Different Susceptibilities of Human Melanoma and Breast Carcinoma Cell Lines to Retinoic Acid-induced Growth Inhibition

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ABSTRACT

The ability of retinoic acid to inhibit the growth of ten cell lines derived from human malignant tumors (six melanomas and four breast carcinomas) and two cell lines derived from nonmalignant breast tissue was investigated in vitro. After a seven-day incubation in the presence of 10^-4 M retinoic acid, the growth of the melanoma cell lines A375 and Hs939 was inhibited by 68 and 62%, respectively; the growth of lines Hs695, Hs852, and SH-4 was not affected, while the growth of the melanoma cell line Hs294 was stimulated two-fold. Breast carcinomas cell lines SK-BR-3 and 734B were inhibited by 83 and 50%, respectively; cell line Hs578T was only slightly inhibited (28%), and the growth of line MDAMB-157 was not affected. The growth of the nonmalignant mammary cell line HBL-100 was slightly inhibited (13%), and that of Hs578Bst was not affected. Further studies, carried out with the more sensitive cell lines (>50% growth inhibited), revealed that the inhibitory effects of retinoic acid were dose dependent. The concentrations of retinoic acid required for 50% growth inhibition of cell lines SK-BR-3, A375, Hs939, and 734B were 1 x 10^-8, 2.5 x 10^-8, 1 x 10^-7, and 3 x 10^-7 M, respectively. When exposed to 10^-4 M retinoic acid, the cells grew at a rate similar to that of control cells for 48 or 72 hr, after which their growth rate decreased. Thus, the population-doubling times of A375, Hs939, and 734B in log phase growth increased from 32, 30, and 67 hr to 48, 75, and 180 hr, respectively. The growth of SK-BR-3 cells was arrested after exposure to retinoic acid for longer than 48 hr. The morphology of all melanoma cells exposed to 10^-4 M retinoic acid was not changed significantly; however, the breast carcinomas 734B and SK-BR-3 cells spread and assumed a flattened morphology after 72 hr in the presence of retinoic acid. These results demonstrate that cell lines derived from tumors of similar histopathological type differ in their responsiveness to retinoic acid.

INTRODUCTION

The crucial role of vitamin A in the control of cellular differentiation in epithelial tissues has long been recognized (26). Vitamin A and its analogs (retinoids) have been under study in recent years both as inhibitors of carcinogenesis and as potential antitumor agents. Their ability to inhibit chemical carcinogen-induced epithelial cancer of the skin, lung, bladder, and breast in experimental animals is well documented (for reviews, see Refs. 35 and 36). Similar effects of retinoids were also noticed in prostate and trachea organ cultures (7, 10, 21) and in mouse 10^1/2 fibroblast cultures (24). The antitumor activity of retinoids has been demonstrated with a few transplantable tumors such as rat chondrosarcoma (37), mouse C3HBA mammary adenocarcinoma (30), and mouse S91 melanoma (14). In addition, retinoids have induced density-dependent growth inhibition in fibroblast cultures of mouse L-929 (12), 3T3 (28), and 3T12-3 cells (1, 11) and hamster NIL and NILpy cells (28). A different type of retinoid-induced growth inhibition, not associated with cell density, was found in cultures of 3T6 mouse cells (19). Recently, we reported that the proliferation of 17 of 31 untransformed, transformed, and tumor cell lines was considerably inhibited (>50% growth inhibition) in vitro by retinoic acid, whereas other cell lines were either slightly inhibited or not affected (23). Cell lines derived from mouse and rat mammary adenocarcinomas and mouse melanomas were found to be very sensitive to the inhibitory effect of retinoic acid (22, 23). Therefore, I tested the ability of retinoic acid to inhibit the growth of cell lines derived from human melanomas and breast carcinomas.

MATERIALS AND METHODS

Cells. The designation of the various cell lines and the type of tumor or the source from which the cells were derived are listed in Table 1. Cell line A375 was generously supplied by Dr. Walter Nelson-Rees of the Naval Biosciences Laboratory, School of Public Health, University of California, Berkeley, Calif. All the other cell lines were the generous gift of Dr. Adeline Hackett, Dr. Abla Craasey, Dr. Martha Stampfar, and Dr. Helene Smith of the Peralta Cancer Research Institute, University of California, Lawrence Berkeley Laboratory, Oakland, Calif.

Tissue Culture Techniques. The growth medium was Dulbecco's modified Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated (56°, 30 min) fetal bovine serum (Flow Laboratories, Ingelwood, Calif.), nonessential amino acids (Grand Island Biological Co.), and 50 μg gentamicin per ml (Schering Corp., Kenilworth, N. J.). The growth medium of all of the breast carcinoma and nonmalignant cell lines contained, in addition, 10 μg insulin per ml (Calbiochem, San Diego, Calif.). The cells were cultured in 75- or 25-sq cm tissue culture flasks (Corning Glass Works, Corning, N. Y.) in 15 or 5 ml growth medium, respectively, at 37° in a humidified atmosphere consisting of 10% CO2 and 90% air. Growth media were changed twice each week, and the cells were subcultured once a week. To prepare each subculture for passage or enumeration, the
cells were detached by a 2- to 5-min treatment with 0.05% trypsin (1:250; Difco Laboratories, Inc., Detroit, Mich.): 0.02% EDTA in 0.15 M NaCl (titrated to pH 6.8 with 1 N NaOH) and suspended in growth medium. Cell counting was performed by using a hemacytometer or an electronic particle counter (Electrozone/Celloscope Model 112 ct; Particle Data, Inc., Elmhurst, Ill.). Cell viability was estimated from the proportion of cells excluding 0.1% trypan blue. The doubling time of the various cell lines was determined by counting viable cells at 24-hr intervals during their growth from an inoculum of 0.3 or 0.5 × 10^6 cells/75-sq cm flask to confluence (usually after 6 to 11 days).

**Treatment of Cells with Retinoic Acid.** All-trans-β-retinoic acid, the gift of Dr. Beverly Pawson of Hoffmann-La Roche Inc. (Nutley, N. J.), was dissolved in ethyl alcohol at 3 mg/ml (10^-6 M) and further diluted in alcohol to obtain a series of 10-fold decreasing concentrations from 10^-2 to 10^-4 M. Immediately before each experiment, the retinoic acid solutions were diluted 1:1,000 or 1:10,000 into the growth medium such that the final alcohol concentration was 0.01% ethyl alcohol with or without 10^-6 M retinoic acid. The cultures were cultivated on Days 3 and 6, and on Day 7 the cells were detached and counted. Percentage of inhibition was calculated as:

\[ 100 - \left( \frac{R}{C} \right) \times 100 \]

where R and C are the numbers of cells in retinoic acid-treated and control cultures, respectively (23).

**RESULTS**

**Effect of Retinoic Acid on the Growth of Human Tumor Cells.** The effects of retinoic acid on the proliferation of various cell lines derived from human melanoma, breast carcinoma, and nonmalignant breast tissue were determined after a 7-day incubation (Table 1). Of the 12 cell lines tested, 2 melanoma cell lines (A375 and Hs939) and 2 breast carcinoma cell lines (SK-BR-3 and 734B) were susceptible to retinoic acid, and their growth was inhibited by 50 to 83%. The growth of lines Hs578T and HBL-100 was only slightly inhibited, whereas no effect was observed on the growth of lines Hs695, Hs852, SH-4, MDA-MB-157, and Hs578Bst. Surprisingly, the growth of the melanoma cell line Hs294 was stimulated 2-fold over the control in the presence of 10^-2 M retinoic acid.

The inhibition of the sensitive cell lines was apparently not due to cytotoxic effects of retinoic acid, since no decrease in viability was detected in the treated cultures compared to control cells with one exception. Cell line SK-BR-3 exhibited decreased viability (−70%) after prolonged incubation with retinoic acid as compared to >90% viability in control cultures after 7 days. The cells that had not been affected by 10^-4 M retinoic acid were also insensitive to higher retinoic acid concentrations. However, at 10^-4 M retinoic acid, the growth medium of both treated and control cultures contained 1% ethyl alcohol, and many of the cell lines, notably Hs294 and A375, were sensitive to alcohol at this concentration. The combination of high alcohol concentration and 10^-4 M retinoic acid was very cytotoxic, and cell viability was below 40%.

The melanoma cell line Hs939 produced melanin under the culture conditions used in this study. When grown in the presence of 10^-4 M retinoic acid for 6 days, the Hs939 cells produced 3-4 times more melanin than did the control cells on a per cell basis. No induction of melanin production was observed with the other melanoma lines including amelanotic melanomas used in this study.

**Dose-Response Relationship of Retinoic Acid-Induced Growth Inhibition.** Since the growth of the melanoma cell lines A375 and Hs939 and breast carcinoma cell lines 734B

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**Table 1**

<table>
<thead>
<tr>
<th>Cell line designation</th>
<th>Type of tumor and source of cells</th>
<th>Doubling time in culture^b^ (hr)</th>
<th>% of growth inhibition by retinoic acid^b^</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Melanoma</strong></td>
<td></td>
<td></td>
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<tr>
<td>Hs695 Primary tumor (6, 9)</td>
<td>67 ± 4</td>
<td>NSI</td>
<td></td>
</tr>
<tr>
<td>Hs852 Primary tumor (6, 9)</td>
<td>53 ± 3</td>
<td>NSI</td>
<td></td>
</tr>
<tr>
<td>A375 Primary tumor (16)</td>
<td>32 ± 2</td>
<td>68 ± 3</td>
<td></td>
</tr>
<tr>
<td>SH-4 Metastasis, pleural fluid (33)</td>
<td>36 ± 3</td>
<td>NSI</td>
<td></td>
</tr>
<tr>
<td>Hs294 Metastasis, lymph node (6, 9)</td>
<td>32 ± 1</td>
<td>(−100)^d^</td>
<td></td>
</tr>
<tr>
<td>Hs939 Metastasis, mediastinum</td>
<td>30 ± 2</td>
<td>62 ± 4</td>
<td></td>
</tr>
<tr>
<td><strong>Breast carcinoma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-MB-157 Metastasis, pleural effusion (38)</td>
<td>44 ± 4</td>
<td>NSI</td>
<td></td>
</tr>
<tr>
<td>734B Metastasis, pleural effusion (34)</td>
<td>67 ± 3</td>
<td>50 ± 2</td>
<td></td>
</tr>
<tr>
<td>SK-BR-3 Metastasis, pleural effusion (15)</td>
<td>68 ± 4</td>
<td>83 ± 3</td>
<td></td>
</tr>
<tr>
<td>Hs578T Primary carcinoma (17)</td>
<td>48 ± 2</td>
<td>28 ± 5</td>
<td></td>
</tr>
<tr>
<td><strong>Nonmalignant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hs578Bst Breast myoepithelial tissue (17)</td>
<td>83 ± 7</td>
<td>NSI</td>
<td></td>
</tr>
<tr>
<td>HBL-100 Epithelial cell from human milk (29)</td>
<td>32 ± 2</td>
<td>13 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

^a Mean ± S.E. of data obtained in 3 independent experiments, each performed in duplicate.

^b The assay for growth inhibition is described in "Materials and Methods." Values are the mean ± S.E. of at least duplicate flasks. Similar data were obtained in 3 independent experiments.

^c NSI, no significant inhibition (<10% inhibition of growth).

^d The growth of Hs294 was not inhibited by retinoic acid; instead, cell proliferation was enhanced, and on Day 7 twice as many cells were counted in retinoic acid-treated cultures as in control cultures.

^e This cell line was derived from a metastatic melanoma of a 24-year-old female (Caucasian) in 1977 at the Naval Biosciences Laboratory, Oakland, Calif.
and SK-BR-3 was inhibited by at least 50% at $10^{-8}$ M retinoic acid after a 7-day incubation, the effects of higher and lower concentrations of retinoic acid on cell growth were determined. Cells were cultured in the presence of increasing retinoic acid concentrations in the range $10^{-10}$ to $10^{-5}$ M, and growth inhibition was determined at the end of a 7-day incubation (Charts 1 and 2). Growth inhibition was concentration dependent over a range from $10^{-9}$ to $10^{-6}$ M for most of the cells. However, great differences were noticed in the dose-dependent sensitivity of the various cell lines to retinoic acid. At $10^{-6}$ M, the growth of SK-BR-3, Hs939, and A375 was inhibited by 50, 26, and 21%, respectively, whereas the growth of 734B was not affected. At $10^{-5}$ M retinoic acid, the growth of SK-BR-3, A375, 734B, and Hs939 was inhibited by 90, 86, 68, and 56%, respectively. The concentrations of retinoic acid required for 50% growth inhibition of cell lines SK-BR-3, A375, Hs939, and 734B were $1 \times 10^{-9}$, $2.5 \times 10^{-8}$, $1 \times 10^{-7}$, and $3 \times 10^{-7}$ M, respectively.

**Time Course of Retinoic Acid-induced Growth Inhibition.**

The growth curves of the various cell lines in the absence and in the presence of $10^{-6}$ M retinoic acid are shown in Charts 3 and 4. The number of cells in retinoic acid-treated
and in control cultures was almost identical for the first 48 or 72 hr; this indicated that the plating efficiencies and the initial growth rates of the treated cells were not decreased by retinoic acid. However, longer exposure to the drug resulted in decreases in the growth rates of the various cell lines such that the doubling times of A375, Hs939, and 734B changed from 32, 30, and 67 hr to 48, 65, and 180 hr, respectively. The growth of SK-BR-3 cells in the presence of retinoic acid was inhibited in a different pattern; after 48 hr in culture, cell growth was completely arrested, and the total cell number per culture decreased from 0.4 to 0.25 × 10^6 due to cell death.

**Effect of Retinoic Acid on Cell Morphology.** All but 2 of the cell lines tested in this study retained their normal morphology during their growth in the presence of 10^-8 M retinoic acid. The breast carcinomas 734B and SK-BR-3 exhibited morphological changes after exposure to retinoic acid for longer than 72 hr (Fig. 1). At this time, the cells had spread and assumed a more flattened morphology. In addition, the flattened cells were also more strongly attached to the substratum than were control cells, and longer incubation with trypsin was required to detach them.

**DISCUSSION**

The data presented in this paper show that growth inhibition by retinoic acid was observed in only 2 of 6 melanomas and 2 of 4 breast carcinomas. It is difficult to explain the diverse response to retinoic acid among human cell lines derived from tumors of similar histopathological type. Previous studies have described differences in responsiveness to anticancer drugs among patients with cancers of identical type and among cultured cells derived from these cancers (32). Furthermore, it was found that several cell lines of human malignant melanoma, all isolated from a single biopsy sample, expressed different in vitro survival sensitivities to several cytotoxic and cytostatic drugs (2,4).

We have observed heterogeneity in growth inhibition by retinoic acid of metastatic murine B16 melanoma clones and in vivo-selected cell variants. Several possible explanations were suggested for the differential sensitivities of various rat hepatoma cell lines to anticancer drugs; these included alterations in cell transport of the drug, variability in the drug conversion to an "inactive" form or inability of the cells to "activate" the drug, and finally the presence of alternative biochemical pathways to overcome the drug-inhibited step (3). All these could also be plausible explanations for the differential responsiveness of the human cell lines to retinoic acid. Since several studies have detected specific intracellular, retinoic acid-binding protein in human lung and breast carcinomas, it has been suggested that the antitumor activity of retinoic acid may be mediated via this cellular protein (8, 27). Therefore, differences in sensitivity to retinoic acid may result from variations in the level of the binding protein, and resistance could represent total lack of such a protein.

The stimulation of the proliferation in cultures of the melanoma cell line Hs294 exposed to retinoic acid was not expected, since we had not encountered a similar effect in our previous studies with numerous cell lines (23). However, preliminary results have been reported which described enhancement of DNA synthesis by approximately 60% at 6 hr after a 30-min exposure of human HeLa and CV-1 cells to 10^-8 M retinoic acid (20).

Evaluation of the inhibitory effect of retinoic acid on the growth of the melanoma cell lines A375 and Hs939 and on the breast carcinoma cell lines SK-BR-3 and 734B indicates that inhibition was achieved at low and noncytotoxic retinoic acid concentrations, was dependent on drug concentration, and was detectable only after the cells had been exposed to retinoic acid for 48 or 72 hr. This was long before the treated cells reached high densities. Analysis of the growth curves of cell lines A375, Hs939, and 734B in the absence and in the presence of 10^-8 M retinoic acid suggests that, after 48 to 72 hr, retinoic acid induced slowing of the growth rate. Since retinoic acid altered the slope of the growth curves and the doubling times, it follows that the inhibitory effects were due to a uniform decrease in the growth rates of most, if not all, of the cells in the population (18). This pattern of growth inhibition is almost identical to the retinoic acid inhibition of the growth of the murine melanoma lines B16 and S91 (22). However, inhibition of the breast carcinoma cell line SK-BR-3 appears to be completely different from the above pattern of growth inhibition. The growth of the SK-BR-3 cells is completely arrested after 48 hr of exposure to retinoic acid.

The flattened morphological appearance induced by retinoic acid in cultures of 734B and SK-BR-3 breast carcinoma cell lines is reminiscent of similar changes that were described for spontaneously transformed mouse 3T12-3 (1, 11) and virally transformed hamster NILPy fibroblasts (28). However, in the latter 2 studies, cell growth slowed in the presence of retinoic acid only after the cells attained confluence at a lower saturation density than did untreated cells, whereas the growth of the human breast carcinoma cell lines tested here were inhibited even at very low cell density. The changes in cell morphology after exposure to retinoic acid and the increased adherence to the substratum may be the result of retinoic acid-induced changes in glycolipid and/or glycoprotein synthesis or their exposure on the cell surface (1, 11, 28).

Clinical trials in man using topically or p.o. administered retinoic acid demonstrated its therapeutic value in the treatment of actinic keratoses and basal cell carcinomas (5), urinary papillomas (13), and leukoplakias of the oral cavity (31). More recently, it was reported that retinoic acid was effective in therapy of unresectable metastatic squamous cell carcinoma of the lung in humans (25). In the latter report, it was concluded that the antitumor effect was a combination of stimulation of the immune response and direct effect on the tumor tissue itself (25). The studies described here indicate that each tumor may respond differently to retinoic acid therapy. This could limit the use of the drug to those tumors which will be found to be sensitive to retinoic acid in vitro. Further studies on the mechanism of growth inhibition in culture and in immuno-suppressed experimental animals will be required before it can be established whether in vitro susceptibility to retinoic acid of a human melanoma or breast carcinoma may be indicative of a potentially effective chemotherapy.

3 R. Lotan and G. L. Nicolson, submitted for publication.
ACKNOWLEDGMENTS

The support and encouragement of Dr. Garth L. Nicolson and the excellent technical assistance of Dafna Lotan are gratefully acknowledged. I am also greatly indebted to Dr. Adefine Hackett, Dr. Abia Creasey, Dr. Martha Stamper, and Dr. Helene Smith of the Perlat Caneer Research Institute and to Dr. Walter Nelson-Rees and John Weaver of the Naval Biosciences Laboratory for generously providing the cells and expert advice. The cells were produced with support from the National Cancer Institute, Viral Oncology Program, under the auspices of the Office of Naval Research and the Regents of the University of California. My thanks are also extended to Dr. Beverly Pawson of Hoffmann-La Roche Inc. for supplying the retinoic acid, to Tony Neri for help with the photography, and to Adele Brodginski for assistance in preparation of the manuscript.

REFERENCES

Fig. 1. Morphological changes in breast carcinoma cells after a 7-day exposure to retinoic acid (10^{-6} M). SK-BR-3 cells cultured in control growth medium (a) or in the presence of retinoic acid (b). 734B cells cultured in control growth medium (c) or in the presence of retinoic acid (d). Phase contrast, ×200.
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