Retinoic Acid Metabolism and Inhibition of Cell Proliferation: An Unexpected Liaison

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Abstract
The rationale for the use of all-trans-retinoic acid (RA) as an anticancer agent is based on its ability to inhibit growth and promote differentiation of some neoplastic cells. However, RA is not effective in all conditions of cell culture, and in some cases, it may stimulate cell growth. We used a serum-free culture system to study the effect of RA on cell proliferation. Following 2 days of RA exposure, 9 of a total of 15 cell lines showed an inhibition of cell growth (RA-sensitive), while 6 of 15 cell lines showed resistance to RA (RA-resistant cells). Metabolic studies and high-performance liquid chromatography analysis of the cell-associated and medium extracts from cells incubated with [3H]RA revealed that all nine RA-sensitive cells showed a very high activity to metabolize RA to polar metabolites found in the medium. In sharp contrast, RA-resistant cells retained about 60% of the original RA at 76 h. However, conditioned medium from the sensitive cells was without activity on the growth of sensitive and resistant cells. We conclude that a relationship exists between RA inhibition of cell growth and intracellular RA metabolism. These data may help design useful strategies in cancer therapy by retinoids and dispel the notion that RA itself is responsible for the inhibition of cell growth.

Introduction
RA2 is a potent modulator of growth and differentiation (1–4). It binds to its nuclear receptors (RARs) (5) and as the 9-cis-isomer to retinoid X receptors (6) to affect gene transcription. RA is active in differentiation therapy (7, 8) of acute promyelocytic leukemia, and it inhibits the growth of several neoplastic cells (9, 10). However, RA has achieved as yet only a limited success in controlling cancer cell growth. This limitation might have resulted, in part, from pharmacological factors in RA administration, such as inappropriate tissue distribution or toxicity. It is clear that not all transformed cells are inhibited, nor is susceptibility limited to malignant transformed cells (11), since some untransformed cells were found to be growth inhibited. There seems to be no correlation between the growth rate of the cells and their susceptibility to retinoid-induced growth inhibition. The growth of some malignant cell lines was found to be stimulated by retinoids (11).

The growth and differentiation of various normal and malignant cells in culture are modulated (stimulated or inhibited) by RA. It has been shown that there are RA-sensitive (growth-inhibited) and RA-resistant cells, although the reason for different RA responses is not clearly understood. Culture media supplemented with 10% FCS usually stimulates cell growth, while retinoids have been shown to affect cell growth in serum-free medium (11). The toxicity of a high dose of retinoids is enhanced in serum-free conditions, possibly because of the absence of serum albumin, which binds retinoids. Therefore, we investigated cell proliferation in serum-free culture condition and the possible relationship between metabolism and proliferation. Metabolites of RA include 13-cis-RA, 9-cis-RA, 4-hydroxyretinoic acid, and 4-oxo retinoic acid (12). Some of these metabolites are active in mediating RA function, whereas others are probably catabolic products (13). In view of our expectation that RA itself is active in the inhibition of malignant cell growth, it was surprising to find that only cells that were very active in metabolizing RA to oxidation products were growth inhibited and that cells that retained the intact RA were stimulated to grow.

Materials and Methods

Cells and Cell Culture. Most normal and cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). The K-308 cell line was obtained from Dr. S. H. Yuspa, National Cancer Institute (Bethesda, MD). The NIH 3T3, NIH ram-3T3, BEAS-2B, HT-144, HL 60, BXPC-3, MCF-7, T-47D, MDA-MB-231, MDA-MB-453, SW-620, BT-474, ZR-75-1, and K-308 cell lines were maintained in DMEM 90–1 supplemented with 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD). The HS-578-Bst cell line, a normal breast epithelial cell, was maintained in DMEM as above but supplemented with 10% fetal bovine serum with 10–8 M insulin. Stock cells were routinely cultured as adherent monolayers in 150 cm2 Falcon tissue culture flasks at 37°C in a humidified atmosphere of 5% CO2 in air. Dulbecco’s PBS without Ca2+ or Mg2+ (pH 7.5) was used for all washes.

Cell Proliferation Experiments. Cells from subconfluent cultures were harvested by treatment with trypsin (0.05% trypsin and 0.2% EDTA; Biofluids, Inc., Rockville, MD). Cells were seeded in a series of 60 mm-diameter tissue culture dishes at 1 × 105 cells/dish in medium with 10% FCS. After 24 h, to allow for cell attachment, the culture medium was replaced with experimental serum-free medium (Biofluids, Inc.) containing the indicated concentrations of RA. Control cultures contained the ethanol vehicle in the same amount. The cells were cultured at 37°C in a humidified atmosphere composed of 95% air and 5% CO2. Dishes were removed from the incubator at each of the indicated times (24–96 h). Cultures were rinsed twice with 5 ml PBS (pH 7.4) and pipetted gently; then the cells were detached after a brief exposure to 0.05% trypsin and suspended repeatedly to give a single-cell suspension. The number of cells was measured using an electronic Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Results are presented as the means ± SDs of triplicate cultures after 48 h.

Measurement of Radioactivity from all-trans-3H-RA In the Cell Extracts and Medium. Cells were removed from the surface of the tissue culture flask with trypsin/EDTA (0.05% trypsin and 0.53 mm EDTA in HBSS without Ca2+ or Mg2+ (GIBCO) and were seeded in a series of 100 mm-diameter tissue culture dishes at 1 × 105 cells/dish in medium with 10% FCS. After 24 h, to allow for cell attachment, the culture medium was replaced with serum-free medium. [3H]all-trans-RA (DuPont, New England Nuclear; 53 Ci/mmol) was added to each plate in the same serum-free medium containing the indicated concentration of 30 nM [3H]RA (5 mCi/dish). At the appropriate times, the media were collected, and the cells were rinsed twice with PBS containing 30 nM cold RA (Sigma); then cells were scraped into 1 ml of PBS. The cell suspension was centrifuged at 1200 rpm for 5 min. The cell pellet was extracted with 1 ml methanol, vortexed, and stored at −70°C for 24 h (cell
extracts). Aliquots (0.1 ml) of the media from culture dishes were also saved and extracted with 0.9 ml of methanol; extracts were stored at -20°C (medium extracts). Aliquots of these extracts and of the supernatant were counted for recovery; the rest was used for HPLC analysis. Recovery of radioactivity was at least 85% of initial amount. Total radioactivity of cell extract and medium extract samples were measured on a liquid scintillation counter.

HPLC Analysis. The supernatant of the cell extracts were analyzed for radioactivity RA and its metabolites by HPLC. Each sample was dried and dissolved in 50 μl of methanol for analysis. The analysis was performed on a Partisil 10 ODS-2 column [4.6 mm (inside diameter) × 25 cm; Whatman, NJ] fitted with a precolumn of Pellicular ODS (Whatman). A Beckman model 110A pump was connected to a Gilson 116 UV detector monitoring at the fixed wavelength of 340 nm (Gilson Medical Electronics, Middleton, WI). The mobile phase was acetonitrile:1% ammonium acetate in water (65:35), according to the procedure of Frolik et al. (12). The flow rate was 2.2 ml/min, and the total time for the separation was 25 min. Effluent from the HPLC column flowed directly into a flowthrough scintillation spectrometer (Beta-One; Radiomatic Instruments and Chemical Co., Inc., Tampa, FL) to determine radioactivity.

Results and Discussion

We tested 15 cell lines, mostly derived from normal epithelia and epithelial cancers, to study the effect of RA on their growth. NIH3T3, K-308, HL60, BXPC-3, Hs-578-Bst, MCF-7, T-47D, BT-474, and ZR-75-1, i.e., 9 of 15 cell lines, were growth inhibited (29 to 78%) by RA-sensitive and resistant cells after 72 h incubation. Conditioned medium (48 h) from sensitive cells was without activity on both sensitive and resistant cells after 72 h incubation.

Peak [3H]RA levels were achieved in 2 to 6 h. Cell-associated [3H]RA levels decreased sharply in all nine RA-sensitive cell lines (Fig. 1A). Most radioactivity was found in the medium. All six RA-resistant cell lines retained more than 50% intact [3H]RA, even at the 72 to 96 h (Fig. 1B). Fig. 2 shows a representative radioactivity profile obtained by HPLC analysis of the cell extracts at 2 and 48 h. RA-sensitive and -resistant cells showed qualitatively similar metabolites (Fig. 2). HPLC profiles of metabolites in RA-sensitive cells at 2 and 48 h are representative of all nine sensitive cells (Fig. 2, A and B). After 2 h, cell extracts contained 89 ± 6% of the radioactivity as [3H]RA (peak I), 5 ± 1% as 9-cis-RA (peak II), and 6 ± 1% as 13-cis-RA (peak III). After 48 h, however, only 12 ± 3% was RA, while 56 ± 4% was recovered as polar and very polar metabolites (peaks IV), 8 ± 2% was 9-cis-RA, and 26 ± 5% was 13-cis-RA.

Table 1 Source of cell lines and influence of all-trans-RA on their growth

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
<th>Pathological characterization</th>
<th>Cell number (% of control)</th>
<th>RA sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH 3T3</td>
<td>Mouse embryo</td>
<td>Fibroblast</td>
<td>22 ± 2%</td>
<td>Sensitive</td>
</tr>
<tr>
<td>K-308</td>
<td>Mouse skin</td>
<td>Keratinocyte</td>
<td>68 ± 2%</td>
<td>Sensitive</td>
</tr>
<tr>
<td>HL 60</td>
<td>Human</td>
<td>Myeloid leukemia</td>
<td>58 ± 2%</td>
<td>Sensitive</td>
</tr>
<tr>
<td>BXPC-3</td>
<td>Human pancreas</td>
<td>Adenocarcinoma</td>
<td>71 ± 7%</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Hs-578-Bst</td>
<td>Human breast</td>
<td>Epithelial normal</td>
<td>67 ± 3%</td>
<td>Sensitive</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human breast</td>
<td>Ductal carcinoma</td>
<td>47 ± 7%</td>
<td>Sensitive</td>
</tr>
<tr>
<td>T-47D</td>
<td>Human breast</td>
<td>Ductal carcinoma</td>
<td>44 ± 2%</td>
<td>Sensitive</td>
</tr>
<tr>
<td>BT-474</td>
<td>Human breast</td>
<td>Carcinoma</td>
<td>69 ± 6%</td>
<td>Sensitive</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Human breast</td>
<td>Carcinoma</td>
<td>69 ± 6%</td>
<td>Sensitive</td>
</tr>
<tr>
<td>NIH ras-3T3</td>
<td>Mouse embryo</td>
<td>Transformed fibroblast</td>
<td>97 ± 3%</td>
<td>Resistant</td>
</tr>
<tr>
<td>HT-144</td>
<td>Human skin</td>
<td>Malignant melanoma</td>
<td>107 ± 4%</td>
<td>Resistant</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>Human bronchia</td>
<td>Transformed epithelial</td>
<td>106 ± 4%</td>
<td>Resistant</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Human breast</td>
<td>Adenocarcinoma</td>
<td>97 ± 4%</td>
<td>Resistant</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>Human breast</td>
<td>Carcinoma</td>
<td>96 ± 5%</td>
<td>Resistant</td>
</tr>
<tr>
<td>SW 620</td>
<td>Human colon</td>
<td>Adenocarcinoma</td>
<td>125 ± 2%</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

*a* Tumors from which the cell lines were derived.

*b* Determined after 48 h in 500 nM RA or solvent control (0.05% ethanol). Values are the means ± SD of duplicate samples from two experiments (n = 4).

The difference between control and RA-treated cells was statistically different (P < 0.05) in all cell lines.

**Fig. 1.** Cell-associated radioactivity from [3H]RA in RA-sensitive (A) and RA-resistant cells (B). Cultures were incubated with [3H]RA (5 μCi/dish), and at the indicated times, cells were harvested; then the total cell-associated [3H] was determined. Each point value is the mean of duplicate determinations performed on at least two experiments for each cell line.
When we attempted to repeat these data using the 10% serum-containing culture assay, radioactive RA was not rapidly taken up by the cells, and we could not observe a significant difference in metabolism between RA-sensitive and -resistant cells, nor was an effect on cell growth detected (data not shown).

It is relevant to our findings that the growth of murine sarcoma virus-transformed 3T3 cells is not altered by RA in serum-containing medium but is stimulated by BA in serum-free medium (11). This phenomenon is known to occur with other cells and differentiation inducers (13, 14). It is also relevant that removal of serum from the culture medium has permitted the discovery of growth factors and the definition of their biological activity (15, 16).

Clinical trials have demonstrated that BA, although initially effective against acute promyelocytic leukemia (7, 18–20), is not able to maintain patients in remission, because of the emergence of BA-resistant clones (8). Cells from patients with leukemia at relapse show high levels of CRABP, the cellular RA-binding protein (21) not detected prior to RA therapy (8). Moreover, CRABP-II has been suggested as a protein linked to RA cellular metabolism, possibly acting as a transporter to the endoplasmic reticulum, where RA is metabolized (22). Our preliminary data show that RA-sensitive cells also display low CRABP-I level (data not shown). But it remains to be established whether the low metabolic activity of RA-resistant cells may be due to the high CRABP level.
A second category of proteins relevant to RA metabolism is the cytochrome P-450s. These molecules have been shown to be involved in the metabolism of RA (12, 13, 23–25), and ketoconazole and liarozole were shown to inhibit the cytochrome P-450-dependent oxidation of RA (26, 27). Therefore, differences in cytochrome P-450 between RA-sensitive and -resistant cells may also play a role in the observed differences in RA metabolism.

Finally, recent work (28) has demonstrated that disruption of the RARα and RARγ receptors results in receptor-specific alterations in RA metabolism. Thus, RA resistance may result from changes in the expression of any or all these three categories of proteins and remains to be understood.

In conclusion, our results open up the possibility to predict therapeutic outcomes in tumor cells, depending on their ability to metabolize RA. However, this possibility is tempered by the consideration that our data were obtained in serum-free culture conditions and thus may not apply to the in vivo situation, where the cells, although not bathing in serum, are nonetheless exposed to intestinal fluid and proteins. Our results also suggest that a metabolite of RA, rather than the parent compound itself, may be responsible for the observed inhibition of cell growth. Our effort is presently focused to identify such metabolites.

Acknowledgments

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References

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