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Journal
Cancer research, 39(10)

ISSN
0008-5472

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Publication Date
1979-10-01

Peer reviewed

[CANCER RESEARCH 39, 4055–4057, October 1979]
0008-5472/79/0039-0000$02.00

Inhibition of Human Melanoma Colony Formation by Retinoids[1]

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ABSTRACT

We studied the effects of retinoids on the *in vitro* survival of melanoma colony-forming cells in biopsies obtained from ten patients with metastatic melanoma. The results indicate that specific retinoids reduce the ability of fresh human melanoma cells to form colonies in soft agar. The retinoids studied had differential effects on the survival of clonogenic melanoma cells, and these effects vary from patient to patient. The data provide support for the clinical trial of selected retinoids in micrometastatic and advanced melanoma.

INTRODUCTION

Vitamin A has several important functions for normal differentiation of epithelial cells (6, 25, 26). Retinoids (vitamin A and its synthetic analogs) can also prevent the development of epithelial cancer in several organ sites in experimental animals by modifying preneoplastic states during the latent period of cancer development (25). Inhibitory noncytotoxic effects of β-all-*trans*-RA[3] on the proliferation of murine melanoma *in vitro* have been noted (13, 15). Short-term *in vitro* treatment of hamster and mouse fibroblasts with retinol results in marked changes in glycolipid synthesis patterns (17). These studies in animal systems suggest that retinoids have important biochemical and biological effects on cells. Two years ago, our research group reported a relatively simple soft-agar technique for direct cultivation of human tumor stem cells from a variety of neoplasms (8–10, 12), an approach which has recently been confirmed by at least 3 other groups (1, 20, 29). An analysis demonstrating applicability of the soft-agar culture system for melanoma colony formation was recently completed (16). We report here on the effect of retinoids on the survival of human melanoma colonies in this clonogenic assay system.

MATERIALS AND METHODS

Retinoids. Retinol and β-all-*trans*-RA were obtained from Sigma Chemical Co. (St. Louis, Mo.). An aromatic RA ethyl ester analog (RO-10-9359) and 13-*cis*-RA (RO-43780) were kindly provided by E. Miller of Hoffmann-LaRoche Inc. (Nutley, N. J.). All retinoids were suspended at 10^{-3} M in dimethyl sulfoxide and kept at 4° in light-protected tubes until just before use.

Patient Material. All patients (identified by code letter) had metastatic malignant melanoma confirmed by histopathological examination of the excised tissue studied. The 10 patients and sites of metastases studied were as follows: Patient A, lung; Patient B, inguinal node; Patient C, axillary node; Patient D, supraclavicular node; Patient E, axillary node; Patient F, subcutaneous nodule; Patient G, nasal mucosa; Patient H, axillary node; Patient I, inguinal node; and Patient J, skin nodule. This protocol has been approved by the University of Arizona Committee for the Protection of Human Subjects.

Cell Culture Techniques. Excisional biopsies of malignant melanoma were mechanically dissociated into a single-cell suspension and processed as described elsewhere (9, 16). Retinoid sensitivity was assessed with the same basic assay approach which our laboratory has previously applied for measurement of tumor stem cell sensitivity to cytotoxic anticancer drugs (24). The melanoma cell suspension was incubated with either 1.0×10^{-5} M or various concentrations of the appropriate retinoid at 37° for 1 hr. The cells were then washed twice with serum-free medium, and 5.0×10^5 cells were mixed in a 1.0-ml volume of 0.3% agar containing 10% heat-inactivated horse serum in enriched Connaught Medical Research Laboratories Medium 1066 and plated over a 1.0-ml nutrient feeder layer of 0.50% agar in 35-mm plastic Petri dishes. Conditioned medium was not required in the feeder layer.

Plates were incubated at 37° in a humidified atmosphere containing 6% CO_2 for 10 to 21 days. Melanoma colonies were counted when they reached the 30-cell stage, generally about 14 days. All control and drug assays were done in triplicate. Melanoma colonies from these patients all expressed melanin pigmentation, which served as a marker of neoplastic origin of the colony-forming cells (16). Morphology of the neoplastic melanoma cell colonies was further defined with our new dried-slide technique and a combination of Papanicolaou's and Lillie's melanin staining (23).

RESULTS

The effects of the retinoids on melanoma colony formation varied with the patient and the type of retinoid. Marked inhibition of melanoma colony formation was noted in some patients with different retinoids, but in no instance was survival of melanoma colony-forming cells reduced to less than 20% of the control. The effect of the different retinoids varied from patient to patient. Dose-response curves for patients A through E are presented in Chart 1.

Results for 10 Patients Tested at 10^{-5} M Concentration of Specific Retinoids. The effect of a single concentration of retinoid on colony formation was tested on cells obtained by biopsies from 10 patients with melanoma (Table 1). This concentration was selected because it is considered to be pharmacologically achievable. Although the mean inhibition by retinol was 49%, the effects on survival of colony formation ranged from 80% inhibition to mild stimulation in one case. Similar results were seen with the other 3 retinoids tested.

Effects of Retinoids on Human Melanoma Colony Formation: Dose-Response Curves. Retinol was tested in 2 patients, and survival of melanoma colony formation was reduced 70% (A) and 80% (E) at a concentration of 10^{-9} M, but a further

[1] This investigation was supported by NIH Grants CA 17094 and CA 21839.
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[3] The abbreviation used is: RA, retinoic acid.
Received February 20, 1979; accepted July 12, 1979.

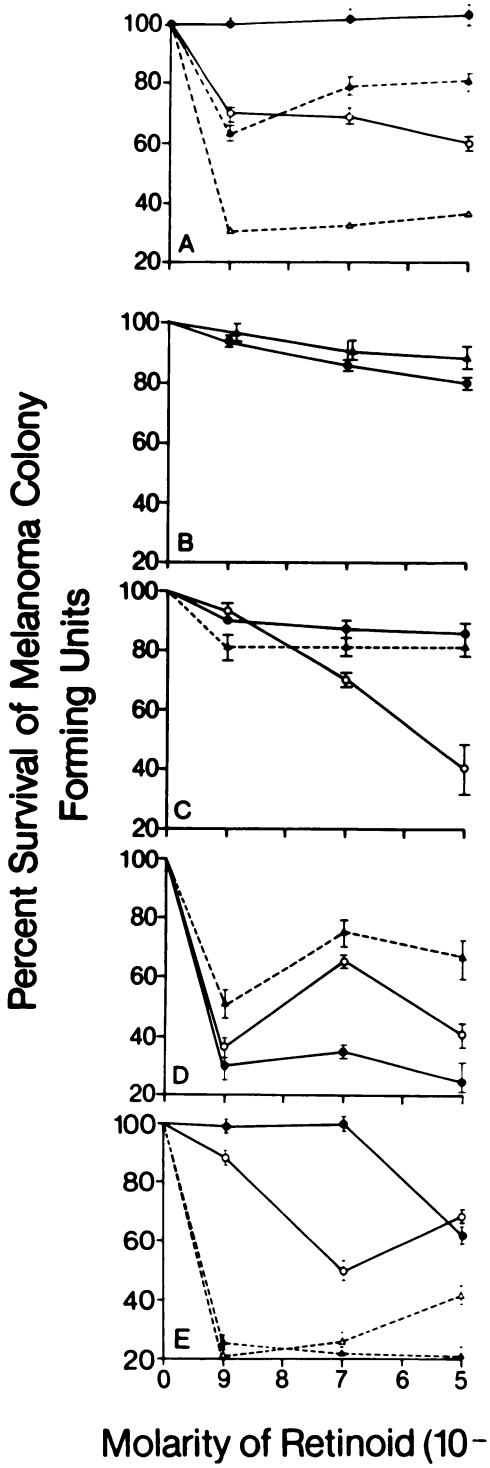

Chart 1. Effect of retinoids on human melanoma colony formation. ●, *cis*-RA (RO-43780); ○, β-all-*trans*-RA; ▲, aromatic RA ethyl ester analog (R-10-9359); and △, retinol.

decrease in survival was not achieved at higher concentrations.

β-All-*trans*-RA was tested in 4 patients, and 3 different types of dose-response curves were observed over the dose range tested. In 2 patients, survival of melanoma colonies was reduced at a concentration of 10^{-9} M (Patient A, 30%; Patient D, 60%), but a plateau occurred where no further decrease in survival was achieved at higher concentrations. In one patient (Patient C), a dose-dependent effect was evident, and survival of melanoma colonies was reduced 60% at the highest concentration (10^{-5} M) used. In Patient D, a dose dependence at lower concentrations and plateau at a higher concentration was evident.

13-*cis*-RA was tested in all 5 patients, and 3 different types of survival curves were seen. In 3 patients (Patient A, B, and C), no reduction (20%) in survival was observed. In one patient (Patient E), no reduction in survival was seen except at the highest concentration tested (10^{-5} M, 40% reduction in survival). However, in one patient (Patient D), survival was reduced at 10^{-9} M, but survival was not further reduced at higher concentrations.

Aromatic RA ethyl ester analog was tested in 5 patients, and 2 different types of effects on melanoma colony formation were seen. In 2 patients (Patients B and C), no response was seen. In 3 patients, melanoma colony survival was reduced at low concentrations, but survival was not further reduced at higher concentrations (Patient A, 30 to 40% decrease; Patient D, 30 to 50% decrease; Patient E, 75 to 80% decrease).

Thus, in the multiple concentrations studied, 4 different types of general responses were noted: no response at any concentration; a response only at high concentrations; a concentration dose-dependent effect; and a response at low concentrations with no further reduction in tumor colony formation with increasing molarity of the retinoid. Additionally, the specific retinoid causing one or more of these responses varied from patient to patient.

DISCUSSION

The studies reported here indicate that retinoids can reduce the ability of fresh human melanoma cells to form colonies in soft agar and that differential sensitivities are expressed. This observation indicates that melanoma colony formation is heterogeneous with respect to responsiveness to retinoids. Whether these effects on the cells capable of forming melanoma colonies are working through the same or different mechanisms, or on different groups of cells has yet to be established. We have yet to study effects of combinations of these agents, which may partially answer this question.

Table 1

Percentage of survival of human melanoma colony formation after retinoid exposure at 10^{-5} M[a]

	Retinoid			
Patient	Retinol	13-*cis*-RA	All-*trans*-RA	Aromatic RA ethyl ester analog
A	33	105	60	80
B		81		91
C		88	35	81
D		24	40	67
E	41	61	64	21
F	46	65		
G	20			46
H	45		18	
I	38	45	97	
J	133		113	
Mean survival	51 ± 3.7[b]	67 ± 2.7	61 ± 3.4	64 ± 2.6

[a] Based on the available published data, this concentration of retinoid is a pharmacologically achievable level (5) and produces biological effects (18).
[b] Mean ± S.D.

Retinoids are not conventionally considered to be directly cytotoxic to normal or tumor cell populations (13, 15, 19, 25). Either the retinoids have cytotoxic effects on a small subpopulation of sensitive cells which have clonogenic properties, or are altering some fundamental property necessary for cells to clone *in vitro*. The exposure time to the retinoid is short (1 hr), which suggests that the retinoids induce a long-standing change in the proliferative capacity of the sensitive population of clonogenic melanoma cells. The mechanism responsible for this effect has yet to be established.

Changes in RNA metabolism after prolonged retinoid treatment have been studied in several systems (2, 27, 30). We have recently examined the short-term (1 hr) effects of retinol, *trans*-, *cis*-, and aromatic RA on human melanoma cell lines, and find rapid and profound effects on labeled precursor incorporation into RNA and protein.[4] Ornithine decarboxylase, a possible regulator of RNA metabolism, has been noted to change rapidly in response to retinol (7) and conceivably could be playing an important role. Alternatively, alterations in surface proteins induced by retinoids (17) may lead to changes in cloning ability.

While the *in vivo* relevance of these observations remains to be established, a wide variety of retinoids are known to interact directly with diverse normal and malignant cell types (4, 19). For example, retinoids have been noted to inhibit the growth and development of certain transplantable tumors, including rat chondrosarcomas (11, 28), murine mammary adenocarcinoma (19), and murine S91 melanoma (4). Also, these agents increase host-immune responses (4), possibly acting through stimulation of T-killer cells (3, 14). Additionally, direct effects of retinoids on the *in vitro* proliferation of murine melanoma cell lines S91 and B16 have been noted (13, 15), which supports our direct observations on human melanoma.

In prior studies with known cytoxic agents (*e.g.*, melphalan, Adriamycin), our group has reported excellent correlation between *in vitro* sensitivity or resistance in this agar culture assay system and clinical response *in vivo* (24). We have recently made similar correlative observations in melanoma patients with cytotoxic drugs (16, 21, 22). Therefore, the results of the current *in vitro* studies showing marked inhibitory effects of relatively low doses of selected retinoids on human melanoma colony formation provide evidence in support of initiating clinical trials of selected retinoids in patients with malignant melanoma. In view of the fact that retinoids are already on trial as chemopreventative agents in normal subjects, their use in patients with known diagnosed cancer in the adjuvant or metastatic setting appears most reasonable. Such trials would ideally be carried out in conjunction with *in vitro* study, so that retinoids which we would predict to have clinical activity in specific patients would be selected and the predictive capability of this assay system for retinoids could be directly tested.

ACKNOWLEDGMENTS

We thank B. Soehnlen and L. Young for excellent technical assistance and E. Eifrig for secretarial preparation of this manuscript.

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Cancer Res 1979;39:4055-4057.