[CANCER RESEARCH 39, 4767-4771, December 1979]
0008-5472/79/0039-0000$02.00

Heterogeneity in Growth Inhibition by β-*trans*-Retinoic Acid of Metastatic B16 Melanoma Clones and *in Vivo*-selected Cell Variant Lines[1]

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ABSTRACT

A malignant B16 melanoma parental cell line, four unselected clones derived from this line which possess varying lung colonization potentials (clones 9 and 14 > parental B16 > clones 12 and 15), four variant cell lines selected *in vivo* for increased ability to colonize preferentially lungs (B16-F10 > B16-F1, parental B16), brain (B16-B10n > B16-F1, parental B16), or ovaries (B16-O10 > B16-F1, parental B16), and a line selected *in vitro* for resistance to lymphocyte cytotoxicity (B16-F10^{Lr-6}) have been examined in culture for their susceptibilities to growth inhibition by retinoic acid. The proliferation of all clones and cell lines was inhibited in the presence of noncytotoxic concentrations of retinoic acid (10^{-5} and 10^{-6} M); however, the extent of inhibition varied significantly. The parental B16 line and parental clone 14, as well as the *in vivo*-selected variant cell lines B16-F1 and B16-B10n, were extremely sensitive to retinoic acid, and their growth was inhibited by 75 to 90% after a 5-day incubation in the presence of 10^{-5} M retinoic acid. Under similar conditions, the growth of parental clones 9, 12, and 15 and of selected cell lines B16-F10, B16-F10^{Lr-6}, and B16-O10 was inhibited only moderately (40 to 60%). A more striking difference in sensitivity was observed when cells were exposed to a low retinoic acid concentration (10^{-9} M). While the parental B16 line and B16-F1 were inhibited by nearly 40%, parental clones 12 and 14 and selected cell line B16-F10^{Lr-6} were inhibited by about 20%, and parental clones 9 and 15 and lines B16-B10n, B16-O10, and B16-F10 were not significantly affected (0 to 10% inhibition). These results demonstrate that the parental B16 melanoma line is heterogeneous with respect to sensitivity to retinoic acid and includes some cell variants with reduced susceptibility to the drug.

INTRODUCTION

The metastasis of tumors is one of the most troublesome facets of cancer and is often responsible for inefficiency of postsurgical therapy. Fidler (12) has suggested that efforts to design effective therapeutic agents and protocols should be directed toward the few but fatal metastatic cell subpopulations that exist within a malignant tumor and has proposed that highly metastatic clones obtained from tumor cell lines and variant lines selected *in vivo* for enhanced metastatic potential could be useful for testing new therapeutic approaches. In order to obtain malignant cell lines of differing metastatic potential, Fidler (11) utilized a murine B16 melanoma line with moderate *in vivo* metastatic potential (defined by the number of experimental pulmonary tumor colonies that formed after i.v. administration of single-cell suspensions of B16 cells) to select variants from tumor cell populations with high lung colonization abilities.

Starting with the unselected, uncloned B16 melanoma cell line of moderate *in vivo* metastatic potential (parental B16), variant lines were selected for their abilities to implant, invade, survive, and grow to form gross lung tumor colonies after i.v. injection into syngeneic C57BL/6 mice. After one selection for lung colonization, a line was obtained (B16-F1) which, when subjected to 9 further *in vivo* selections, eventually yielded a more metastatic line[4] (B16-F10) compared to either parental B16 or B16-F1 melanoma lines (11). Using a similar selection approach, Brunson and Nicolson obtained variant B16 lines with increased propensity to colonize brain (B16-B10n) (5, 6) or ovaries (B16-010) (26).

Clonal variability in the phenotypic properties of the parental B16 melanoma line has been proposed to account for the success in selecting highly metastatic variants from the initial cell population (12, 14, 15, 25, 26). That this heterogeneity in phenotypic properties preexisted in the unselected parental tumor cell population has been shown by *in vitro* cloning experiments where clones of widely differing metastatic potential have been obtained from unselected tumor lines (12, 14, 25, 26).

Retinoids, analogs of vitamin A, have been used as potential anticarcinogenesis and antitumor agents. Their ability to prevent or delay the development of chemical carcinogen-induced epithelial tumors has been demonstrated in several studies (for reviews, see Refs. 22, 30, and 31), but there are only a few reports on retinoid effects on transplantable animal (3, 10, 28, 32) or spontaneous human (4, 24, 27) tumors. The mechanisms of retinoid inhibition of carcinogenesis and tumor growth are unknown; however, it has been suggested that they may enhance or induce differentiation processes in premalignant or malignant cells. An indirect effect on tumor growth mediated by retinoid stimulation of the immune response of the host has also been considered (10, 19, 24).

We have found that retinoids inhibit the proliferation of a variety of transformed and tumor cells in culture by decreasing their growth rates (18, 20, 21). In an attempt to understand the mechanism of retinoid antitumor activity, subsequent studies were performed with the highly sensitive murine B16 and S91 melanoma cell lines (20). This investigation demonstrated that growth inhibition by retinoic acid was time and dose dependent and resulted from a decrease in growth rate (20). In the present investigation, we have examined the ability of retinoic acid to inhibit the proliferation of the parental B16 melanoma as well as clones and *in vivo*-selected variant cell lines of varying metastatic potentials derived from the parental B16 cell line.

[1] Supported by USPHS Grants R01-CA-22823 and R01-CA-15122 and Contract N01-CB-74153 from the National Cancer Institute.

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Received October 5, 1978; accepted August 21, 1979.

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We report that, although all of the clones and cell lines tested were inhibited by retinoic acid, differences existed in the *in vitro* sensitivities of these cell lines to growth inhibition by the drug.

MATERIALS AND METHODS

Cells and Culture Techniques. All of the cells used in this study were derived from a single cell line (parental) established by Dr. I. J. Fidler (National Cancer Institute-Frederick Cancer Research Center, Frederick, Md.) from the transplantable malignant murine B16 melanoma. The properties and derivation of the different clones and cell variants are described in Table 1. The metastatic cell lines B16-B10n and B16-O10 were developed by K. W. Brunson in our laboratory from cell line B16-F1 obtained from Dr. Fidler (5, 6, 26), and the other clones and cell lines were the gift of Dr. Fidler. All the experiments were carried out with low passage cells (<10) cultured in 10-cm-diameter tissue culture dishes (Falcon Plastics, Oxnard, Calif.) with Dulbecco's modified Eagle's minimal essential medium containing 10% heat-inactivated fetal bovine serum (Flow Laboratories, Inglewood, Calif.), nonessential amino acids, and 50 μg gentamicin per ml. Cultures were incubated at 37° in a humidified atmosphere of 8% CO_2:92% air. For passage and enumeration, cells were detached with 2 mM EDTA in a phosphate-buffered NaCl solution, containing (per liter H_2O): 8.0 g NaCl, 0.2 g KCl; 1.15 g Na_2HPO_4; and 0.2 g KH_2PO_4. Cell counting was performed using an electronic particle counter (Electrozone/Celloscope Model 112 ct; Particle Data, Inc., Elmhurst, Ill.).

The doubling time of the various cell lines was determined after following their growth from an inoculum of 5×10^4 cells/dish to confluence (1 to 2×10^7 cells/10-cm dish) during 5 days with medium changes on alternate days (20, 21).

Viability was determined from the proportion of cells excluding trypan blue (0.1%), and plating efficiency was calculated from the ratio of the number of colonies counted after 5 days in culture to the initial number of cells plated on Day 0.

Treatment of Cells with Retinoic Acid. All-*trans*-β-retinoic acid, a gift from Dr. B. Pawson of Hoffmann-La Roche Inc. (Nutley, N. J.), was dissolved in ethyl alcohol to obtain a series of 10-fold decreasing concentrations from 10^{-2} to 10^{-6} M. These solutions were stored at −20° for up to 1 week under N_2. Immediately before each experiment, retinoic acid solutions were diluted 1:1000 into the culture medium such that the final alcohol concentration was 0.1%. Control cultures received medium containing 0.1% alcohol. All procedures were carried out in subdued light (20, 21).

Assay for Growth Inhibition. Cells were plated at 5×10^4 cells/dish in 10 ml medium in the absence or in the presence of retinoic acid (at concentrations ranging from 10^{-9} to 10^{-5} M). Medium was changed on Day 3, and the cells were detached and counted on Day 5. The percentage of growth inhibition by each retinoic acid concentration was calculated from the equation:

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

where *R* and *C* are the numbers of cells in retinoic acid-treated and in control cultures, respectively (20, 21).

Assay for Inhibition of Colony Growth. Cells from the same culture of B16-F1 were plated at 10^2, 10^3, or 10^4 cells/dish in a series of 6-cm-diameter dishes with 5 ml growth medium in the absence or presence of 10^{-5} M retinoic acid. At 24-hr intervals thereafter, duplicate cultures at the different cell densities were removed from incubation, the cells were fixed and stained (Diff-Quik; Harelco, Gibbstown, N. J.), and the number of cells per colony was determined. Under these conditions, there was no decrease in the plating efficiencies of cells plated in the presence compared to the absence of retinoic acid. Percentage of growth inhibition was calculated as:

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

where *R* and *C* are the number of cells per colony in retinoic acid-treated and control cultures, respectively.

RESULTS

Effect of Retinoic Acid on the Proliferation of B16 Melanoma Cell Lines in Culture. The metastatic behaviors of the B16 cell lines are listed in Table 1. In culture, these cell lines proliferate rapidly with a doubling time of 14 to 16 hr, except for the lines B16-B10n and B16-O10 which multiply at somewhat slower rates (Table 1). The plating efficiencies of the different B16 cell lines are 70 to 80%.

Subculturing the B16 cell lines in the presence of 10^{-5} M retinoic acid did not decrease their viabilities or plating efficiencies; instead, incubation with retinoic acid causes reductions in the growth rates of all of the cell lines. Some of the cell lines seem to be less affected by the drug. At the end of a 5-day exposure to 10^{-5} M retinoic acid, the parental B16 line, B16-F1, parental clone 14, and B16-B10n are strongly inhibited (75 to 90%), whereas the other cell lines and parental clones are only moderately growth inhibited (Table 1). Cell viabilities at the end of this treatment are similar to those found in untreated cultures, suggesting that 10^{-5} M retinoic acid is not cytotoxic. The sensitivity of B16-F1 cells to retinoic acid-induced growth inhibition remained stable and did not change by more than 10% during weekly subculture in the continuous presence of 10^{-5} M retinoic acid for 3 months.

To further characterize the differences in the sensitivity of the various cell lines to retinoic acid, we examined their growth in the presence of decreasing concentrations of the drug. As observed previously (20), growth inhibition by retinoic acid is concentration dependent, and, even at very low concentrations, growths of some of the cell lines are dramatically inhibited. However, certain cell lines are not affected (Chart 1). At the lowest retinoic acid concentration tested (10^{-9} M), where the growths of the parental line and B16-F1 are still inhibited by nearly 40%, the growths of parental clones 12 and 14 and line B16-F10$^{Lr\text{-}6}$ are inhibited by about 20%, and parental clones 9 and 15 and lines B16-O10, B16-B10n, and B16-F10 are not significantly inhibited. The dose-response relationships are quite linear for most cell lines except B16-F10n, which is insensitive at the lowest retinoic acid concentration and very sensitive at the higher concentrations. A comparison of the retinoic acid concentrations required for 50% growth inhibition of the various B16 cell lines (Table 1) indicates that differences in the drug sensitivities are much greater than the 2-fold values obtained when percentage of growth inhibition is monitored at high (10^{-6} to 10^{-5} M) retinoic acid concentrations. By this

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Table 1

Characteristics of clones and variant cell lines of metastatic B16 melanoma

B16 melanoma line	Source	Metastatic potential	Organ colonization preference	Doubling time in culture (hr)	% of growth inhibition by 10^{-5} M retinoic acid	Approximate concentration (μM) required for 50% growth inhibition[a]
Parental	s.c. C57BL/6 tumor established in culture (11, 12)	Moderate	Lung > lymph node > ovary > liver > kidney	14–16[b]	88 ± 4[c]	0.01
Clone 12	*In vitro* cloning of the parental B16 line (14)	Low	Lung	13–14	62 ± 3	0.3
Clone 15		Low	Lung > lymph node	14–15	40 ± 2	>10.0
Clone 9		High	Lung ≫ lymph node > brain	14–15	51 ± 3	1.0
Clone 14		High	Lung ≫ ovary	14–15	85 ± 3	0.06
F1	B16 parental selected once *in vivo* for lung colonization (11)	Moderate	Lung > lymph node > brain > ovary	14–16	89 ± 4	0.01
F10	B16 parental selected 10 times *in vivo* for lung colonization (11)	High	Lung	14–16	42 ± 4	>10.0
$F10^{Lr-6}$	B16-F10 selected for resistance to lymphocyte-mediated cytotoxicity *in vitro* (13)	Low	Lung	14–15	54 ± 3	1.0
B10n	B16-F1 selected 10 times *in vivo* for brain colonization (5, 6)	Moderate	Brain	17–18	75 ± 3	0.07
O10	B16-F1 selected 10 times *in vivo* for ovary colonization (26)	High	Ovary > lung	17–19	48 ± 2	10.0

[a] Calculated from curves presented in Chart 1.
[b] Range of doubling times obtained in 3 independent experiments.
[c] Average ± S.E. of duplicate cultures. Similar results were obtained in 3 independent experiments.

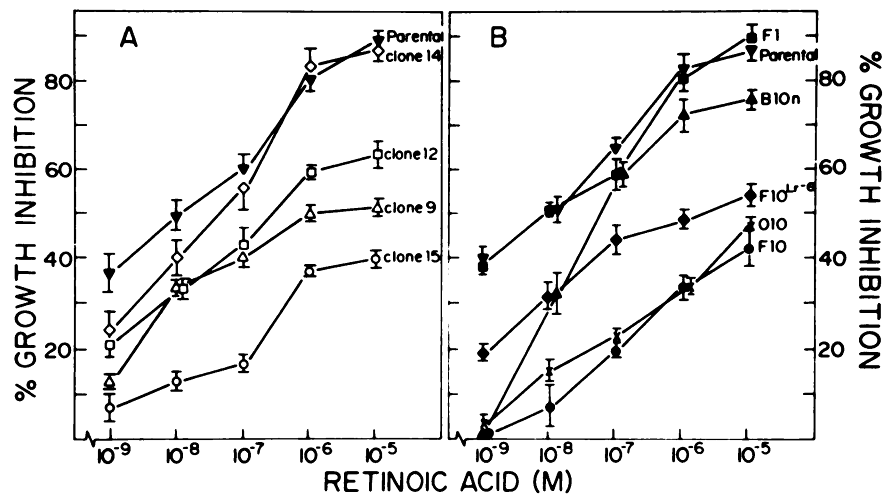

Chart 1. Dose-response relationship of retinoic acid-induced growth inhibition of B16 melanoma parental clones (*A*) and *in vivo*-selected cell variants (*B*). Values represent the average of duplicate cultures from 3 independent experiments; *bars*, S.E.

criterion, the ratio of sensitivities of the most sensitive to the most resistant lines is about 1000.

Effect of Retinoic Acid on the Growth of Individual Colonies of B16 Melanoma Cells. The heterogeneity observed in the response to retinoic acid among the various *in vivo*-selected B16 cell lines is greater than that expected from cells originating from a sensitive population. To test whether this heterogeneity can be readily observed among subpopulations of the cell line from which *in vivo* selections were derived, we examined the effect of retinoic acid on the proliferation of cells within randomly selected colonies of B16-F1 (Table 2). As previously observed (20), growth inhibition by retinoic acid becomes apparent after 3 days of treatment. The small standard error of the mean number of cells per colony in cultures exposed to retinoic acid for 4 or 5 days indicates that most colonies consist of cells that are similarly inhibited by retinoic acid. However, the range of values suggests the presence of a few, less sensitive cell subpopulations.

DISCUSSION

In previous studies, we found a wide range of responses to retinoic acid-induced growth inhibition among malignant cell lines of different or similar histopathological types (18, 20, 21). Dion *et al.* (9) recently reported that continuous treatment with retinoids can inhibit the formation of clones (in semisolid medium) from certain established tumor cell lines. Meyskens and Salmon (23) found that a brief (1-hr) exposure to 10^{-5} M of several retinoids reduces the ability of fresh metastatic human melanoma cells to form colonies in soft agar. Although the sensitivities to retinoids vary from patient to patient, the authors suggest that sensitivity to retinoids *in vitro* may be used to

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Table 2
Inhibitory effects of retinoic acid on B16-F1 colony growth

Days in culture	No. of cells/colony				% of inhibition of colony growth
	Control		Retinoic acid		
1	3.2 ± 1.2[a]	(1-6)[b]	3.3 ± 1.2	(1-6)	0
2	7.0 ± 1.7	(4-12)	6.8 ± 1.9	(4-12)	0
3	15.6 ± 2.1	(8-22)	11.2 ± 2.2	(8-16)	28.2
4	43.6 ± 13.2	(20-66)	16.8 ± 3.0	(8-20)	61.5
5	103.5 ± 20.7	(61-138)	18.7 ± 3.5	(12-56)	81.9

[a] Mean ± S.E. in 50 individual colonies in each duplicate culture.
[b] Numbers in parentheses, range.

select patients with malignant melanoma for clinical trials in the adjuvant or metastatic setting (23). We used a unique collection of clones and variant cell lines with different metastatic properties (but all derived from the same parental murine B16 melanoma population) and found heterogeneous responses to retinoic acid. No correlation was noted between the metastatic potentials of the B16 cell lines and their sensitivities to retinoic acid. For example, the highly metastatic parental clone 14 was nearly as sensitive to retinoic acid as was the parental line, whereas clone 15 and cell line B16-F10 were considerably less sensitive than were the parental line and B16-F1.

The finding that various unselected clones exhibit a range of retinoid sensitivities indicates that heterogeneity in the response of the *in vivo*-selected variant cell lines was not introduced during the selection process. Our observations that cloning and selection yielded a relatively high proportion of less sensitive clones and variants derived from sensitive parental cell population may have been fortuitous, since analysis of growth inhibition in numerous individual colonies of B16-F1 cultures demonstrated that most of the colonies consist of cells which are similar in inhibition.

The mechanism by which retinoic acid inhibits the proliferation of certain tumor cell lines including B16 melanoma is not yet understood. Chytil and Ong (7) suggested that the antitumor activity of retinoic acid may be mediated via specific intracellular retinoic acid-binding proteins. The presence of such a binding protein was recently detected in s.c.-grown B16 melanoma and in lungs of mice bearing B16 melanoma metastases (29). This finding raised the possibility that heterogeneity in the response of the various B16 melanoma cell lines to retinoic acid is due to different levels of intracellular retinoic acid-binding protein. Quantitative analysis of B16-F1 cell homogenates for intracellular binding proteins revealed the presence of approximately 0.08 pmol of retinoic acid-binding protein per 10^6 cells and no detectable retinol-binding protein.[3] The very low level of retinoic acid-binding protein in the B16-F1 cell line, which is very sensitive to retinoic acid, renders it difficult to determine whether variations in the levels of retinoid-binding proteins account for differential drug sensitivity.

Previous studies have encountered heterogeneity among subpopulations or clones of various tumors with respect to their properties *in vivo* and in culture (see discussion in Refs. 8 and 12), including differences in susceptibility to cytotoxic drugs (1, 2, 17). However, to our knowledge, the present study is the first to demonstrate heterogeneity in drug response among clones as well as variant cell lines, which exhibit distinct metastatic properties. In spite of differences in susceptibilities to retinoic acid, all of the B16 cell lines tested were significantly inhibited at the higher (10^{-6} to 10^{-5} M) retinoic acid concentrations which are pharmacologically achievable (16). Therefore, our results do not negate the potential applications of retinoids for therapy of certain tumors. They do suggest, however, that heterogeneity in drug responses among highly malignant subpopulations of a tumor may limit the successful *in vivo* application of *in vitro* drug testing. Further drug testing of cells from multiple metastatic sites may enhance the predictive value of *in vivo* responses.

[3] R. Lotan, D. Ong, and F. Chytil, unpublished data.

ACKNOWLEDGMENTS

We thank Dafna Lotan for excellent assistance and Adele Brodginski for help in preparing the manuscript.

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