gene, compared to RA. Fig. 1A shows that 13-cis-RA is nearly as effective (6.1-fold) as RA in stimulating TGase II activity in cultured human breast cancer MCF-7 cells.

Inhaled 13-cis-RA Stimulates TGase II Activity in Rat Lung but Not Liver Tissue. The details of this experiment are given in Table 3 and Fig. 1B. This figure shows a significant (2.9-fold) stimulation by inhaled 13-cis-RA of lung TGase II activity. The increase was evident with a dose as low as 69 μg/kg given daily for 14 days and reached a maximum at an inhaled dose of 1012.3 μg/kg, i.e., a total calculated daily deposited dose of 351 μg/kg reached after 45 min of inhalation of the aerosol. It then decreased down to 1.2-fold with larger amounts of inhaled retinoid.

Next, we were interested in studying whether inhaled 13-cis-RA had any effect on liver RARs. Western blot analysis of rat liver samples from the same rats as shown in Fig. 2A failed to show any increase in RARs after administration of 13-cis-RA by inhalation (not shown), supporting the concept that topical administration is an effective means of local biomarker enhancement, but the systemic concentration of 13-cis-RA that results from inhaled drug delivery is insufficient to induce liver RARs.

Furthermore, rats were made to inhale different amounts of the same solution of 13-cis-RA by varying the exposure time between 5 and 240 min, resulting in different inhaled doses between 115.0 and 5935.6 μg/kg body weight every day for 14 consecutive days (Table 3). Western blot analysis of these rat lung tissues is shown in Fig. 3A, and its densitometry is shown in Fig. 3B. As in the previous experiment, inhaled 13-cis-RA effectively increased the amount of RAR proteins between 1.2- and 38.8-fold for RARα, 1.6- and 30.6-fold for RARβ, and 2.2- and 74.0-fold for RARγ (Fig. 3B). However, there was a complex dose-response relationship, and it appeared that the most effective exposure was the shortest one (i.e., for 5 min at 115.0 μg/kg body weight). In contrast to the observed stimulation for lung RARs, liver RARs were not responsive to inhaled 13-cis-RA (not shown).

Inhaled 13-cis-RA Stimulates RARα, RARβ, and RARγ Proteins in Rat Lung but Not Liver Tissue. This experiment was conducted to study the specific effect of inhaled 13-cis-RA on lung tissue of the rat. The details of this experiment are shown in Table 4. Inhalation exposure to 13-cis-RA (Fig. 2A) at high (Lanes 2 and 3) or middle (Lane 5) doses as specified in Fig. 2 legend caused an increase of between 3.4- and 4.7-fold over solvent control (Lanes 1 and 4) at different times of daily exposures to the retinoid for RARα, an increase of between 7.2- and 10-fold for RARβ, and an increase of between 8.1- and 12.9-fold for RARγ (Fig. 2B). Therefore, RARs appear to be highly responsive to inhaled 13-cis-RA in the rat lung tissue.

Dietary RA Stimulates TGase II Activity in SENCAR Mouse Liver. The details of this experiment are shown in Table 1. We tested the hypothesis that dietary RA might be effective in stimulating TGase II activity in SENCAR mouse liver tissue. We used SENCAR mice fed either a physiological RA diet (3 μg/g of diet) or a pharmacological RA diet (30 μg/g of diet) for 75 weeks. Fig. 1D shows that dietary RA (3 μg/g of diet) is effective in stimulating TGase II activity in liver from male SENCAR mice by 5.0-fold over physiological RA (3 μg/g of diet).

Dietary RA Increases Liver RARs. Next, we tested the hypothesis that dietary RA might be effective in increasing liver RARs. We used SENCAR mice fed either a physiological RA diet (3 μg/g of diet) or a pharmacological RA diet (30 μg/g of diet) for 75 weeks. Dietary RA (30 μg/g of diet) up-regulated
RARs (Fig. 4A) in liver from male SENCAR mice by 21.8-fold for RARα, 13.5-fold for RARβ, and 12.5-fold for RARγ (Fig. 4B). Fig. 4C shows a representative immunohistochemical analysis of male SENCAR mouse liver samples using polyclonal antibody to RARα as explained in “Materials and Methods.” A marked increase in staining was observed in the nuclei of mice consuming the pharmacological RA diet compared to physiological RA.

We then tested the ability of dietary RA to increase RARs at shorter times of dietary consumption of physiological and pharmacological levels of RA, as indicated in Table 2. Fig. 5, A and B, shows an induction of 1.4–4.4-fold for liver RARα, 2.2–14.3-fold for RARβ, and 1.3–8.9-fold for RARγ. In sharp contrast, no effect of dietary RA was observed on lung tissue RARs and TGase II (not shown).

DISCUSSION

Retinoids are key regulators of lung epithelial cell differentiation (15–19) and act as ligands of the nuclear receptors RARs (20, 21). They have been used in chemoprevention approaches in different tissues, and 13-cis-RA has been shown to be effective against leukoplakia (22) as well as against head and neck cancer (6). However, systemic administration presents considerable problems if one takes into account the interactive nature of the retinoid molecules and the high affinity of albumin for retinoids in the blood (3, 4). In fact, we have previously shown that the uptake of serum retinoids in cultured cells is inversely related to the concentration of albumin in the culture medium (3, 23). The high affinity interaction of retinoids with albumin and possibly other proteins may limit attainment of effective concentrations of retinoid in lung epithelium and impede chemopreventive activity. Therefore, we have suggested an alternative approach (7), i.e., the possibility that topical delivery to the lung by inhalation may permit more efficacious chemopreventive approaches.

With the type of efficient delivery system described (24), the amount of drug that is required to achieve critical retinoid dose concentration in bronchial epithelium is a small fraction of
the doses that have been used clinically. Because only a small amount of drug would be administered per dose, both potential for side effects and the cost economy of the drug should be improved compared to the standard oral drug delivery approach.

In this paper, we have tested the hypothesis that 13-cis-RA, when delivered topically by inhalation, may be more effective than when given in the diet to elicit up-regulation of key target genes at the target site. Our experiments are consistent with this hypothesis. Inhaled 13-cis-RA increased lung TGase II activity \( (P < 0.001) \) without significant effect on liver enzyme activity \( (P < 0.544) \), whereas dietary RA has a significant effect on liver TGase II enzyme activity of SENCAR mice \( (P < 0.003) \) but is without effect on lung TGase II (not shown). Furthermore, inhaled 13-cis-RA greatly stimulated pulmonary RAR\( \alpha \), RAR\( \beta \), and RAR\( \gamma \) expression at the protein level, whereas it failed to have any significant effect on liver RARs.

Interestingly, a marked stimulation of RARs was already observed with repeated exposures of 5 min each to inhaled 13-cis-RA (Fig. 3A). The stimulation of RAR\( \alpha \), RAR\( \beta \), and RAR\( \gamma \) in the lung samples confirms that the aerosol apparatus effectively delivered 13-cis-RA to the lungs and therefore permitted the immediate response in biomarker up-regulation. The complex dose-response effect of aerosolized 13-cis-RA on RAR expression in the lung suggests that retinoid metabolism is occurring. Also, the lack of effect on RARs after 28 days of dietary retinoids may be explained on the basis of autoinduction of retinoid metabolism. This is obviously an important point,
because metabolism has been a major problem with prolonged administration of dietary retinoids (25). Another consideration is that RAR turnover may explain these effects. For these reasons, our future work will focus on measuring lung retinoid levels achieved in a dose- and time-dependent manner with prolonged administration of aerosolized and dietary 13-cis-RA. However, this approach needs a large number of animals, if performed on small rodents. We are planning to use an alternative approach based on a reporter gene assay, which measures down to 0.1 ng of retinoid (26, 27).

Furthermore, we have shown in this paper that dietary RA at pharmacological concentration enhances liver RARs quite early (i.e., as early as after 1 day of feeding), without any significant effect on lung tissue RAR proteins. Interestingly, a recent report has shown an increase in RARβ by oral 13-cis-RA, albeit at the mRNA level (28). In this paper, the 13-cis-RA had been fed for a much longer period of time (6 months compared to our 1-, 14-, and 28-day study), and this may explain the observed effect on lung tissue. Also, their baseline levels for RARβ mRNA may have been very low, because cigarette smoking has been shown to reduce RARβ (29) and this may have favored a detectable effect at the mRNA level after reverse transcription-PCR.

Ethanolic solutions of 13-cis-RA were aerosolized with particle sizes calculated to provide substantial pulmonary deposition. The vehicle vapors were not removed from the exposure air and may have had an effect on biomarkers, as the vehicle-exposed animals had higher levels of some markers than unexposed controls. However, the effect was small and may have been influenced by the stress of handling and exposure. Stress has significant effects on some parameters, including tumorigenesis (30), and may have contributed to decreased tumor multiplicity in mice exposed to 13-cis-RA (24) and budesonide (31). In any case, the addition of 13-cis-RA to the aerosol at the...
Retinoid Inhalation Up-Regulates Lung RARs

To explain the effect of retinoid inhalation on lung RARs in SENCAR mice, the study measured RARα, RARβ, and RARγ levels at two time points: 3 and 30 days. Western blot analysis was conducted on liver samples using polyclonal antibodies to RARα, RARβ, and RARγ. Mice were fed RA for different time periods: 1, 14, and 28 days; and liver from SENCAR mice fed a physiological RA diet (30μg/g) for 1, 14, and 28 days. The densitometric analysis of Western blots shown in Fig. 5 illustrates the up-regulation of RARs in liver from SENCAR mice fed RA (30μg/g) for 1, 14, and 28 days. The vertical axis represents integrated density value (IDV).

Finally, we observed a significant increase in biomarker expression relative to vehicle-only aerosols. This observation suggests a close connection between carcinogen exposure and retinoid utilization and an increase in the utilization with increased environmental exposure. These speculations will be addressed in our future work.

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Topical Delivery of 13-cis-Retinoic Acid by Inhalation Up-Regulates Expression of Rodent Lung but not Liver Retinoic Acid Receptors

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