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Inhibition of Human Melanoma Colony Formation by Retinoids

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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 patients with metastatic melanoma. The results indicate that melanoma colony-forming cells in biopsies obtained from ten patients with metastatic 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.

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 80% inhibition to mild stimulation in one case. Marked inhibition of melanoma colony formation was reduced 70% (A) and 80% (E) at a concentration of $10^{-5} \text{ M}$ or various concentrations of the appropriate retinoid at $37^\circ$ for 1 hr. The cells were then washed twice with serum-free medium, and $5.0 \times 10^5$ 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 $37^\circ$ 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. Morphology of the neoplastic melanoma cell colonies was further defined with our new dried-slide technique and a combination of Papanicolaou's and Lilie's melanin staining.

MATERIALS AND METHODS

Retinoids. Retinol and $\beta$-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 $10^{-3} \text{ M}$ 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 $1.0 \times 10^{-5} \text{ M}$ or various concentrations of the appropriate retinoid at $37^\circ$ for 1 hr. The cells were then washed twice with serum-free medium, and $5.0 \times 10^5$ 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.

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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 80% inhibition to mild stimulation in one case. Marked inhibition of melanoma colony formation was reduced 70% (A) and 80% (E) at a concentration of $10^{-5} \text{ M}$, but a further

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Molarity of Retinola (10^{-N})

Chart I. Effect of retinol on human melanoma colony formation. • cis-RA (RO-43780); O, β-all-trans-RA; A, aromatic RA ethyl ester analog (R-10-9359); and Δ, retinol.

decrease in survival was not achieved at higher concentrations.

β-All-trans-RA was tested in 5 patients, and 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

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>Patient</th>
<th>Retinol</th>
<th>13-cis-RA</th>
<th>All-trans-RA</th>
<th>Aromatic RA ethyl ester analog</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>33</td>
<td>105</td>
<td>60</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>81</td>
<td>35</td>
<td>67</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>88</td>
<td>40</td>
<td>67</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>41</td>
<td>64</td>
<td>21</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>46</td>
<td>65</td>
<td>46</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>20</td>
<td>18</td>
<td>46</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>45</td>
<td>97</td>
<td>46</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>38</td>
<td>113</td>
<td>46</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Mean survival: 51 ± 3.7b 67 ± 2.7 61 ± 3.4 64 ± 2.6

Based on the available published data, this concentration of retinol 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.\(^*\) 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 clonogenic ability.

While the \textit{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 \textit{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 cytotoxic agents (e.g., melphalan, Adriamycin), our group has reported excellent correlation between \textit{in vitro} sensitivity or resistance in this agar culture 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 \textit{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 clinical trials of selected retinoids in patients with malignant melanomas appears most reasonable. Such trials would ideally be carried out in conjunction with \textit{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.

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\textbf{REFERENCES}

\textit{\textsuperscript{*}F. Meyskens, manuscript in preparation.}


24. Salmon, S. E., and Swarty, G. Inhibition of the growth and changes in glycolipids and LETS in 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 \textit{in vitro} proliferation of murine melanoma cell lines S91 and B16 have been noted (13, 15), which supports our direct observations on human melanoma.


