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Dexamethasone, Prostaglandin A, and Retinoic Acid Modulation of Murine and Human Melanoma Cells Grown in Soft Agar[1,2,3]

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ABSTRACT—The cloning efficiencies of a murine melanoma cell line (S91 CCL 53.1) and a human melanoma cell strain (C8146c) were inhibited by dexamethasone (DEX), prostaglandin A_1 (PGA_1), and *β-all-trans*-retinoic acid (RA) in a dose-dependent manner. Murine melanoma tumor colony-forming units (MTCFU) were inhibited more than 99% by DEX (1×10^{-7} *M*) and RA (1×10^{-7} *M*) with a concentration needed to produce a 50% reduction in colony formation for both hormones of 5×10^{-9} *M*. Combinations of DEX and RA effected a synergistic inhibition on colony formation, which was reflected by a 1½ log reduction in the hormone concentration needed to produce a greater than 99% inhibition of colony formation. When PGA_1 was added to DEX and RA, a greater than additive reduction in colony formation was observed. Human MTCFU from cell strain C8146c were inhibited more than 85% at an RA concentration of 1×10^{-7} *M*, but they were reduced only to 40% of control at a DEX concentration of 1×10^{-6} *M*. DEX–RA produced an additive inhibition of colony formation. Addition of submaximal amounts of PGA_1 to DEX–RA combinations or to either hormone alone resulted in synergistic reduction of human MTCFU. These results demonstrated that the proliferative potential of human and murine melanomas can be simultaneously regulated by DEX, PGA_1, and RA.—JNCI 1983; 71:927–932.

Several hormones including steroids, retinoids, and prostaglandins inhibit melanoma cell proliferation and/or induce differentiation (*1–8*). Glucocorticoids inhibit the proliferation of hamster melanoma cells (*4*) and induce differentiation in murine melanoma cells as measured by changes in tyrosinase activity (*3*). The inability of glucocorticoids to produce complete regressions of melanomas in vivo may be due to resistant mutants, as suggested by a recent study in which nonresponsive clones were isolated from a glucocorticoid-sensitive melanoma cell line (*4*). The high incidence of glucocorticoid receptors in cells from biopsy samples obtained from patients with melanoma suggests that human melanoma cells may also be responsive to this hormone (*9*).

Retinoids inhibit the proliferation and induce differentiation of murine melanoma cells in vitro (*1, 10*). Some human melanoma cells in culture are inhibited by RA, but the response is not uniform, with the growth of some cell lines being either insensitive to or even stimulated by RA (*11*). Even clones selected from individual human (*12*) and murine (*13*) melanoma cell lines displayed differential sensitivities to RA. We have found that cells from biopsy specimens of melanoma tissue grown in agar also demonstrated marked heterogeneity in response from patient to patient, ranging from a substantial inhibition of melanoma colony formation to no effect (*14*).

Prostaglandins also inhibit the growth of murine melanoma tumors in vivo (*15, 16*). PGA_1 is a strong inhibitor of DNA synthesis (*6, 17*), and we have found the growth of murine melanoma cells in soft agar to be strongly inhibited by PGA_1 (*7*). Turner et al. (*18*) recently reported that irreversible inhibition of murine melanoma growth occurs only if the cells are exposed continuously to PGA_1 for more than 72 hours. Our laboratory has also found that PGA_1 is a potent inhibitor of human melanoma colony formation and that prostaglandins B_1, $F_{1\alpha}$, and $F_{2\alpha}$ were inactive (*19*).

Previous studies have measured the activity of a single hormone such as DEX or retinoids on melanoma cell growth. The use of antiproliferative hormones in combination may lead to an enhanced biological response. In this communication we measured the combined effects of DEX, RA, and PGA_1 on human and murine clonogenic tumor cells in vitro. The reduction of MTCFU in soft agar was enhanced by continuous exposure to the combined hormones.

MATERIALS AND METHODS

Chemicals.—DEX and RA were purchased from Sigma Chemical Co., St. Louis, Mo. Ham's F-10 medium was obtained from GIBCO, Santa Clara, Calif. FCS and HS were from KC Biological, Lenexa, Kans. PGA_1 was obtained from The Upjohn Co., Kalamazoo, Mich.

Cell cultures.—The Cloudman S91 murine melanoma clone CCL 53.1 was obtained from the American Type Culture Collection, Rockville, Md., and was maintained by serial transplantation in DBA/2J mice. The tumors were harvested, and single-cell suspensions were obtained as previously described (*7*). The cells were added to a flask containing F-10 media with 10% HS and 2% FCS. CCL 53.1 cells readily formed a monolayer and were subsequently subcultured. All experiments were performed on cells that had been subcultured no more than 10 times after isolation from mouse melanomas.

ABBREVIATIONS USED: DEX-dexamethasone; FCS=fetal calf serum (heat-inactivated); HS=horse serum; ID_{50}=concentration needed to produce a 50% reduction in melanoma colony formation; MTCFU=melanoma tumor colony-forming units; PGA_1=prostaglandin A_1; RA=*β-all-trans*-retinoic acid.

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The human melanoma cell strain C8146c was developed in our laboratory from cells obtained from biopsies of subcutaneous nodules and is described in detail elsewhere (*19*). Early subcultures of C8146c were stored in the vapor phase of liquid nitrogen. C8146c was grown in Ham's F-10 medium containing 10% FCS and was not used for more than 10 subculturings. C8146c has tyrosinase activity (*19*), and the cells grown in agar contain melanosomes (Hendrix M: Personal communication). In culture the doubling time of C8146c was 51 hours. These human melanoma cells when implanted in nude mice generated tumors within 6 weeks.

Clonogenic assay.—The assay system is a simplification of the bilayer agar assay developed by Salmon and coworkers (*20, 21*), as we have previously described (*22*). A bilayer of agar in medium without additional additives was constructed in 35-mm-diameter petri dishes. For human melanoma cell lines the medium used in both agar layers was Ham's F-10 with 10% FCS, and 10,000–20,000 cells were plated per dish. The murine cell line was plated at a concentration of 5,000 cells/ml in Ham's F-10 medium containing 10% HS and 2% FCS. The petri dishes were incubated at 37°C in a humidified atmosphere containing 6% CO_2 for 10–14 days. The colonies were counted and grouped into size classes based on colony diameter with the use of an optical image analyzer (Omnicon FAS II; Bausch & Lomb, Rochester, N.Y.) (*23*). The colony size cutoff was arbitrarily chosen as 60 μm, which corresponded to 28 cells for CCL 53.1 on day 10 and 10 cells for C8146c on day 14.[6] PGA_1, DEX, and RA were added at various concentrations with the cells on day 0. The experiments were done in the dark, and all stock solutions were protected from light with a foil wrap.

Data analysis.—Cloning efficiencies were calculated from the total number of cells plated. Visual inspection of the dishes through a microscope on day 0 found the top agar layer contained only single cells. The in vitro hormone effects in the combination studies were quantitated according to the methods of Valeriote and Lin (*24*) and Drewinko et al. (*25*). The surviving fraction (*SF*) of MTCFU resulting from each hormone individually and the surviving fraction of the hormone combination were determined experimentally. If the SF_{A+B} was equal to $(SF_A)\cdot(SF_B)$, the combined drug effects were additive. If the SF_{A+B} was less than $(SF_A)\cdot(SF_B)$, the combined effect was defined as greater than additive or synergistic. If the SF_{A+B} was greater than $(SF_A)\cdot(SF_B)$ but less than (SF_A) and (SF_B), the combination interaction was defined as subadditive. If the SF_{A+B} was greater than $(SF_A)\cdot(SF_B)$ and greater than or equal to the lowest value SF_A or SF_B, the combination interaction was defined as antagonistic (*26*). Statistical comparisons between the experimental and calculated data were done for each of the hormones.

For each combination of hormones, the difference for the observed minus expected percentage survivals of MTCFU was calculated for each experiment. The *z*-statistic calculated for the hormone combination is the difference (observed minus expected) of MTCFU survival divided by the square root of the observed variance of the surviving fraction for the hormone combination, yielding a standard normal *z*-statistic with corresponding *P*-values according to the method of Drewinko et al. (*25*). With the use of this approach, drugs that produce a perfectly additive response give a *z*-value of 0. We have designated a *P*-value of .05 or less as indicating an antagonistic or synergistic effect of the combination (depending on the direction of the *z*-value) and a *P*-value of greater than .05 but less than .20 as indicating a subadditive or subsynergistic response.

[6]The number of cells per colony size was derived either by computation or exact measurement (Meyskens FL Jr, Thomson SP, Moon TE: Unpublished observations).

RESULTS

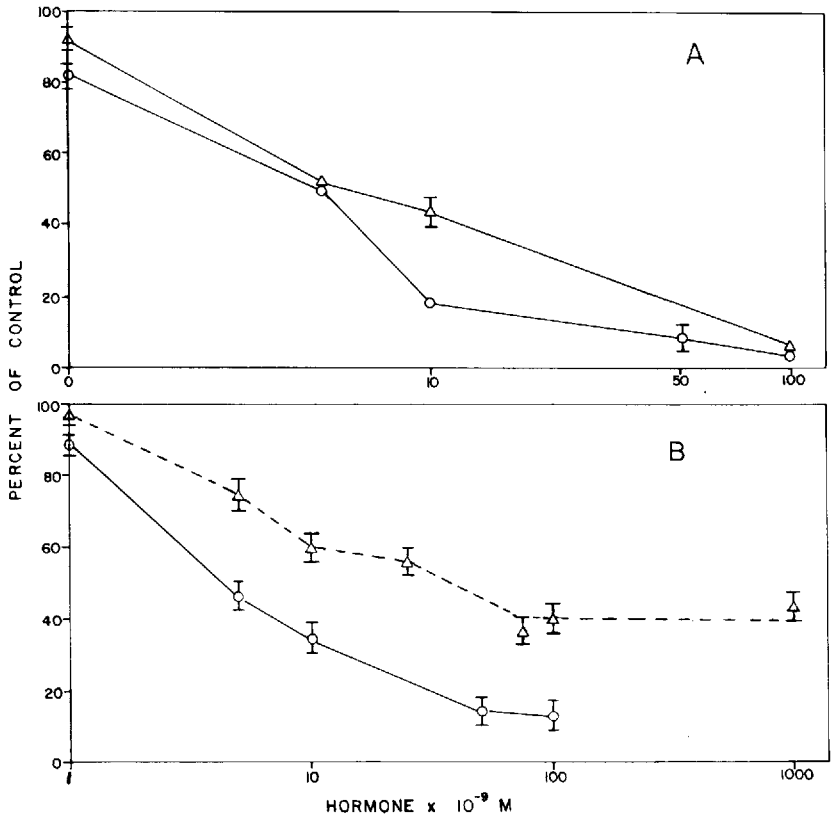
Effect of DEX and RA on Melanoma Colony Formation

CCL 53.1 murine melanoma cell growth in soft agar was sensitive to RA in a dose-dependent fashion (text-fig. 1). The ID_{50} of RA was 5×10^{-9} *M* (table 1). A greater than 95% reduction in colony formation was achieved at an RA concentration of 1×10^{-7} *M*. This murine cell line responded in a like manner to DEX with an ID_{50} occurring at 5×10^{-9} *M*. The inhibition of colony formation by DEX was dose-dependent with more than 95% inhibition occurring at a concentration of 1×10^{-7} *M*.

The sensitivity of human melanoma cells of strain C8146c to RA (text-fig. 1) was comparable to the response obtained with the murine melanoma cell line. An RA concentration of 5×10^{-9} *M* produced a 50% reduction in MTCFU, and a greater than 85% inhibition was obtained at a concentration of 1×10^{-7} *M* (table 1). When the treated cells were grown for an additional 5 days, the remaining colonies were larger, but no additional colonies appeared. Thus reduction in colony number effected by RA was not due to an increase in doubling time but due to a total inhibition of growth. The human melanoma cells were not as sensitive to DEX as the murine cells. A 50% reduction in MTCFU was achieved with a DEX concentration of 5×10^{-8} *M*. This concentration was a log higher than that needed to produce a 50% reduction in MTCFU from the murine line. A complete reduction in MTCFU from C8146c could not be achieved at any concentration tested. DEX concentrations of 1×10^{-7} to 1×10^{-6} *M* resulted in only a 60% reduction in colony formation (table 1), demonstrating that a large portion of the MTCFU from C8146c was insensitive to this hormone.

Effect of DEX and RA in Combination on Melanoma Colony Formation

When both RA and DEX were added to murine melanoma cells in soft agar at nanomolar concentrations, there was a synergistic inhibition of the number of colonies formed (table 2). For example, at a concentration of 1×10^{-9} *M*, DEX and RA reduced MTCFU 8 and



TEXT-FIGURE 1.—Effect of increasing concentrations of DEX (△---△) and RA (○——○) on the anchorage-independent growth of CCL 53.1 (panel A) and increasing concentrations of DEX (△---△) and RA (○——○) on C8146c (panel B). Control plates were seeded with 5,000 CCL 53.1 and 15,000 C8146c cells and contained 2,750±56 and 2,676±86 colonies, respectively. The experiments were repeated two more times, and similar results were obtained.

18%, respectively. The expected inhibition was 25%; however, the observed reduction in MTCFU was much greater than additive (table 2). This synergism occurred in a dose-dependent manner between the concentration range of 1–10 n*M*. When RA and DEX were used together, the ID_{50} was slightly greater than 1×10^{-9} *M*. This was a half-log decrease over the concentration needed when either DEX or RA was added alone. Also, when RA and DEX were added simultaneously, there was a decrease of 1–1½ logs over the concentration needed for each hormone alone to elicit greater than 98% inhibition (table 2).

TABLE 1.—*Hormone sensitivity of established murine (CCL 53.1) and human (C8146c) melanoma cells in soft agar*

Cell line	Treatment	ID_{50}, *M*	Maximum inhibition of MTCFU	
			Percent control	Concentration, *M*
CCL 53.1[a]	DEX	5×10^{-9}	3	1×10^{-7}
	PGA_1	2×10^{-6}	0	7×10^{-6}
	RA	5×10^{-9}	4	1×10^{-7}
C8416c[b]	DEX	5×10^{-8}	40	1×10^{-6}
	PGA_1	2×10^{-6}	0	7×10^{-6}
	RA	5×10^{-9}	14	1×10^{-7}

[a]Single-cell suspensions of CCL 53.1 were plated in quadruplicate as described in "Materials and Methods." Control plates yielded 3,056± 52 colonies >60 μm on day 10. Cloning efficiency was 61%.

[b]Single-cell suspensions of C8146c were plated in quadruplicate as described in "Materials and Methods." Control plates yielded 1,640± 56 colonies >60 μm on day 14. Cloning efficiency was 16%.

The data in table 2 show that DEX and RA can act together to inhibit the MTCFU from the human melanoma cell strain C8146c. An additive reduction in MTCFU was obtained with all concentrations up to 1×10^{-8} *M*. The sensitivity of these human melanoma cells to the combined action of DEX–RA was not as dramatic as those inhibitions seen with the murine cell line. DEX–RA at 5×10^{-9} *M* effected a significant 70% reduction in colony number. Only 60% of the MTCFU in this human melanoma cell line were sensitive to DEX (text-fig. 1). Yet when DEX was added with RA at 5×10^{-9} *M*, a further 40% reduction in colony number was observed over that obtained with RA alone.

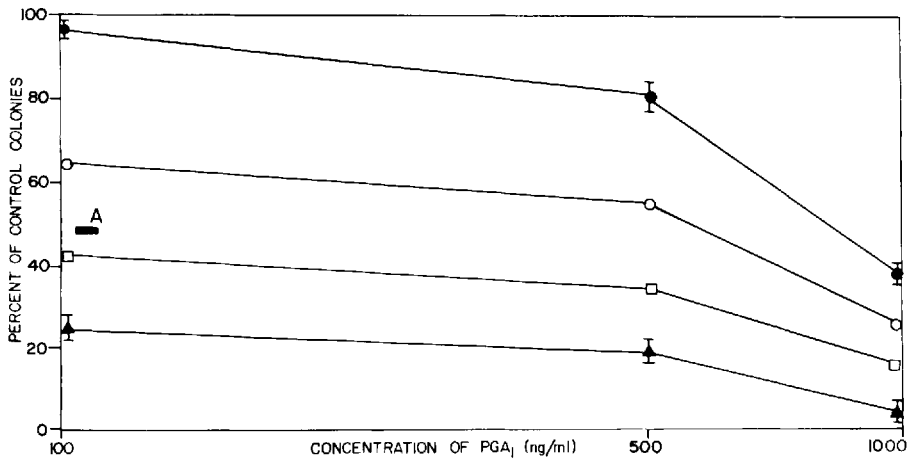
Effect of PGA_1 on DEX and RA Inhibition of MTCFU

We have previously demonstrated that PGA_1 irreversibly inhibited the anchorage-independent growth of murine melanoma (*19*), but the concentration needed to produce complete inhibition was very large (7 μ*M*). Because PGA_1 may be inhibiting melanoma cell growth through an as yet undefined biological mechanism, the effect of lower concentrations in combination with other hormones was tested. As shown in text-figure 2, submaximal concentrations of PGA_1 resulted in a dramatic decrease in murine melanoma colony formation in the presence of RA and DEX. The observed inhibition curve was greater than the calculated additive effects (text-fig. 2). DEX–RA used at a low concentration (1×10^{-9} *M*) or PGA_1 at a low concentration (100 ng/ml) produced only a minor reduction in MTCFU. The addition of PGA_1 to

TABLE 2.—*Effect of DEX-RA combination on melanoma colony formation*

Cell line	Treatment (concentration)	Percent inhibition[a]		Statistical evaluation	
		Expected	Observed effect,[b] ±SE	z	P
CCL 53.1	RA–DEX (both 1×10^{-9} M)	25	46±1 S	−10.5	.0001
	RA (1×10^{-9} M) + DEX (5×10^{-9} M)	44	84±1 S	−20.0	.0001
	RA (1×10^{-8} M) + DEX (5×10^{-9} M)	91	98±0.5 S	−7.00	.0001
	RA–DEX (both 1×10^{-8} M)	93	100 S		0
C8146c	RA–DEX (both 1×10^{-9} M)	33	32±1 A	0.50	.61
	RA–DEX (both 2.5×10^{-9} M)	53	53±1 A	0	1
	RA–DEX (both 5×10^{-9} M)	70	70±2 A	0	1
	RA (1×10^{-8} M) + DEX (5×10^{-9} M)	75	75±0.5 A	0	1
	RA–DEX (both 1×10^{-8} M)	78	79±1.5 A	−0.33	.73

[a]The effect of the expected and observed inhibition was calculated as described in "Materials and Methods."
[b]Results are expressed as standard errors relative to mean of control. A z-value of 0 indicates that the expected and observed survival percentages of the combination were equal. The larger a positive value, the greater the chance the combination was antagonistic (A). The larger a negative value, the greater the chance the combination was synergistic (S).



TEXT-FIGURE 2.—Effect of 1×10^{-9} M DEX and 1×10^{-9} M RA in combination with increasing concentrations of PGA_1 on MTCFU of murine melanoma CCL 53.1 in soft agar. CCL 53.1 melanoma cells were plated in quadruplicate as described in "Materials and Methods," and the hormone(s) were placed in the culture dish. Control plates had 3,160±62 colonies >60 μm. Colonies >60 μm contained at least 28 cells, 54% of the colonies were >104 μm and contained at least 105 cells, and 4% of the colonies were >149 μm on day 10 and contained at least 247 ells. ●=PGA alone; ○=expected additive result of all three hormones; □=observed effect of RA plus DEX plus the calculated effect of PGA_1; ▲=DEX and RA plus an increasing concentration of PGA_1. Point A (—) shows the reduction in colony formation elicited by the DEX–RA combination. This experiment was done two times, and similar results were obtained.

DEX–RA produced an additional 50% reduction of murine MTCFU.

A similar result was also obtained with the human melanoma cell line (table 3). The addition of PGA_1 synergistically enhanced the inhibition of MTCFU in the presence of DEX and RA. A concentration of 100 ng PGA_1/ml was very effective in potentiating the inhibitory effect of 2.5-nM concentrations of DEX and RA. The addition of PGA_1 to DEX–RA increased the observed inhibition in this experiment (table 3) by more than 50%, which is more than the 11% one would expect for an additive result. Note that PGA_1 did not enhance the inhibition in the presence of DEX–RA at 1×10^{-9} M. However, this low dose of DEX–RA did interact synergistically with higher PGA_1 concentrations. The combination of PGA_1 and DEX or RA also resulted in a

TABLE 3.—*PGA_1 effect on human MTCFU inhibition by DEX and RA*[a]

Treatment (concentration)	Total colony percent inhibition ± SE	Effect[b]	Statistical evaluation		Percent inhibition of colonies, 104 μm[c]
			z	P	
PGA_1 (100 ng/ml)	11±0.5				1
Plus DEX–RA (both 1×10^{-9} M)	33±1	A	7.00	.0001	53
Plus DEX–RA (both 2.5×10^{-9} M)	61±1.0	S	−8.94	.0001	89
PGA_1 (500 ng/ml)	45±1				41
Plus DEX–RA (both 1×10^{-9} M)	79±0.5	S	−16.00	.0001	94
Plus DEX–RA (both 2.5×10^{-9} M)	81±0.5	S	−14.00	.0001	95
PGA_1 (1 μg/ml)	75±0.5				87
Plus DEX–RA (both 1×10^{-9} M)	92±0.5	S	−8.75	.0001	99
Plus DEX (1×10^{-7} M)	94±0.5	S	−4.28	.0001	99
Plus RA (1×10^{-8} M)	94±0.5	S	−7.14	.0001	99

[a]Single-cell suspensions of C8146c were plated in soft agar in continuous contact with the compounds as described previously (*19*).
[b]A=antagonistic; S=synergistic.
[c]Total colonies >60 μm, 2,622, contained at least 10 cells, and 804 colonies were >104 μm on day 14 and contained at least 36 cells.

significant increase in the observed inhibition of colony formation and demonstrated that DEX and RA need not both be present for PGA_1 to express its synergistic interactions (table 3). For example, the maximal inhibition effected by DEX at 0.1 μM or higher concentrations was 60%. The addition of PGA_1, which alone produced a 75% inhibition (table 3), resulted in synergistic interaction, with 94% of the MTCFU being inhibited. Visual inspection revealed that most of the cells had failed to divide. There was not a large amount of clusters less than 60 μM, which one would expect if the hormone combination was retarding only the growth rate.

DISCUSSION

The growth of melanoma cells from established murine and human cultures can be strongly inhibited both in vitro and in vivo by DEX, PGA_1, and RA (*1, 2, 4–7, 14, 16*). However, some melanoma cells are insensitive to either DEX or RA (*4, 11, 13*).

The murine melanoma cell line used in this study represents an ideal case. More than 96% of the colony-forming cells were totally responsive to single-agent treatments by DEX or RA. These results are comparable to those of a previous report in which the dose-dependent effects of RA on the monolayer growth of S91 melanoma were demonstrated (*1*). The human melanoma cell strain represents a more complicated situation. The colony-forming units were highly responsive to RA, but a large portion of these cells was resistant to DEX.

The combined use of DEX–RA in murine melanoma cells resulted in a dramatic reduction of MTCFU expression. The observed inhibition was much larger than that predicted for an additive response. The data in table 2 clearly demonstrated that nanomolar combinations of DEX–RA produced a synergistic inhibition of MTCFU expression. The enhanced reduction of colony formation may be the result of two processes. The second hormone could generate an enhanced response by acting on melanoma cells that are resistant to the first hormone, or it could act in concert with the first hormone at the cellular level. For example, at a concentration of 1 nM most of the murine melanoma cells did not respond to either DEX or RA. Since all the MTCFU can be inhibited by either hormone alone at reasonable concentrations (table 1), we speculate that the second hormone is not acting on cells resistant to the first hormone. At 1×10^{-9} M a subthreshold response was generated in most cells by either DEX or RA. Yet when DEX and RA are added together, about half of the MTCFU were blocked from forming colonies. DEX and RA may independently inhibit the expression of the same cellular process and when DEX and RA are combined, the integrated subthreshold effects lead to a block in cellular division. It remains to be elucidated which processes are regulated either independently or concurrently by these hormones.

The interpretation of the combined DEX–RA effects on the human melanoma cell strain is more complicated, since a large proportion of melanoma colony-forming cells did not respond to DEX. If one assumes that DEX and RA do not work together on the MTCFU to inhibit colony formation, then the maximal inhibition that could be achieved would be an additive effect. Since only an additive effect was observed, one cannot know whether RA and DEX were acting independently on different cell populations or if the hormones interacted at the cellular level to comodulate the human melanoma cells. At least 60% of the melanoma colony-forming cells were responsive to both DEX and RA, so the lack of synergism suggests that the proliferation of human melanoma may not be as tightly regulated by these hormones in comparison to murine melanoma. Nevertheless, the addition of DEX does contribute to the further suppression of anchorage-independent growth. For example, at an RA concentration of 10 nM only 32% of the melanoma colony-forming cells formed colonies, and the addition of 10 nM DEX reduced this survival fraction by another 35% (table 2).

In this report we have demonstrated that DEX, RA, and PGA_1 comodulate melanoma cell growth and produce a synergistic inhibition of MTCFU expression. For the combination DEX–RA, these effects were observed at readily achievable plasma concentrations (*27, 28*). The other beneficial consequence of using these two hormones in concert is that they lowered the effective inhibitory threshold concentration, which is very important for a solid tumor, inasmuch as the effective concentration of hormone to which the tumor is expressed may be much lower than measured plasma levels. The additive effects of the DEX–RA combination on the MTCFU of the human melanoma cell strain suggest that the hormones may be useful in vivo, particularly since a significant number of cells from fresh biopsy specimens yielded at least partial inhibitory responses to RA (*14*). These results suggest that a subpopulation of patients may exist with adequate sensitivity to DEX and RA to allow a significant response to these hormones.

Previous clonogenic assay studies (*19*) also showed that PGA_1 can act as a single agent to irreversibly inhibit melanoma cells, but only at a very high concentration (7 μM). In the present study a PGA_1 concentration 25-fold lower greatly enhanced the inhibitory effects of DEX and RA. Little is known about the pharmacokinetics of PGA_1 in vivo. A preliminary study is under way in which PGA_1 has been administered to breast cancer patients (*29*). Our data suggest the potential usefulness of PGA_1 at this low concentration with DEX and RA. Hopefully, an analogue of PGA_1 may be developed with a high therapeutic index for patient studies.

Our observations demonstrate that the clonogenic capacity of melanoma cells in soft agar is under the control of several hormones. None of these hormones individually elicited a major response at useful concentrations, but in combination and at low concentrations they substantially inhibited MTCFU. The results presented here provide a rationale for a multihormone regimen for advanced melanoma. The coupling of antiproliferative hormones may also be applicable to other tumor types.

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