Expression of a Dominant-Negative Retinoic Acid Receptor Construct Reduces Retinoic Acid Metabolism and Retinoic Acid-Induced Inhibition of NIH-3T3 Cell Growth

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Abstract

We have previously reported an unexpected relationship between retinoic acid-induced inhibition of cell growth and the ability of various cell lines to metabolize the retinoid. Here, we report that stable expression of the truncated retinoic acid receptor RARα403, transduced in NIH-3T3 cells by a retroviral vector, rendered the cells resistant to retinoic acid for growth inhibition and reduced their ability to metabolize the retinoid at the same time as it blunted the induction of the target gene transglutaminase II. The data suggest that retinoic acid receptors mediate the growth-inhibitory action of retinoic acid as well as its metabolism and the induction of transglutaminase II.

Introduction

Cell growth and differentiation are regulated by vitamin A and its derivatives, the retinoids (1). We have recently reported an unexpected relationship between the ability of RA to inhibit cell growth and its metabolism in 15 different cell lines (2). Nine of the 15 cell lines were able to metabolize RA and responded to it by cell growth inhibition. Resistant cells were unable to metabolize RA, although uptake was apparently unaffected. In this study, we asked whether inhibition of cell growth and RA metabolism depend on RAR. We addressed this question in NIH-3T3 cells expressing a truncated form of the RAR, the RARα403, which is dominant negative over the function of RARs (3).

Materials and Methods

[11,12-3H(NH)]all-trans-RA (53 Ci/mmol) was from DuPont NEN Research Products. All-trans-RA was from Sigma Chemical Co. DMEM, heat-treated FBS, antibiotic-antimycotic solution, 0.05% trypsin, and 0.02% Versene in Earle’s balanced salt solution without calcium and magnesium were all from Biofluids, Inc. Dulbecco’s PBS without calcium or magnesium (pH 7.5) was used for all washes and was from BioWhittaker, Inc. Aquasol and acetonitrile were prepared by Packard Instrument Co., Inc., and Baxter Scientific Products, respectively.

Infection of NIH-3T3 with Retroviral Vectors. The retroviral vector was a gift from Dr. S. J. Collins (Fred Hutchinson Cancer Research Center, Seattle, WA). In this construct, a truncated RARα gene is inserted into the retroviral vector LXSN (3). Cells were seeded at 50% confluency into 100-mm dishes. After 24 h, they were infected with the LXSN vector or the LXRαSN retroviral vector in the presence of 4 μg/ml Polybrene. After overnight incubation, the medium was replaced and cells were grown for 36–48 h before G418 (1 mg/ml) was added. G418-resistant cells were isolated. Cells were maintained in DMEM with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate, in a 6% CO2 atmosphere at 37°C.

Northern Blot Analysis. Isolation of total RNA was performed using the total RNA isolation kit (Tel-Test Inc., Friendswood, TX). The full-length fragments of the mouse RARs were excised from the expression plasmid pBSG-RAR (4). The probes were labeled with [32P]dCTP using random primer labeling methods. Total RNA (40 μg) was fractionated on a 1% agarose gel and blotted overnight onto Schleicher & Schuell-nitrocellulose. The membranes were hybridized and other procedures were as described previously (4).

Cell Proliferation. Cells were harvested with 1 mM EDTA-0.05% trypsin at the subconfluent state and seeded in 60-mm tissue culture dishes at 1 × 105 cells/dish. After 24 h, the growth medium containing 10% FBS was removed and replaced with medium containing 2% FBS and either 1 μM RA in DMSO or DMSO alone. Cells were observed at 0, 24, 48, 60, 72, and 96 h, and growth was monitored by counting the suspended cells with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

HPLC. The LXSN-3T3 and LRARα403SN-3T3 cells were trypsinized and plated in duplicate for each time point at a density of 5 × 105 cells in 60-mm dishes. After 24 h, the 10% FBS medium was replaced with medium containing 2% FBS and either [3H]RA (0.5 μCi/ml; 1 μM final concentration in the medium) or 1 μM RA alone for determination of cell number. Cells were incubated for the following times: 2, 24, 48, 60, 72, and 96 h. At each time point, medium was removed, and a 20-μl aliquot was measured in Aquasol (10 ml) in a Beckman Liquid Scintillation Counter. Cells were washed twice with 2 ml of cold PBS containing 30 μM RA, extracted with 1.0 ml of methanol, and spun for 5 min at 14,000 × g, and radioactivity was measured. To calculate cell-associated radioactivity at each time point (starting at 5 × 104, 0 time), aliquots of medium (500 μl) were lyophilized, and the residue was redissolved in 500 μl of methanol. Medium and cell extracts were stored at −70°C until analyzed on HPLC as described previously (2).

Transglutaminase Assay. Cells were plated at 1 × 105/dish in 60-mm culture dishes and treated with 1 μM RA for 72 h. Cells were harvested in 2 mM sodium phosphate (pH 7.2), 10 mM DTT, 0.5 mM EDTA, and 50 μg/ml phenylmethylsulfonyl fluoride, and they were then sonicated for 10 s. Cell lysates were assayed for TGase II by measuring (2,3,5-triphenyltetrazolium chloride) incorporation into dimethyl casein at pH 9.0 for 20 min at 28°C, according to Lichti and Yuspa (5). Protein concentration was determined by the Bradford method (6).

Results

Expression of the RARα403 Construct. Northern blot analysis was performed to detect the expression of the typical 4.7-kb retroviral transcripts containing the RARα403 mRNA (Fig. 1). The expression of the endogenous RARα transcript at 3.7 kb can barely be observed in the vector LXSN-transfected cells at this time of exposure. The dominant-negative activity of the mutated receptor was confirmed in LRARα403 NIH-3T3 cells by transient reporter assays as shown previously (4).
Cell Proliferation. We studied the effect of 1 μM RA on cell number over a period of 4 days in the LRARα403SN-NIH-3T3 cells that stably expressed retrovirus-transduced dominant-negative RARα403 (4). Fig. 2 shows that RA failed to significantly inhibit the growth of the LRARα403SN-NIH-3T3 cells. In sharp contrast, an inhibitory effect of RA on cell proliferation was observed in control LXSN-NIH-3T3 cells (Fig. 2), similar to wild-type NIH-3T3 cells, which are inhibited 35–40% by 1 μM RA under similar conditions (7).

Cell-associated Retinoid. Next, we studied the effect of RARα403 expression on RA metabolism because of the observed relationship between growth inhibition and RA metabolism (2). LXSN-NIH-3T3 and LRARα403SN-NIH-3T3 cells were incubated in the presence of [3H]RA (0.5 μCi/ml, 30 nm), and cell-associated radioactivity was quantified over time (Fig. 3). The LRARα403-NIH-3T3 cells contained 6- to 8-fold higher radioactivity after 24 h, compared to LXSN-NIH-3T3 cells (Fig. 3), and the majority of the radioactivity (at least 70% at 60 h) was in the form of intact RA in the resistant cells (Fig. 4), whereas the sensitive cells showed nearly complete depletion of the retinoid by 60 h (t1/2 = 26 h for sensitive cells versus 50 h for resistant cells).

Medium Retinoid. There was significantly more radioactivity in methanolic extracts from LXSN-NIH-3T3 cells compared to LRARα403SN-NIH-3T3 cells at the later time points (Fig. 5A), as expected from the higher cell-associated radioactivity in the unresponsive cells. HPLC analysis of the medium extracts indicated a half-life for medium RA of about 30 h for the sensitive cells and a two-slope curve for the resistant cells with a similar initial...
Fig. 4. Expression of the dominant-negative RARα403 construct reduces the rate of RA loss from the cells. LXSN-NIH-3T3 and LRARα403SN-NIH-3T3 cells were treated with [3H]RA. Radioactivity eluted at the position of all-trans-RA was measured by radioactivity flow detector connected with the HPLC column. Differences were significant at 2, 48, 60, and 72 h (P < 0.05). Data are means; bars, SE.

Fig. 5. Analysis of medium radioactivity. A, total cpm from medium extracts of LXSN-NIH-3T3 and LRARα403SN-NIH-3T3 cells. Differences are statistically different at 72 and 96 h. Data are means; bars, SE. B, [3H]RA in medium from LXSN-NIH-3T3 and LRARα403SN-NIH-3T3 cells.
half-life of 30 h and a much slower rate of loss, probably resulting from lack of metabolism, in the medium from RA-resistant LRARα403SN-NIH-3T3 cells and RA-responsiveness of NIH-3T3 cells. Therefore, the dominant negative in this system may act mainly on RARγ (4). It is therefore tempting to speculate that because TGase II activity was assayed as described in “Materials and Methods.” Results were significantly different (P < 0.01) between DMSO- and RA-treated NIH-3T3 cells and between RA- and DMSO-treated LRSN-NIH-3T3. In sharp contrast, there was no difference in TGase activity between RA- and solvent-treated LRARα403SN-NIH-3T3 cells.

Dominant-Negative Effect of the Truncated Receptor on TGase II. Table I shows that RA induced TGase II activity by more than 20-fold in both the NIH-3T3 as well as the LXSNN-NIH-3T3 cells. In sharp contrast, LRARα403SN-NIH-3T3 cells failed to respond to RA and showed a lower basal level of TGase II activity.

Table I  Expression of the dominant negative RARα403 construct blunts the induction of TGase II by RA

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TGase activity (cpm/µg protein)</th>
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<tbody>
<tr>
<td>NIH-3T3</td>
<td>57,478 ± 8,607</td>
</tr>
<tr>
<td>LXSN-NIH-3T3</td>
<td>39,659 ± 6,721</td>
</tr>
<tr>
<td>LRARα403SN-NIH-3T3</td>
<td>620 ± 410</td>
</tr>
<tr>
<td>RA:DMSO</td>
<td>380 ± 260</td>
</tr>
</tbody>
</table>

TGase II activity was assayed as described in “Materials and Methods.” Results were significantly different (P < 0.01) between DMSO- and RA-treated NIH-3T3 cells and between RA- and DMSO-treated LXSNN-NIH-3T3. In sharp contrast, there was no difference in TGase activity between RA- and solvent-treated LRARα403SN-NIH-3T3 cells.

Discussion

Retinoids are chemopreventive agents (8, 9) as well as differentiation agents (10) and, as such, occupy a pivotal position in cancer prevention and treatment. RAs bind to their nuclear receptors RARs (11) and retinoic acid receptors (12), which are thought to mediate their various effects (1).

RARs display cell type-specific transcript expression (13) and have been shown to be down-regulated during the process of carcinogenesis, and reexpression of RARs reactivates sensitivity to RA and growth inhibition in resistant mouse epithelial tumors (14). In NIH-3T3 fibroblasts, we have reported the expression of RARα and RARβ, which we could detect only at the mRNA level (4). RA treatment of these cells caused a marked reduction in RARα protein, an induction of RARβ transcripts, and no effect on RARγ (4). It is therefore tempting to speculate that because TGase II expression is increased in these RA-treated cells, this induction may take place via RARγ, because RARβ expression is very low in these cells. Therefore, the dominant negative in this system may act mainly as an inhibitor of RARγ.

One of the major effects of RA is its ability to inhibit cell growth in normal (7) as well as in neoplastic cells when given in pharmacological concentrations (2, 7, 15). At physiological concentrations or in vitamin A-deficient cells, the retinoid may enhance cell growth (16), and, in fact, morphogenesis and cell growth in the embryo require vitamin A (17), although teratogenic effects of retinoids are observed readily in excessive administration schedules (18). Transfection with the RARα cDNA (19) rendered two retinoid-resistant, estrogen receptor-negative human breast carcinoma cell lines responsive to RA by growth inhibition. However, in this study, no correlation was noted between cell growth inhibition and RA metabolism. This may be because monitoring of metabolism was done with nonradioactive RA. In another study of breast cancer cell lines, the expression of dominant-negative truncation mutants of RARα also reduced RA inhibition of MCF-7 cell growth (20). Gudas’ group has shown that knockout of the expression of the RARα gene actually led to an increased RA metabolism in embryonal carcinoma F9 cells, whereas the RARγ knockout caused reduced metabolism (21). Our results with the dominant negative support the notion that RARs are involved in both the inhibition of cell growth as well as RA metabolism and further reinforce the concept that the two are somehow connected.

Self-induced RA metabolism is thought to be responsible for the very large reduction in the concentration of RA in patients treated for acute promyelocytic leukemia, who eventually may develop RA resistance (22). Zhang et al. (23) have established the involvement of RARα in the RA-mediated induction of TGase II and apoptosis in the tracheobronchial epithelial cell line SPOC-1, using the dominant-negative construct RARα403 as well as RAR- and retinoid X receptor-selective retinoids.

Our work shows that dominant-negative RARα expression blunts TGase II induction and reduces its endogenous levels by 70% (Table I), consistent with the findings of Jetten and collaborators (23) and Davies and collaborators (24), who have recently demonstrated the presence of a versatile retinoid response element (RA and retinoid X response elements) in the promoter region of the mouse TGase II gene.

In conclusion, this work demonstrates for the first time that RARs are mediators of RA-induced inhibition of cell growth and RA metabolism in fibroblast cells.

References


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