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# Characterization of the Effects of Different Retinoids on the Growth and Differentiation of a Human Melanoma Cell Line and Selected Subclones<sup>1</sup>

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## ABSTRACT

The effect of four different retinoids [retinol, 13-*cis*-retinoic acid (Ro4-3780),  $\beta$ -all-*trans*-retinoic acid, and aromatic retinoic acid ethyl ester (Ro10-9359)] on the cellular proliferation (cell number) and biochemical differentiation (tyrosinase activity) of a human melanoma cell line (MIRW) and three subclones was assessed. All four retinoids ( $10^{-6}$  M) inhibited the cellular proliferation (36 to 42%) and stimulated tyrosinase activity (58 to 72%) in the parent cell line to a similar extent. In contrast, the effects of the different retinoids on three derived melanoma clones was dissimilar. For example, in clone A6,  $\beta$ -all-*trans*-retinoic acid stimulated tyrosinase activity by 48% but caused only a 7% inhibition of cellular proliferation. This retinoid caused a more pronounced effect in the other two subclones, stimulating tyrosinase from 135 to 195% and inhibiting growth 19 to 33%. All three melanoma clones demonstrated increased tyrosinase activity (110 to 225%) and reduced proliferation (37 to 52%) following exposure to 13-*cis*-retinoic acid. This retinoid was found overall to be the most effective stimulator of tyrosinase, while retinol was observed to be the least active, stimulating enzyme activity slightly (25%) in only one of three clones. Retinol inhibited proliferation 27 to 33% in two of three melanoma subclones. The aromatic retinoic acid ethyl ester elevated tyrosinase levels in two clones but inhibited the enzyme in one melanoma line. Cellular proliferation, however, was reduced in all three clones. These results suggest that retinoid-induced changes in human melanoma cell growth and differentiation reflect underlying cellular differences and diverse biochemical interactions.

## INTRODUCTION

Vitamin A and its derivatives (retinoids) are compounds which block the promotion of cell transformation initiated by a variety of agents (8, 24, 26, 28) and reverse preneoplastic lesions (6, 24, 26) in sensitive target tissues. Detailed structure-function relationships of retinoids in a variety of systems have been reported (25). In various systems, the effects of retinoids on diverse transcriptional events (2, 7, 25, 27) including RNA synthesis (2, 27) have been studied.

Recently, the effects of  $\beta$ -all-*trans*-retinoic acid and retinyl acetate on the *in vitro* cellular proliferation of 2 different murine malignant melanomas have been extensively characterized (14, 15). Changes in differentiation as measured by tyrosinase activity or melanin synthesis accompanying the inhibition of growth were not delineated although a visual increase in me-

lanogenesis was noted. Inhibition of cellular proliferation of cultured human melanoma cells by  $\beta$ -all-*trans*-retinoic acid has also been shown (13).

We have recently reported that retinoids have potent inhibitory effects on colony formation in fresh cells obtained from biopsies of human melanoma tissue (17). These observations have led us to further investigate the effects of retinoids on human melanoma growth and differentiation. We report here the effects of 4 different retinoids on the cellular proliferation and differentiation of a human melanoma cell line and several derived clones.

## MATERIALS AND METHODS

**Cell Cultures.** The cell line used in these studies was established from cells obtained from excisional biopsy of a metastatic melanoma nodule from a 17-year-old white male (protocol approved by the University of Arizona Human Subjects Committee) and is designated MIRW. This line has been in continuous culture for 18 months; it requires a high serum concentration (20%), grows as an adherent monolayer with the basic cell type being spindle shaped and melanotic, and has a doubling time of 24 hr. Eighteen clones were developed by the limiting dilution technique in microtiter wells, and cell lines were established from the individual clones. Three cloned lines with the features outlined in Table 1 were selected for further study.

All cell lines were grown in F-10 medium containing 20% heat-inactivated fetal calf serum and penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and Fungizone (0.25  $\mu$ g/ml) (Grand Island Biological Co., Grand Island, N. Y.). The cells were subcultured every 5 to 8 days. Cell numbers were obtained with a hemacytometer, and viability was monitored by exclusion of trypan blue.

**Retinoids.** Retinol and  $\beta$ -all-*trans*-retinoic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.). 13-*cis*-Retinoic acid (Ro4-3780) and aromatic retinoic acid ethyl ester (Ro 10-9359) were a generous gift of E. Miller (Hoffman-LaRoche, Inc., Nutley, N. J.). All 4 retinoids were stored at  $-20^{\circ}$  in light-protected tubes as a stock solution at  $10^{-2}$  M in dimethyl sulfoxide and diluted with tissue culture medium just before use.

**Radioisotopes.** L-[ring-3,5-<sup>3</sup>H]Tyrosine (48 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.).

**Tyrosinase Activity.** The assay is a modification of the method of Pomerantz (22). After the appropriate treatment, medium is replaced with fresh medium containing the compound under investigation and [<sup>3</sup>H]tyrosine (1  $\mu$ Ci/ml). Cultures are then incubated for 24 hr, and the spent medium is removed and assayed for the presence of <sup>3</sup>H<sub>2</sub>O as detailed elsewhere.

**Melanin Assay.** The content of melanin was measured by

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solubilizing 1 million cells with 1.0 ml N NaOH and 10% dimethyl sulfoxide for 30 min. The absorbance was read at 470 nm, and melanin content was expressed as  $\mu\text{g}$  melanin per  $10^6$  cells.

**RESULTS AND DISCUSSION**

All 4 retinoids at a concentration of  $10^{-6}$  M inhibited the cellular proliferation [36 to 42% (Table 2)] and stimulated tyrosinase activity [58 to 72% (Chart 1)] and melanin content [93 to 115% (Table 3)] in the parent MIRW to a similar extent. In contrast, the effect of the different retinoids on the derived melanoma clones was diverse. None of the clones tested exhibited as great an inhibition of growth by retinol ( $10^{-6}$  M) as did the parent line (41% versus 0 to 33% inhibition). Also, the levels of enzyme activity (Chart 1) and melanin content (Table 3) in the 3 clones exposed to different retinoids varied markedly although the activity changes in tyrosinase activity and melanin content was parallel in all instances. It is of interest that, in both the parent cell line and the derived clones, no morphological changes were detected. As can be seen in Tables 2 and 3 and Chart 1, the effect of retinoids on human melanoma cell proliferation (cell number) and differentiation (tyrosinase activity and melanin content) is heterogeneous with respect both to the retinoid and to the subclone of melanoma tested. 13-cis-Retinoic acid was found overall to be the most effective stimulator of tyrosinase activity and melanin synthesis, while retinol was observed to be the least effective of all the retinoids in increasing these products of differentiation. All retinoids caused some reduction in proliferation but, again, 13-cis-retinoic acid was found to be the most potent at the concentration used ( $10^{-6}$  M). The differences in inhibition of cellular proliferation of the various MIRW subclones by a particular retinoid, e.g.,  $10^{-6}$  M retinol (parent, 41%; clone A6, 27%; clone A9, 0%; clone A15, 33%) and aromatic retinoic acid ethyl ester (parent, 39%; clone A6, 39%; clone A9, 25%; clone A15, 55%), indicates that the clones contain cells with different capacities to respond to a particular retinoid. Lotan has also noted different susceptibilities of human (13) and murine (14, 15) melanoma to alterations in proliferation by  $\beta$ -all-trans-retinoic acid.

The variability of response of individual clones to the different retinoids is difficult to explain. Distinct retinol- and retinoic acid-binding proteins have been detected in some cells (1, 3, 23); therefore, the differences in inhibition of cellular proliferation in the same clone between retinol and the 3 retinoic acid derivatives may at least partially reflect differences in the presence and/or activity of the 2 binding proteins. Also, retinol is known to have potent detergent-like effects on membranes (9) and on the biosynthesis of glycoproteins (12, 19); therefore, other biochemical effects may be operative in explaining the

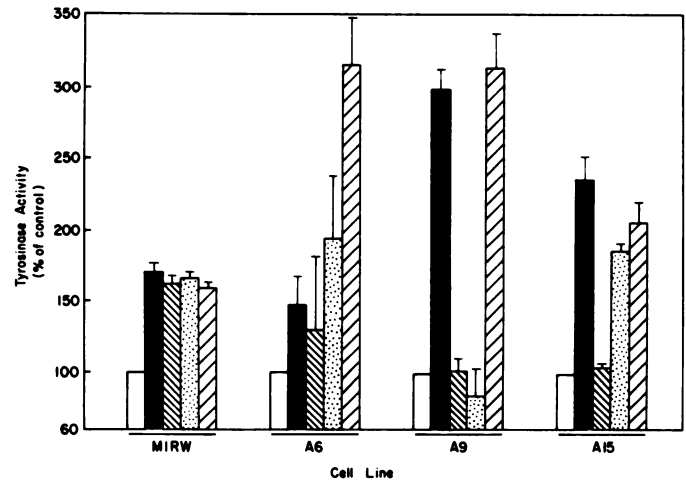


Chart 1. Effect of retinoids on tyrosinase activity in human melanoma lines. Conditions of the experiment are as outlined in Table 2. Retinoid concentration was  $10^{-6}$  M. Tyrosinase activity was measured after 7 days of exposure to the retinoid as detailed in "Materials and Methods." Basal enzyme activity per  $10^6$  cells was: parent, 30, 903 cpm; clone A6, 1247 cpm; clone A9, 9713 cpm; and clone A15, 8943. □, control; ■,  $\beta$ -all-trans-retinoic acid; ▨, retinol; ▩, aromatic retinoic acid ethyl ester; ▧, 13-cis-retinoic acid. Values are the averages of 4 determinations. Bars, S.E.

Table 3

Effect of long-term exposure to retinoids on melanin content of a human melanoma cell line and subclones

The conditions of the experiment were as detailed in Table 2. Melanin content was measured at the end of the 8-day experiment as described in "Materials and Methods." Melanin content ( $\mu\text{g}$ ) per  $10^6$  cells was: parent, 16.0; clone A6, 1.2; clone A9, 6.2; and clone A15, 5.8.

Retinoid	Melanin content (% of control)			
	Parent	Clone A6	Clone A9	Clone A15
Retinol	210 ± 5 <sup>a</sup>	125 ± 6	101 ± 4	88 ± 5
$\beta$ -All-trans-retinoic acid	193 ± 4	145 ± 8	375 ± 8	275 ± 8
13-cis-Retinoic acid	215 ± 8	225 ± 15	25 ± 4	201 ± 6
Aromatic retinoic acid ethyl ester	201 ± 6	405 ± 6	412 ± 15	257 ± 11

<sup>a</sup> Mean ± S.D.

Table 1

Characteristics of human melanoma cell line MIRW and subclones

MIRW cell line	Morphology	Chromosomal range
Parent	Highly melanotic; mixture of cell types	23-146
A6	Amelanotic; cuboid-shaped	55-81
A9	Lightly melanotic; cuboid- and spindle-shaped	27-55
A15	Lightly melanotic; spindle-shaped	32-49

Table 2

Effect of long-term exposure to retinoids on proliferation of a human melanoma cell line and subclones

Cells ( $2 \times 10^4$ ) were seeded in 25-sq cm Corning flasks, and the appropriate retinoid was added at  $10^{-6}$  M. The medium and retinoid were changed every 3 days. After 8 days, the cells were removed with Tyrode-EDTA solution and counted. The average number of cells  $\times 10^4$  per 25-sq cm flask was: parent,  $0.72 \pm 0.12$ ; clone A6,  $1.53 \pm 0.22$ ; clone A9,  $1.14 \pm 0.14$ ; and clone A15,  $2.13 \pm 0.15$ . All experiments were done in subdued light, and the flasks were lightly covered with foil for the duration of the experiment.

Retinoid	Cell no. (% of control)			
	Parent	Clone A6	Clone A9	Clone A15
Retinol	59 ± 2 <sup>a</sup> (80) <sup>b</sup>	73 ± 2 (92)	100 ± 2 (100)	67 ± 2 (92)
$\beta$ -All-trans-retinoic acid	58 ± 1 (81)	93 ± 3 (100)	67 ± 2 (92)	81 ± 3 (100)
13-cis-Retinoic acid	64 ± 2 (85)	63 ± 2 (85)	61 ± 1 (79)	48 ± 2 (69)
Aromatic retinoic acid ethyl ester	61 ± 1 (82)	61 ± 1 (81)	75 ± 3 (94)	45 ± 3 (65)

<sup>a</sup> Mean ± S.D. of 2 independent experiments, each performed in triplicate.

<sup>b</sup> Numbers in parentheses, average percentage of control using  $10^{-6}$  M retinoid.

differences in cellular response to retinol and the 3 retinoic acid derivatives.

The variability of inhibition of cellular proliferation by the 3 retinoic acid derivatives in the same clone may possibly reflect several underlying mechanisms: (a) different transport mechanisms for the individual retinoic acid derivatives; (b) different binding affinities for the cellular retinoic acid-binding protein in different clones; and (c) different biochemical capacities of the individual clones to convert a particular retinoid to an active form. Recent studies of retinoic acid derivatives suggest that all these possibilities may play a role (1, 19, 25).

In murine (20) and human<sup>3</sup> melanoma cell lines, the response of cellular proliferation to a hormonal signal (melanocyte-stimulating hormone) may at least in part be coupled to differentiation. The results presented here indicate that human melanoma cell growth *in vitro* can be inhibited by retinol,  $\beta$ -all-trans-retinoic acid, 13-cis-retinoic acid, and aromatic retinoic acid ethyl ester and is frequently associated with an increase in differentiated function, *i.e.*, tyrosinase activity (Chart 1) and melanin content (Table 3). We have also recently investigated the effects of these 4 retinoid derivatives on the development of human melanoma colonies using fresh cells from biopsies and have found inhibition in most instances (17). Although the mechanism underlying the retinoid-induced stimulation or inhibition of cell proliferation observed in numerous *in vitro* and *in vivo* systems is unknown (11, 13-15, 19, 25), the results with the fresh (17) and cultured (13) melanoma cells suggest that retinoids may act at a cellular target site (perhaps at the genome) which is not only involved in growth regulation but may also be linked to cellular mechanisms controlling the expression of differentiated functions.

Coupled with the known positive immunological effects of these agents (4, 5, 10, 18), these observations give considerable impetus to the clinical use of these agents in early and advanced human melanoma.

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# Cancer Research



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