Effects of Arotinoids upon Murine Embryonal Carcinoma Cells

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ABSTRACT

Five arotinoids have been compared with all-trans- and 13-cis-retinoic acids for their ability to promote differentiation of cells from murine embryonal carcinoma line Nulli-SCC1. Ro-13-7410, which contains a terminal carboxylic acid residue, and Ro-14-9572, the sodium sulfonate derivative, are potent inducers of differentiation. The sodium sulfinate derivative, Ro-14-3899, is somewhat less active, whereas the ethyl sulfone (Ro-15-1570) and Ro-15-0778, an arotinoid lacking a terminal group, have little or no effect on embryonal carcinoma cell differentiation. Competition by the arotinoids with all-trans-retinoic acid for sites on the cellular retinoic acid-binding protein is qualitatively consistent with their capacity for promoting differentiation. This relationship and the response of differentiation-defective embryonal carcinoma cells to Ro-13-7410 support the view that arotinoids and retinoids promote differentiation of embryonal carcinoma cells via the same mechanism.

INTRODUCTION

Retinoids are pleiotropic agents which appear to alter cellular behavior at several different levels (9, 12). One notable effect of retinoids is their ability to promote differentiation of murine embryonal carcinoma cells (5, 17), thereby reducing their tumorigenicity (14, 16, 18). In fact, on a molar basis, retinoids containing terminal carboxylic acid residues are the most potent inducers of differentiation of these cells reported to date. Evidence has been presented (4, 13) to support the view that retinoid-induced differentiation of embryonal carcinoma cells is mediated by a CRABP.1

Recently, a group of compounds called arotinoids has been synthesized. These compounds share structural similarities with retinoids and, when administered to animals in excessive doses, elicit characteristic hypervitaminosis A responses (1, 2, 8). Several arotinoids suppress growth of chemically induced skin papillomas and carcinomas in mice and cause proteoglycan release in fetal rat bone organ cultures, as do retinoids (1, 2, 6, 8).2 It has been shown in these tests that the effective dosages are substantially lower than those for retinoid acid or other retinoid analogues. It has also been observed that some arotinoids compete with retinoid acid for sites on the CRABP.2

Since arotinoids are potent analogues of natural retinoids in other tests, we have assessed their effectiveness in inducing differentiation of embryonal carcinoma cells. We have utilized 5 arotinoids (Chart 1). Ro-13-7410 possesses a carboxylic acid residue and is thereby analogous to retinoid acid. Ro-15-0778 lacks a residue on the aromatic ring in the side chain and would be predicted to have little, if any, affinity for CRABP. Ro-14-3899, Ro-14-9572, and Ro-15-1570 possess sulfur-containing residues on the aromatic ring; their potential for promoting differentiation of embryonal carcinoma cells was unpredictable since retinoids with these terminal groups had not been tested in the embryonal carcinoma system or for binding to CRABP.

MATERIALS AND METHODS

Materials. Unlabeled retinoids and arotinoids, as well as all-trans-[3H]retinoic acid (about 30 Ci/mmol), were obtained from Hoffmann-La Roche Inc., Nutley, N. J., and Basel, Switzerland. Sources of culture media and reagents for plasminogen activator and immunofluorescence analyses have been outlined previously (5, 11).

Cell Lines. H6 embryonal carcinoma cells were obtained from Dr. John Littlefield. Nulli-SCC1 cells (10) were obtained from Dr. Gail Martin. Two differentiation-defective mutants, Nulli(RA)~1 and Nulli(HMBA)~1, were derived (11) from Nulli-SCC1 cells by mutagenesis and selection for lack of response to retinoid acid and HMBA, another inducer of differentiation (3). Analyses have revealed that nulli(RA)~1 cells are refractory to HMBA whereas Nulli(HMBA)~1 cells differentiate normally in response to retinoid acid (11, 15, 20). Culture conditions for these cells and procedures for growth curve determinations have been described (5, 11, 13). Medium with or without added retinoids or arotinoids (dissolved in ethanol at final concentrations of 0.1% or less) was changed at least 3 times/week. Cells were inspected for morphology by phase-contrast microscopy just prior to changing the medium. Cultures were kept in a room with amber filters covering all light sources.

Expression of SV40 T Antigen. After 9 days and 2 passages in Ro-13-7410, Nulli-SCC1 cells were either mock-infected or infected with SV40 at an approximate multiplicity of infection of 200. After adsorption of virus and washing, cells were cultured for 3 days in medium lacking Ro-13-7410 and tested for T-antigen expression by immunofluorescence. Details of these procedures are given in the report of Taketo et al. (20).

Plasminogen Activator Analyses. Conditioned culture media (containing plasminogen-depleted serum) were collected after 24-hr periods and aliquots were tested for plasminogen activator activity by incubation with plasminogen and 125I-fibrin. Details of the assay are given elsewhere (11). Values are corrected for background activity (determined with fresh medium containing plasminogen-depleted serum) and are expressed per 10⁵ cells present in the culture at the time of collection of the medium.

Retinoic Acid-binding Protein Analyses. CRABP analyses were carried out with high-speed supernatants (11, 13) of thoroughly washed H6 embryonal carcinoma cells. We have demonstrated previously that such extracts contain only 2S CRABP activity; i.e., they are devoid of contaminating serum binding protein activity (11, 13). H6 cells were used because they possess greater CRABP levels than other embryonal carcinoma lines tested.3 We have carried out CRABP analyses by a procedure which is as reproducible as the conventional sucrose density gradient technique (4, 13) but which requires substantially less homogeneous protein (100 to 250 μg versus ≥1 mg), takes less time, and is more amenable to multiple sampling. For 14K analysis, cytosolic extracts containing 250 μg protein were incubated in the presence of 10 mM Tris-HCl (pH 7.6), 2 mM MgCl₂, and 7 mM mercaptoethanol, with [3H]retinoic acid at final concentrations between 10 and 100 nM. The total volume was 150 μl. After 4 hr at 4°C, 100 μl of dextran-coated charcoal (13) were

1The abbreviations used are: CRABP, cellular retinoic acid-binding protein; HMBA, hexamethylendiaminobisacetamide.

2M. Klaus, W. Bollag, P. R. Huber, and W. M. König, manuscript in preparation.

3K. I. Matthaei, P. A. McCue, and M. I. Sherman. Retinoid binding protein

added and samples were centrifuged at 3000 rpm for 20 min. This step removes more than 95% of the unbound labeled retinoid. One hundred μl of the supernatant were then passed through a 7-cm Sephadex G-25-150 (Sigma Chemical Co., St. Louis, Mo.) column in a Kimble Pasteur pipet (No. 72050) to remove the remaining unbound [3H]retinoic acid. The bound counts elute in the void volume from this column and are collected directly into scintillation vials. As indicated below (see “Results”), the apparent Ka values obtained herein are similar to those determined by sucrose density gradient analysis (4, 22), suggesting that excessive nonspecific binding is not a complication in this procedure. In fact, when incubation mixtures with H6 cytosol are split and analyzed by sucrose density gradient centrifugation versus the Sephadex procedure, specific binding values are within 10% of each other.

Determination of Ki values was carried out as described above except that unlabeled competing retinoic acid or Ro-13-7410 (2.2 × 10−8 M) was added to the reaction mixtures.

RESULTS

In the absence of inducers, embryonal carcinoma cells grow in tightly packed clusters; the cells are small and rounded with large nuclei and prominent nucleoli (Fig. 1). In the presence of 13-cis- or all-trans-retinoic acid at concentrations between 10−8 and 10−6 M, some Nulli-SCC1 cells underwent morphological alterations within 2 days. Cell packing was reduced, the cells became more stellate, and nucleoli were less intense (Figs. 2 and 3). Similar morphological alterations were observed when cultures were treated with Ro-13-7410, Ro-14-3899, or Ro-14-9572 (e.g., Figs. 4 and 5; Table 1). Only a small proportion of cells had altered morphology in cultures exposed to 10−6 M Ro-15-1570 for 2 days, although a somewhat larger fraction of the cells showed an effect after 4 days (Table 1). Ro-15-0778 at 10−8 M had no effect on cellular morphology for up to 7 days of treatment (Fig. 6; Table 1).

Data from several experiments in which the effects of retinoids and aratoinoids on cell morphology were monitored are summarized in Table 1. Although the percentages of cells with altered morphology are only estimated and although there was variation among experiments, some patterns were apparent: (a) all-trans-retinoic acid and 13-cis-retinoic acid possessed similar potency at the concentrations tested; (b) aratoinoids Ro-13-7410 and Ro-14-9572 were at least as effective as the retinoic acids in inducing morphological alterations, whereas Ro-14-3899 was somewhat less active; and (c) as mentioned, Ro-15-1570 was substantially less effective than the aforementioned aratoinoids, and Ro-15-0778 lacked activity.

In a more limited series of studies, we observed that after 4 days small numbers of cells (5 to 15%) underwent morphological change in response to all-trans- and 13-cis-retinoic acids, Ro-13-7410, and Ro-14-9572, at 10−8 M. Also, Ro-15-1570 appeared to be slightly more effective in altering cell morphology at 10−6 M (10 to 25%) than at 10−8 M (5 to 15%), although Ro-15-0778 was still ineffective at the higher concentration.

To investigate the irreversibility and progression of the observed changes in Nulli-SCC1 morphology, cells were exposed to 13-cis-retinoic acid or Ro-13-7410 at 10−6 M for 14 days and then maintained without inducer for an equal period of time. Figs. 7 to 10 illustrate that cultures originally treated with 13-cis-retinoic acid possessed large areas of cells with diverse and characteristic nonembryonal carcinoma morphologies. Some tight clusters of small cells (e.g., Fig. 10, arrow) might have
contained unchanged embryonal carcinoma cells. Parallel cultures treated with Ro-13-7410 also possessed cells with nonembryonal carcinoma phenotypes although the diversity was not so great as that observed with 13-cis-retinoic acid (Figs. 11 and 12). Clumps of small cells were observed in these cultures as well.

Whereas untreated Nulli-SCC1 cells secrete very low levels of plasminogen activator, they produce markedly elevated amounts of this protease when induced to differentiate with retinoids. Table 2 illustrates that increased secretion could be detected within 48 hr of exposure to all-trans- or 13-cis-retinoic acid at concentrations as low as $10^{-8}$ M. At $10^{-6}$ M, the effect was more pronounced. The 2 retinoids appeared to be equally active. By this assay procedure, arotinoid Ro-14-9572 was at least as potent as the retinoic acids. Ro-13-7410 appeared to be almost as active as the retinoids at $10^{-8}$ M but less so at $10^{-6}$ M, whereas the converse was true of Ro-14-3899. Ro-15-1570 elicited only a minor effect whereas Ro-15-0778 was inactive (Table 2). In general, there is good agreement between results of morphological and plasminogen activator secretion analyses.

Further evidence that the morphological and biochemical changes induced by arotinoids were indicative of differentiation was obtained by studies of infectivity with SV40. Taketo et al. (20) have previously demonstrated that Nulli-SCC1 cells, like other embryonal carcinoma cells (19), fail to express SV40 T antigen following viral infection whereas some differentiated progeny following RA treatment are capable of expressing the viral gene. Fig. 13 illustrates that characteristic nucleoplasmic staining for SV40 T antigen was detected following 9 days of treatment with Ro-13-7410. Mock-infected cells (Fig. 14) showed only nonspecific nucleolar staining commonly seen with the antisemur used (20).

We have utilized a simplified assay procedure to determine the ability of retinoids and arotinoids to compete with all-trans-[3H]retinoic acid for sites on the CRABP. By this assay, we have calculated an apparent $K_d$ of $3.8 \pm 0.4$ (S.E.) $\times 10^{-8}$ M, (8 independent determinations) for the binding of all-trans-retinoic acid to the CRABP in cytosolic extracts of H6 embryonal carcinoma cells. Typical results are illustrated in Chart 2. The $K_d$ value obtained here is close to those reported by Jetten and Jetten (4).

### Table 2

Effects of retinoids and arotinoids on plasminogen activator secretion by Nulli-SCC1 cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>10^{-6} M</th>
<th>10^{-10} M</th>
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<tbody>
<tr>
<td></td>
<td>1-2 days</td>
<td>3-4 days</td>
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<td></td>
<td>1-2 days</td>
<td>3-4 days</td>
</tr>
<tr>
<td>None</td>
<td>14</td>
<td>31</td>
</tr>
<tr>
<td>All-trans-retinoid</td>
<td>212</td>
<td>230</td>
</tr>
<tr>
<td>Ro-13-7410</td>
<td>103</td>
<td>302</td>
</tr>
<tr>
<td>Ro-14-3899</td>
<td>94</td>
<td>79</td>
</tr>
<tr>
<td>Ro-14-9572</td>
<td>252</td>
<td>547</td>
</tr>
<tr>
<td>Ro-15-0778</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Ro-15-1570</td>
<td>28</td>
<td>59</td>
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Table 3

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<th>Relative effectiveness in competing for CRABP binding</th>
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<tr>
<td>Competitor</td>
</tr>
<tr>
<td>All-trans-retinoid</td>
</tr>
<tr>
<td>Ro-14-3899</td>
</tr>
<tr>
<td>Ro-14-9572</td>
</tr>
<tr>
<td>Ro-15-0778</td>
</tr>
<tr>
<td>Ro-15-1570</td>
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</tbody>
</table>

* ND, not determined.
The first of these mutants, Nulli(HMBA)−1, fails to respond to HMBA, another inducer of differentiation, but differentiates normally in the presence of all-trans-retinoic acid; the second mutant, Nulli(RA)−1, fails to differentiate following exposure to HMBA or all-trans-retinoic acid (11). Figs. 15 to 23 illustrate that 13-cis-retinoic acid and Ro-13-7410 had the same effect upon the mutant cells as had been documented for all-trans-retinoic acid; Nulli(HMBA)−1 cells underwent an alteration in morphology whereas Nulli(RA)−1 cells did not. Similarly, Table 4 demonstrates that Nulli(RA)−1 cells did not secrete notably increased amounts of plasminogen activator when exposed to 13-cis-retinoic acid or Ro-13-7410 for 2 days; as has been observed with all-trans-retinoic acid (11), there was some elevation in the levels of secretion after 4 days of incubation, but the values reached were far below those measured for parental Nulli-SCC1 cells (see Table 2). On the other hand, both 13-cis-retinoic acid and Ro-13-7410 caused a substantial elevation in plasminogen activator secretion by Nulli(HMBA)−1 cells (Table 4).

The effects of 13-cis-retinoic acid and Ro-13-7410 on the growth properties of Nulli-SCC1 cells are illustrated in Chart 3A. Both compounds had a slight inhibitory effect (3 to 5 hr) on the doubling time and lowered the final cell density. This is presumably not due only to promotion of differentiation since similar results were observed with Nulli(RA)−1 cells (Chart 3B).

**DISCUSSION**

The conclusion that the active arotinoids in this study promote differentiation of Nulli-SCC1 cells is based upon alteration of morphology, maintenance of nonembryonal carcinoma appearance for many days after removal of inducers, dramatic increase of plasminogen activator secretion, and capability to express T antigen following infection with SV40. Similar responses were observed with all-trans- and 13-cis-retinoic acids. The latter observation supports and extends earlier reports (4, 11, 18) that 13-cis-retinoic acid is an effective inducer of embryonal carcinoma cell differentiation; all-trans- and 13-cis-retinoic acids also possess a similar affinity for CRABP sites.

This laboratory has provided evidence that CRABP mediates retinoic acid-induced differentiation of embryonal carcinoma cells (4, 11, 13). It is notable in this regard that Ro-13-7410, Ro-14-3899, and Ro-14-9572 compete for CRABP sites and are effective inducers of differentiation whereas Ro-15-0778 does neither. Ro-15-1570 is only a poor inducer of differentiation, and it competes inefficiently with [3H]retinoic acid for CRABP sites. These results are consistent with the view that arotinoids promote differentiation in the same way as do retinoids and that interaction with CRABP is involved. Further support for this proposal derives from the similar behavior of 13-cis-retinoic acid and Ro-13-7410 on differentiation-defective embryonal carcinoma mutants.

Ro-13-7410 and Ro-14-9572 are at least as effective as retinoids in inducing differentiation of embryonal carcinoma cells at low concentrations. In some experiments, it appeared as though these arotinoids were even more potent than the natural retinoids. This is consistent with the reported potency of arotinoids relative to retinoids in reversing papilloma and skin carcinoma growth (2, 8), inducing cartilage regression in fetal bone cultures (6), and eliciting hypervitaminosis A symptoms (1, 2, 8).

The affinity of Ro-13-7410 for CRABP sites does not appear to be markedly different from that of 13-cis- and all-trans-retinoic acids, whereas preliminary indications (Table 3) are that Ro-14-9572 is a less effective competitor for binding sites. Furthermore, Ro-14-3899 showed a capacity to compete for CRABP sites similar to that of Ro-14-9572, yet the latter arotinoid was more effective at promoting differentiation than the former. These results affirm previous assertions (21) that the relationship between the affinity of a retinoic acid analogue for CRABP and its ability to promote differentiation of embryonal carcinoma cells, although qualitative, is not necessarily quantitative. Other considerations, such as differential rates of uptake or metabolism of the inducers, could be important.

In their study, Jetten and Jetten (4) found that esterified retinoids generally failed to promote differentiation or bind CRABP. Based upon these observations, it might have been predicted that the arotinoid ethyl sulfone, Ro-15-1570, would be inactive as well. This arotinoid had low and variable activity in both binding and biological assays. Further studies will be required to establish whether the arotinoid per se possessed the activity or whether small amounts were biochemically or nonen-

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**Table 4**

Effects of 13-cis-retinoic acid and arotinoid Ro-13-7410 on plasminogen activator secretion by differentiation-defective embryonal carcinoma cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Addition</th>
<th>Plasminogen activator activity (cpm/10⁵ cells)</th>
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<tbody>
<tr>
<td></td>
<td>1-2 days</td>
<td>3-4 days</td>
</tr>
<tr>
<td>Nulli(RA)−¹</td>
<td>None</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td>13-cis-Retinoic acid</td>
<td>19.2</td>
</tr>
<tr>
<td></td>
<td>Ro-13-7410</td>
<td>30.1</td>
</tr>
<tr>
<td>Nulli(HMBA)−¹</td>
<td>None</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>13-cis-Retinoic acid</td>
<td>97.3</td>
</tr>
<tr>
<td></td>
<td>Ro-13-7410</td>
<td>197.7</td>
</tr>
</tbody>
</table>

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**Chart 3**

Effects of 13-cis-retinoic acid and Ro-13-7410 on growth of 'normal' and differentiation-defective embryonal carcinoma cells. A, Nulli-SCC1 cells seeded at 5 x 10⁵ cells and cultured in control medium (O) or in medium containing 10⁻⁶ M 13-cis-retinoic acid (D) or Ro-13-7410 (I). B, Nulli(RA)−¹ cells treated in the same way. Each point is the average of triplicate determinations.
zymatically modified to an active form.

Finally, when considering the mechanism of a particular activity of retinoids and related compounds, it is important to bear in mind that they are likely to be pleiotropic. Evidence has accumulated that the various biological and pharmacological effects of these compounds might, in fact, be achieved by different mechanisms (see Refs. 9 and 12). A good example is our observation that all-trans-retinoic acid depressed the growth rate of mutant embryonal carcinoma cells even though these cells lack CRABP and fail to differentiate in response to retinoic acid (13). The growth curves in the present study are consistent with these observations and suggest that, like retinoid, Ro-13-7410 can interfere with the growth of embryonal carcinoma cells in a way unrelated to that by which it promotes differentiation.

ACKNOWLEDGMENTS

We are most grateful to Drs. Werner Bollag (Roche, Basel, Switzerland) and Al Boris (Roche, Nutley, N. J.) for suggesting the use of, and supplying, the arotinoids in this study. We also thank Drs. Clark Perry and Arnold Lieberman for providing [3H]retinoic acid and Drs. Beverly Pawson and Stanley Shapiro for reading the manuscript.

REFERENCES

Figs. 1 to 6. Morphology of Nulli-SCC1 cells in the absence and presence of retinoids or arylretinoids. All photographs were taken using phase-contrast optics and are reproduced at the same magnification.

Fig. 1. Untreated cells.
Fig. 2. Cells after 2 days of exposure to $10^{-6}$ M all-trans-retinoic acid.
Fig. 3. Cells after 2 days of exposure to $10^{-6}$ M 13-cis-retinoic acid.
Fig. 4. Cells after 2 days of exposure to $10^{-6}$ M Ro-13-7410.
Fig. 5. Cells after 2 days of exposure to $10^{-6}$ M Ro-13-7410.
Fig. 6. Cells after 7 days of exposure to $10^{-6}$ M Ro-15-0778. Scale marker, 50 μm.
Figs. 7 to 12. Irreversibility of morphological alterations of Nulli-SCC1 cells treated with 13-cis-retinoic acid or Ro-13-7410. Cells were treated with these compounds for 14 days and then maintained for an equal period of time in control medium.

Figs. 7 to 10. Treatment with $10^{-6}$ M 13-cis-retinoic acid.

Figs. 11 and 12. Treatment with $10^{-4}$ M Ro-13-7410. Arrows, clusters of tightly packed cells which might represent embryonal carcinoma cells that had resisted differentiation and were proliferating following removal of the inducers. Scale marker (in Fig. 12), 50 μm.

Figs. 13 and 14. SV40 T antigen expression by Nulli-SCC1 cells following treatment with Ro-13-7410.

Fig. 13. Cells treated with Ro-13-7410 for 9 days were infected with SV40 and tested for T-antigen expression 3 days later. Several of the nuclei in the culture show prominent immunofluorescence with anti-T serum.

Fig. 14. Mock-infected cells. Some nucleoli show nonspecific immunofluorescence (see Ref. 20).
Figs. 15 to 23. Morphology of Nulli-SCC1 cells and differentiation-defective derivatives in the absence and presence of 13-cis-retinoic acid or Ro-13-7410. Photographs were taken 2 days after initiation of the cultures. Top, Nulli-SCC1 cells in control medium (Fig. 15) or after exposure to $10^{-4}$ M 13-cis-retinoic acid (Fig. 16) or Ro-13-7410 (Fig. 17). Middle, Nulli(HMBA)$^+$ cells in control medium (Fig. 18) or after exposure to 13-cis-retinoic acid (Fig. 19) or Ro-13-7410 (Fig. 20). Bottom, Nulli(RA)$^+$ cells in control medium (Fig. 21) or after exposure to 13-cis-retinoic acid (Fig. 22) or Ro-13-7410 (Fig. 23). Scale marker (in Fig. 23), 50 μm.
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