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Effect of Receptor-Selective Retinoids on Growth and Differentiation Pathways in Mouse Melanoma Cells

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ABSTRACT. Treatment of B16 mouse melanoma cells with all-*trans*-retinoic acid (ATRA) results in inhibition of cell proliferation and induction of differentiation. Accompanying these events is an induction of retinoic acid receptor β (RARβ) expression, an increase in protein kinase Cα (PKCα) expression, and enhanced activator protein-1 (AP-1) transcriptional activity. These cells express nuclear RARα and RARγ and nuclear retinoid X receptors (RXR) α and β constitutively. We tested the ability of receptor-selective retinoids to induce the biochemical changes found in ATRA-treated melanoma cells and also tested their effectiveness in decreasing anchorage-dependent and -independent growth. The RXR-selective ligand (2*E*,4*E*)-6-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-3,7-dimethyl-2,4,6-octatrienoic acid (SR11246) was most effective at inhibiting anchorage-dependent growth, whereas the RARγ-selective ligand 6-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)(hydroxyimino)methyl]-2-naphthalenecarboxylic acid (SR11254) was most potent at inhibiting anchorage-independent growth. In contrast, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenecarboxamido)-benzoic acid (Am580), an RARα-selective ligand, was the most effective receptor-selective agonist for inducing RARβ mRNA and increasing the amount of PKCα protein. All of the retinoids induced a concentration-dependent increase in AP-1 transcriptional activity, with little difference in effectiveness among the receptor-selective retinoids. A synergistic increase in the amount of PKCα was found when an RAR-selective agonist was combined with an RXR-selective agonist. One possible explanation for this result is that an RXR–RAR heterodimer in which both receptors are liganded is required for maximum expression of this critical component of the ATRA-induced differentiation pathway. Our data suggest that synthetic retinoids can activate different growth and differentiation pathways preferentially in B16 melanoma cells, due, most likely, to their ability to activate a different subset of receptors. BIOCHEM PHARMACOL **59**;10:1265–1275, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. receptor-selective retinoids; melanoma; proliferation; differentiation

Retinoids have been found to inhibit growth and to induce differentiation in a variety of cancer cell lines [1–5]. These effects are thought to be mediated by nuclear receptors that belong to the superfamily of steroid/thyroid hormone receptors. There are two major classes of retinoid nuclear receptors, the RARs‖ and the RXRs. The RARs bind ATRA and 9CRA, whereas the RXRs bind only 9CRA [6, 7]. There are three subtypes (α, β, and γ) of both the RARs [8–10] and the RXRs [11, 12]. These receptors are retinoid-activated transcription factors and act by binding to specific DNA sequences termed retinoic acid response elements, which often consist of a direct repeat of PuG(G,T)TCA with a 1- to 5-bp spacing between the repeats [13–15]. Both RARs and RXRs can form homodimers (RXR:RXR) or heterodimers (RXR:RAR), with the heterodimer having greater affinity/stability in binding to retinoic acid response elements [16, 17]. RXRs can also form heterodimers with other members of the steroid/thyroid superfamily of receptors [18–20]. By competing for RXRs, these other receptors can influence retinoid-regulated gene transcription. In addition to ligand, the activity of these receptors is also regulated by nuclear co-repressor [21], co-activator [22], and integrator [23] proteins.

Our laboratory has been studying the ATRA-induced growth arrest and differentiation of B16 mouse melanoma cells. This differentiation is characterized by increased melanin production [24], arrest in the G_1 phase of the cell cycle [25], loss of anchorage-independent growth, and acquisition of cell surface nerve growth factor receptors [24]. The pathway that leads to this differentiated phenotype is not known, but induction of PKCα plays a signifi-

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‖ *Abbreviations*: RAR, retinoic acid receptor; RXR, retinoid X receptor; ATRA, all-*trans*-retinoic acid; 9CRA, 9-*cis*-retinoic acid; AP-1, activator protein-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; and PKCα, protein kinase Cα.

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cant role [26]. Retinoic acid treatment also leads to a 4-fold increase in AP-1 transcriptional activity, which, in contrast to tetradecanoyl phorbol acetate-induced AP-1 activity, is sustained for at least 36 hr [27]. B16 cells constitutively express RARα and RARγ mRNA and can be induced to express RARβ mRNA by treatment with ATRA [28]. They also express RXRα and RXRβ mRNA, but do not express RXRγ [29]. In light of the expression of multiple retinoid nuclear receptors, the question arises as to whether different nuclear retinoid receptors mediate the various biochemical and phenotypic changes induced in these melanoma cells by ATRA. The availability of receptor-selective retinoids [30] provides one method to examine the relative importance of these different receptors in the regulation of B16 cell differentiation. We found that of the receptor-selective retinoids evaluated, the RXR-selective compound (2*E*,4*E*)-6-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-3,7-dimethyl-2,4,6-octatrienoic acid (SR11246) was most potent in inhibiting anchorage-dependent growth, whereas 6-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)(hydroxyimino)methyl]-2-naphthalenecarboxylic acid (SR11254) (RARγ-selective) was most potent in inhibiting anchorage-independent growth. In contrast, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenecarboxamido)-benzoic acid (Am580), an RARα-selective retinoid, was more effective in inducing RARβ mRNA and increasing PKCα protein levels. There was not a major difference in the ability of the various receptor-selective retinoids to increase AP-1 transcriptional activity. When we treated B16 cells with a combination of an RAR-selective and an RXR-selective retinoid, we observed a synergistic induction of PKCα. This result suggests that for the regulation of this step of the differentiation pathway, RXR is not a "silent partner" in the receptor heterodimer, but can increase the response when occupied by ligand.

MATERIALS AND METHODS

Retinoids

ATRA was purchased from the Sigma Chemical Co. 9CRA, Am580 (RARα-selective), SR11254 (RARγ-selective), SR11246 (RXR-selective), and 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)(cyclopropylidenyl)methyl]benzoic acid (SR11346) (RARβ/RXR-selective) were synthesized as previously described [31, 32]. All retinoids were dissolved in DMSO and kept under nitrogen gas at −80°. Fresh stocks were prepared for each experiment. Manipulations with retinoids were performed under low light conditions to minimize photo-oxidation.

Cell Culture

B16 mouse melanoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum (Sterile Systems), 2 mM l-glutamine, 2 mM sodium pyruvate, 50 U/mL of penicillin G, and 50 μg/mL of streptomycin sulfate. Cells were incubated at 37° in a 5% CO_2/95% air, humidified atmosphere. New cultures were initiated from frozen stock every 6 weeks to prevent phenotypic drift, which can occur with prolonged culture of these cells.

Anchorage-Dependent and -Independent Growth

For monolayer growth studies, cells were seeded at 5×10^4/well in 6-well tissue culture plates. One day after seeding, cells were refed with tissue culture medium containing different concentrations of retinoids. Cells were incubated with the retinoids for 48 hr and then harvested, and cell number was determined using a hemocytometer. Each concentration of the different retinoids was assayed in triplicate dishes of cells, and the entire experiment was repeated three times. For anchorage-independent growth, 5000 cells were mixed with 0.35% agarose in tissue culture medium, and this mixture was overlaid onto a 1% agarose base in 60-mm tissue culture dishes. Finally, a small amount of tissue culture medium was added to cover the top layer. All three layers contained retinoids at the concentrations indicated in the figures. Every 2 days, the top layer of medium with or without the appropriate concentration of retinoid was replenished, and after 10 days of incubation the number of colonies containing more than 25 cells was determined. Each concentration of the different retinoids was assayed in triplicate dishes of the soft agarose cell suspension. The entire experiment was repeated three times.

Northern Blotting

B16 cells were treated with the various retinoids for 24 hr. Control cells received the solubilization agent DMSO. Total RNA was isolated from cells using the TRI reagent (Sigma) according to the manufacturer's protocol. Thirty to forty micrograms of total RNA was separated on 1% formaldehyde agarose gels and transferred to Hybond NX membranes (Amersham). Following prehybridization, blots were probed simultaneously for RARβ and GAPDH. Full-length GAPDH cDNA and a 615-bp *Eco*RI fragment from mouse RARβ cDNA were used as probes. These DNAs were labeled using the Prime-a-Gene labeling system (Promega) according to the manufacturer's protocol. Unincorporated nucleotides were removed using a QIAquick Nucleotide Removal kit (Qiagen). Hybridization of the probes was performed at 68° for 1 hr using Express Hyb solution (Clontech). Following hybridization, blots were washed for 40 min at room temperature in 0.3 M NaCl, 30 mM sodium citrate (pH 7.0), 0.05% SDS, followed by a 40-min wash at 50° in 15 mM NaCl, 1.5 mM sodium citrate (pH 7.0), 0.1% SDS. Then blots were exposed to x-ray film at −80° for various times to generate a signal in the linear range for densitometry (Molecular Dynamics). Intensity of the RARβ bands was normalized to the corresponding GAPDH band, and the results were expressed as fold induction relative to the RARβ mRNA signal in control cells.

TABLE 1. Transcriptional activation activity of retinoids on the (TREpal)$_2$-*tk*-CAT reporter construct normalized to 10^{-6}M ATRA on RARα as 100% for the RARs and 10^{-6}M 9CRA on RXRα as 100%

	Activation (%)															
	RARα				RARβ				RARγ				RXRα			
Retinoid	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-9}	10^{-8}	10^{-7}	10^{-6}M
ATRA	6	37	70	100	30	74	91	105	43	96	104	106	0	11	18	61
9CRA	10	37	84	100	32	80	106	110	26	83	105	120	6	57	93	100
Am580		41	76	70		9	79	113		9	22	28		5	11	10
SR11254	7	7	8	13	6	10	24	46	17	34	80	84	11	10	10	13
SR11246		−1	−2	2		4	16	33		9	11	15		39	82	102
SR11346	3	4	7	19	8	44	72	85	10	10	34	47	2	68	101	102

Cells were transfected with the indicated reporter gene, and the indicated receptor gene in the pSG-5 vector and with a SV β-gal construct to correct for transfection efficiency. After the transfection medium was removed, the cells were refed with growth medium containing the indicated concentrations of the various retinoids. Following a 48-hr incubation, cells were harvested and assayed for CAT activity and β-gal activity as described previously [30]. After correction for transfection efficiency, the data were normalized to the CAT activity in cells ectopically expressing the RARα receptor and treated with 1 μM ATRA. The entire experiment was repeated several additional times with similar results.

Western Blot Analysis of PKCα

Following a 24-hr treatment with different concentrations of the various retinoids, B16 cells were washed twice with PBS, scraped into PBS, and transferred on ice to a 15-mL centrifuge tube. Cells were pelleted at 1000 *g* for 5 min and resuspended in 200 mL of protein lysis buffer (20 mM Tris–HCl, pH 7.5, containing 2 mM EDTA, 1% Triton X-100, 2 mM dithiothreitol, 50 mM NaF, 10 mM $NaHPO_4$, 1 μg/mL of leupeptin, 1 μg/mL of pepstatin, 50 μg/mL of aprotinin, 87 μg/mL of phenylmethylsulfonyl fluoride). Cells were vortexed and disrupted by sonication. The cell lysate was transferred to 1.5-mL microfuge tubes and centrifuged for 5 min at 12,000 *g*. Protein was determined by the bicinchoninic acid method (Pierce Chemical Co.) according to the manufacturer's instructions. Bovine serum albumin was used to construct a standard curve. Equal amounts of protein were separated by SDS–PAGE on 10% polyacrylamide gels and then electrophoretically transferred onto nylon membrane (Hybond C, Amersham) using a semi-dry blotting apparatus (Bio-Rad). The nylon membrane was incubated with 5% nonfat dry milk overnight at room temperature. The membrane was washed three times with TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 5 min each, at room temperature. Next, the membrane was incubated with a 1:500 dilution of antibody to PKCα (UBI) for 1 hr at room temperature. Subsequently, the membrane was washed as described above and incubated further with horseradish peroxidase-conjugated goat anti-mouse IgG at room temperature for 1 hr. The membrane was washed three times with TBST and developed using an ECL kit (Amersham).

Transient Transfection and AP-1 Activity

B16 cells were transfected using the lipofectin reagent with 1.5 μg of pGL-2-AP-1 DNA or the pGL-2 vector alone (1.0 μg) + 0.5 μg of SV40-β gal DNA to correct for transfection efficiency as described previously [27]. After an overnight incubation the transfection medium was removed, and the cells were incubated with different concentrations of the various retinoids as indicated in the results. Cells were harvested 48 hr after transfection and assayed for luciferase and β-galactosidase activity using kits from Promega. Luciferase activity in each individual sample was normalized by the amount of β-galactosidase activity in the same sample, to correct for the transfection efficiency between dishes of cells. All transfections were performed in triplicate dishes, and the experiments were repeated three to five times.

RESULTS

Transcriptional Activation Activity of Retinoids

Retinoids bind and activate the transcriptional activity of their nuclear receptors. We tested the ability of various retinoids to stimulate the transcription of a reporter gene containing a retinoid response element. Both ATRA and 9CRA had similar potency for activating RARα, β, and γ (Table 1). However, 9CRA was much more effective than ATRA in activating RXRα. This agrees with the poor binding affinity of the RXRs for ATRA. Activation of RXRα by ATRA at higher concentrations was probably due to its metabolism to 9CRA. At 10^{-8} M, Am580 specifically activated RARα; however, at higher concentrations, it also activated RARβ. SR11254 had greater selectivity for activating RARγ versus all the other receptors, whereas SR11246 was the most specific retinoid, with activity mostly limited to activating RXR. SR11346 is an interesting retinoid, since it activated both RARβ and RXRα, with a somewhat greater sensitivity for RXRα activation. Based on these results, we compared the effectiveness of the various retinoids for inhibiting anchorage-dependent and -independent growth, inducing RARβ and PKCα, and increasing AP-1 transcriptional activity at a 10^{-7} M concentration. The choice of this concentration was a compromise between inducing a measurable change in the parameter under investigation and retaining receptor selectivity.

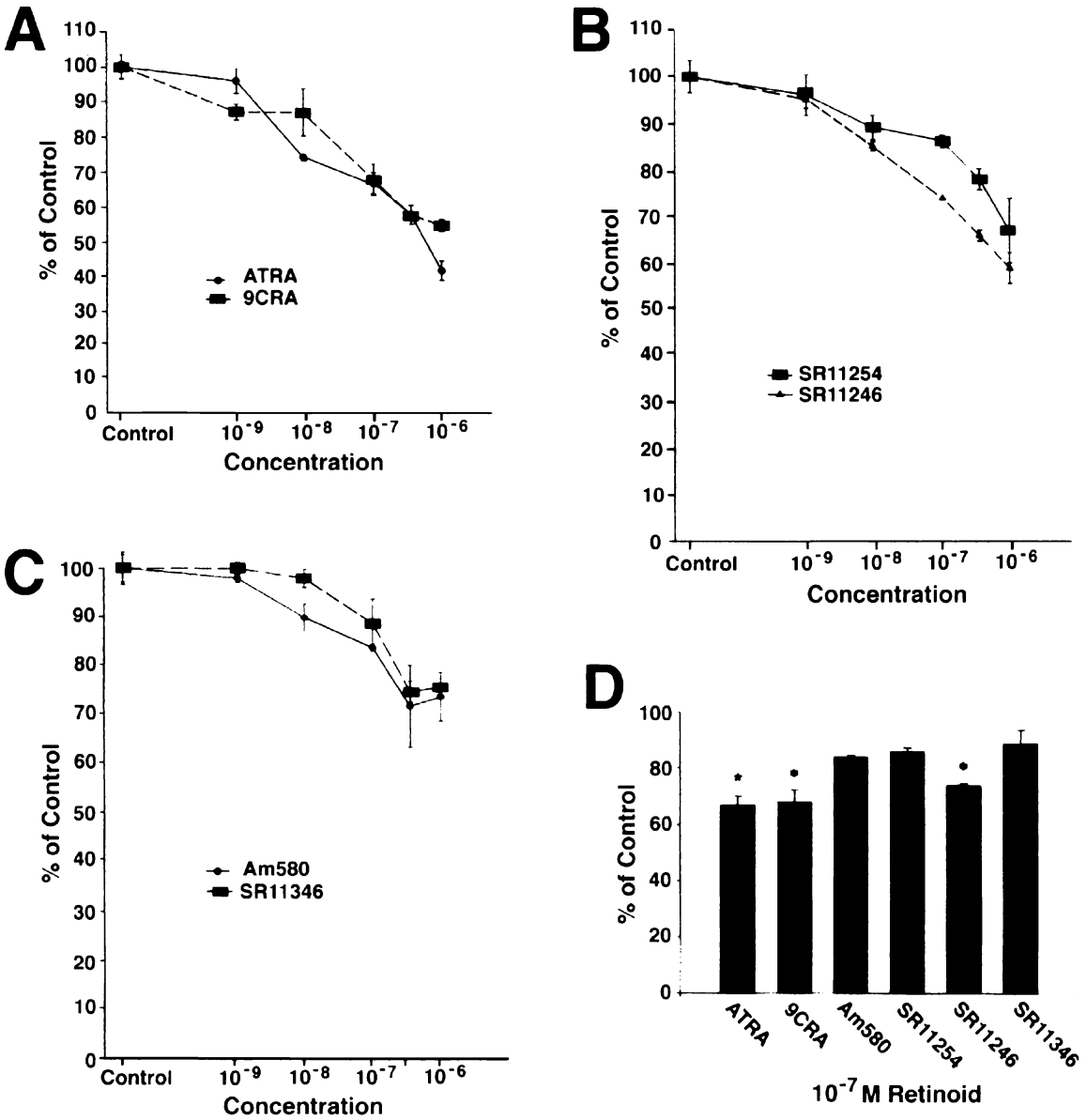

FIG. 1. Effect of various retinoids on B16 mouse melanoma anchorage-dependent growth. B16 cells were seeded at 5 × 10^4/60-mm tissue culture dish. After a 24-hr attachment period, cells were refed with growth medium containing the indicated concentrations of retinoids. After an additional 48 hr of incubation, cells were harvested, and their number was determined with a hemocytometer. Panels A, B, and C are the concentration–response curves for growth inhibition plotted as a percent of control cells (these cells only received the solubilization vehicle DMSO). The error bars represent the SEM of triplicate dishes of cells at each concentration of retinoid. Panel D compares the relative growth inhibition of all the retinoids tested at 10^{-7} M. Key: (*) significantly different from control at $P < 0.05$, using ANOVA followed by Newman–Keuls multiple comparisons. The experiment was repeated two additional times with similar results.

Regulation of Anchorage-Dependent Growth by Receptor-Selective Retinoids

We found previously that ATRA inhibits the growth of B16 mouse melanoma cells in a concentration-dependent manner [24]. Using both a pan-RAR/RXR agonist (9CRA) and receptor-selective retinoids, we determined whether any of the receptor-selective retinoids played a more dominant role in mediating the inhibition of anchorage-dependent growth. At the highest concentration tested (1 μM), we found that ATRA gave the greatest amount of inhibition of cell proliferation (Fig. 1A). Among the receptor-selective retinoids, SR11246 (RXR-selective) gave the greatest inhibition of growth (60% of control) at a 1 μM concentration (Fig. 1, B and C). When all of the retinoids were compared at a 10^{-7} M concentration, only ATRA, 9CRA, and SR11246 inhibited cell proliferation significantly (Fig. 1D).

Effect of Retinoids on Anchorage-Independent Growth

Normal somatic cells require substrate attachment to proliferate, whereas tumor cells acquire the ability to replicate without attachment to a substrate. This property of trans-

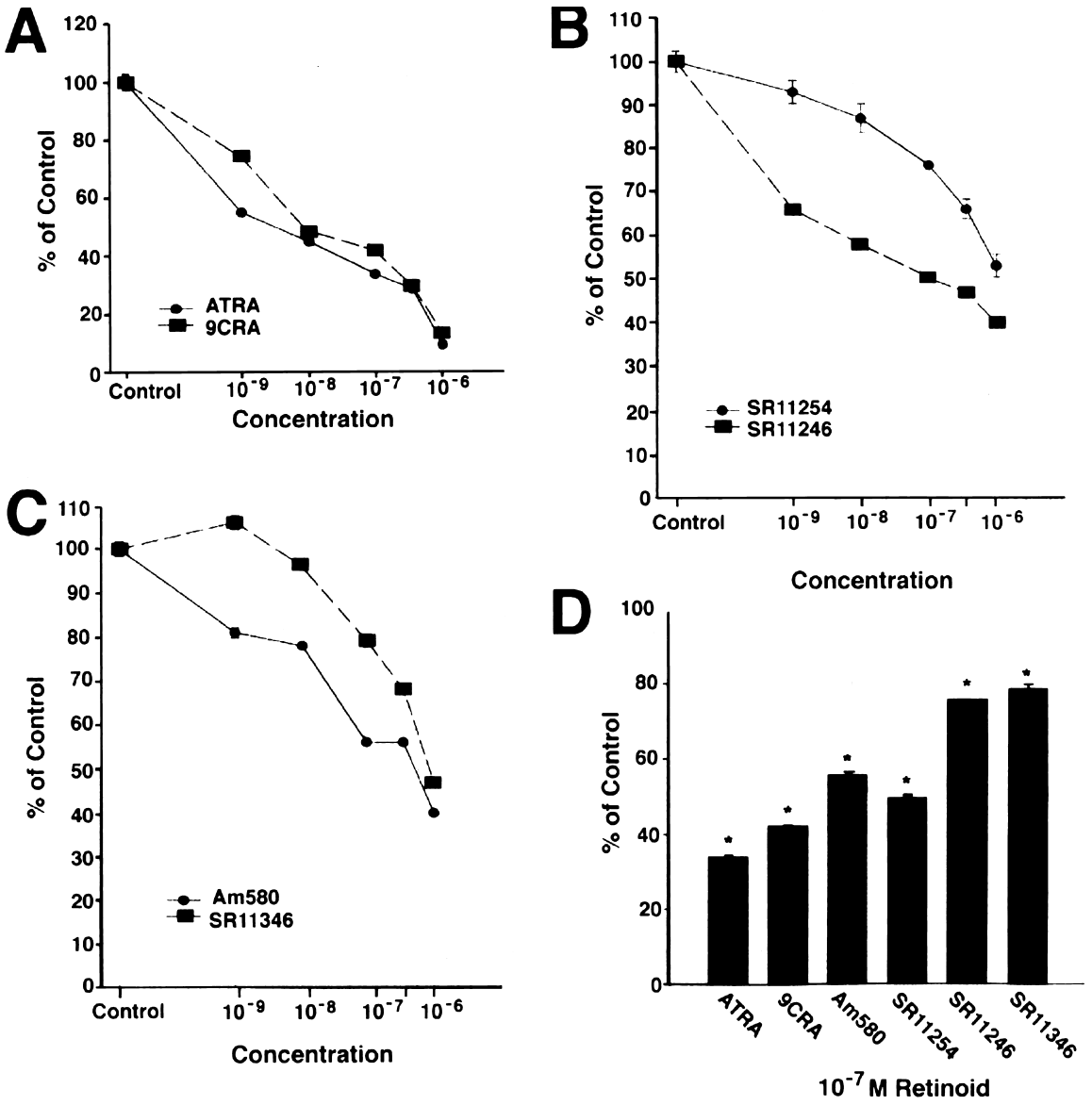

FIG. 2. Inhibition of B16 anchorage-independent growth by retinoids. B16 cells (5000) were mixed with 0.35% agarose in growth medium and overlaid onto a 1% agarose base in 6-well (35-mm) plates. All layers contained the indicated concentrations of retinoids. At the end of 10 days of incubation, the number of colonies containing more than 25 cells (colonies > 3 mm diameter) was determined. Panels A, B, and C depict the average number of colonies, with the error bars representing the SEM of triplicate dishes of cells for each concentration of retinoid tested. Data points without apparent error bars indicate that the SEM was smaller than the symbol. Panel D compares the inhibitory effect of all retinoids tested at a 10^{-7} M concentration. Key: (*) significantly different from control at $P < 0.05$ using ANOVA followed by Newman–Keuls multiple comparisons. This experiment was repeated two additional times with similar results.

formed cells is perhaps the best indicator of tumorigenicity. ATRA inhibits anchorage-independent growth of a number of tumor cell lines [33, 34]. We measured anchorage-independent growth in B16 cells by their ability to form spheroid colonies containing more than 25 cells in 0.35% agarose. All of the retinoids inhibited anchorage-independent growth in a concentration-dependent manner (Fig. 2). When compared at an equimolar concentration (10^{-7} M), all the retinoids inhibited colony formation in soft agarose significantly (Fig. 2D). Overall, ATRA was the most potent compound at this concentration (65% inhibition of colony formation), whereas among the receptor-selective retinoids, SR11254 (RARγ-selective) was somewhat more effective than Am580 (RARα-selective), with both of these compounds being considerably more effective than either SR11246 (pan-RXR) or SR11346 (pan-RXR, RARβ).

Induction of RARβ mRNA by Retinoids

Induction of RARβ is one of the early responses to ATRA treatment of B16 cells [28]. An increase in the level of this message can be detected within 1 hr of treatment. RARβ mRNA was induced at least 15-fold by the lowest concentration of ATRA tested (1 nM, Fig. 3A). This particular

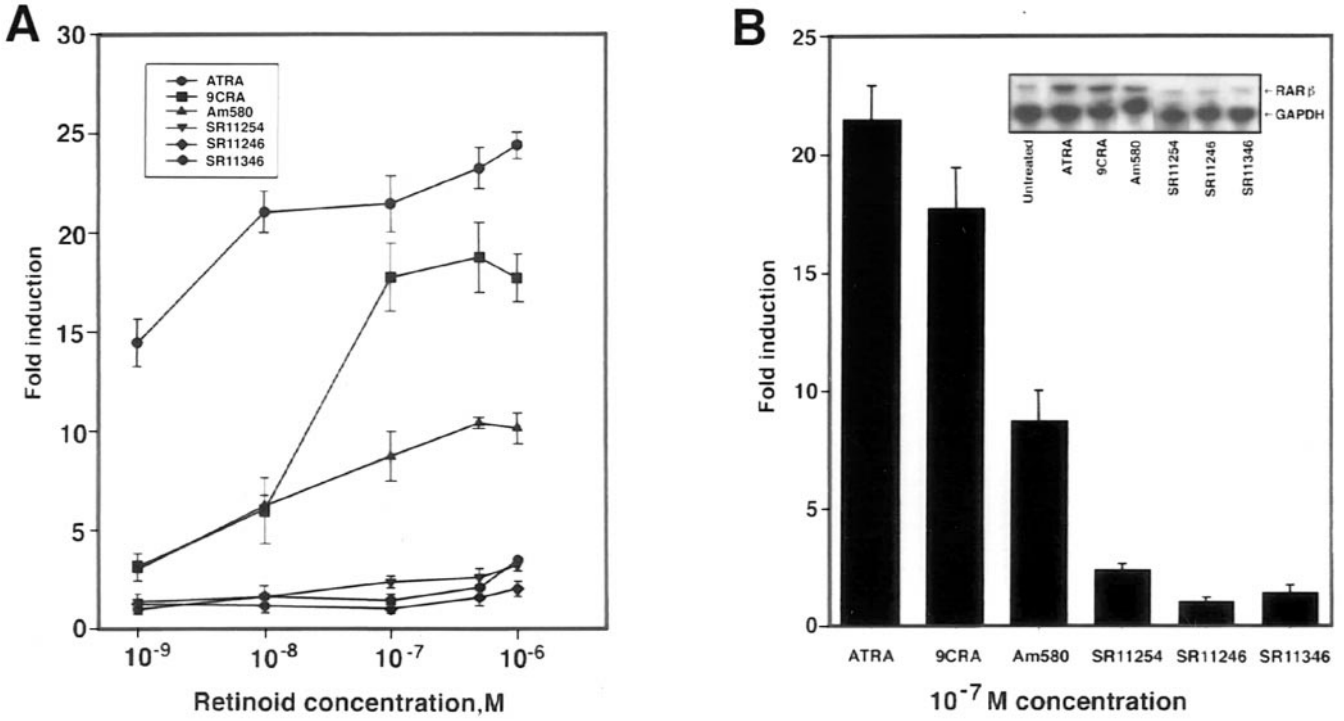

FIG. 3. Induction of RARβ mRNA by retinoids. B16 cells were grown to about 70% confluence in 100-mm tissue culture dishes, then refed with culture medium containing the indicated concentrations of the various retinoids, and incubated for 24 hr. Cells were harvested and processed for RNA purification and northern blotting as described in Materials and Methods. (A) Relative fold induction of RARβ mRNA by different concentrations of retinoids. Autoradiograms from the northern blots were quantitated using a Molecular Dynamics laser densitometer, and after correcting for the internal standard GAPDH a relative increase in RARβ mRNA was calculated using control cells treated only with solubilization vehicle (DMSO). The data are plotted as the average of three separate experiments ± SEM (error bars). (B) Relative fold induction of RARβ mRNA by various retinoids at 100 nM concentrations and representative northern blot (insert). Data are presented as the means ± SEM (error bars) from three separate experiments.

response was already maximal at 10 nM ATRA. 9CRA required a concentration of 100 nM to reach the maximal induction of RARβ mRNA (18-fold, Fig. 3A). Among the receptor-selective retinoids, only Am580 (RARα-selective) was able to achieve a substantial increase in the RARβ mRNA. When compared at a concentration of 10^{-7} M, the following ranking for inducing RARβ mRNA was observed (Fig. 3B): ATRA > 9CRA ≫ Am580 ≫ SR11254 > SR11346 > SR11246.

Increased Expression of PKCα Protein Induced by Retinoids

ATRA induces a large increase in PKCα mRNA and protein [35]. This increase in PKCα appears to play an important role in the ATRA-induced differentiation pathway, since overexpression of PKCα in untreated cells can mimic several of the properties of ATRA-treated cells, whereas down-regulation of PKC by phorbol esters antagonizes ATRA-induced differentiation [26]. To determine the role of RAR and RXR in PKCα induction, cells were treated for 24 hr with different concentrations of the various retinoids, and PKCα protein levels were determined by western blots. The most effective retinoid was ATRA, which induced a 6-fold increase in PKCα at a concentration of 1 nM (Fig. 4A). Surprisingly, the RAR/RXR pan-agonist 9CRA was not as effective, requiring a concentration of 100 nM to achieve an 8-fold induction of PKCα (Fig. 4B). However, at the maximum concentration tested (1 μM), 9CRA resulted in the highest fold increase in PKCα (9-fold). Among the receptor-selective retinoids, at concentrations where specificity is maintained (10^{-7} M), Am580 (RARα-selective) was the most effective and at 1 nM induced a 3- to 4-fold increase in PKCα (Fig. 4A).

Increase in AP-1 Activity Induced by Retinoids

We have reported that ATRA increases AP-1 transcriptional activity in B16 mouse melanoma cells [27]. This finding is in contrast to other cell types where retinoic acid inhibits AP-1 DNA binding and transcriptional activity [36–38]. To determine which receptors might mediate this response, we transfected B16 cells with an AP-1 reporter plasmid and then treated the transfected cells for 24 hr with different concentrations of the various retinoid analogs. 9CRA was the most effective compound, inducing a 2.6-fold increase in AP-1 activity at a concentration of 10^{-7} M (Fig. 5B). In contrast to other retinoid-induced changes, there was not a significant difference between any of the receptor-selective retinoids in their ability to increase AP-1 activity (Fig. 5, A and B).

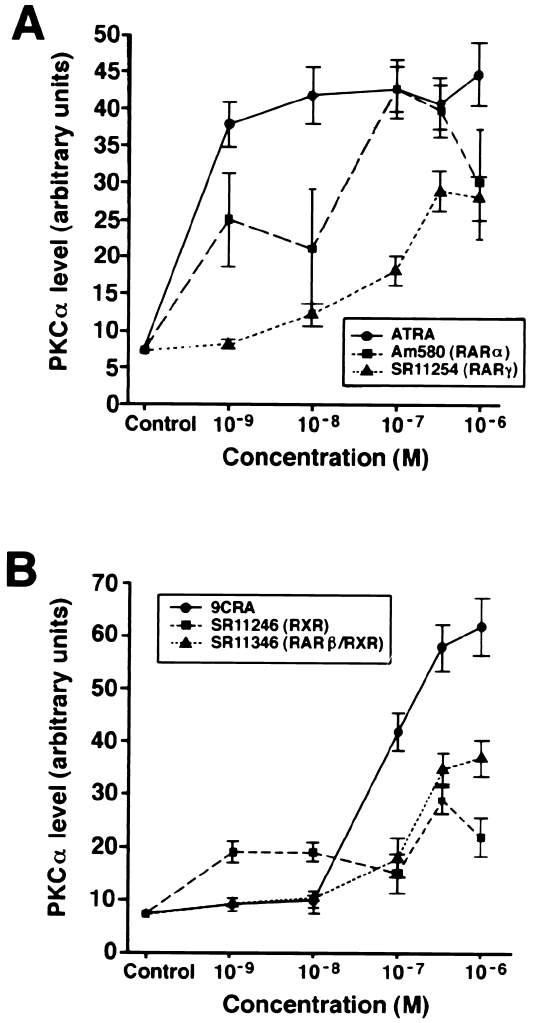

FIG. 4. Increase in PKCα protein levels in B16 cells treated with different retinoids. Cells were seeded at 2 × 10^5/100-mm tissue culture dish and allowed to attach for 24 hr. Then they were refed with growth medium containing the retinoids at the concentrations indicated in the figure. After a 24-hr incubation with these compounds, cells were harvested and assayed for the relative level of PKCα by western blotting as described in Materials and Methods. Autoradiograms were quantitated using a Molecular Dynamics laser densitometer and plotted as O.D. units. This experiment was repeated three additional times with similar results.

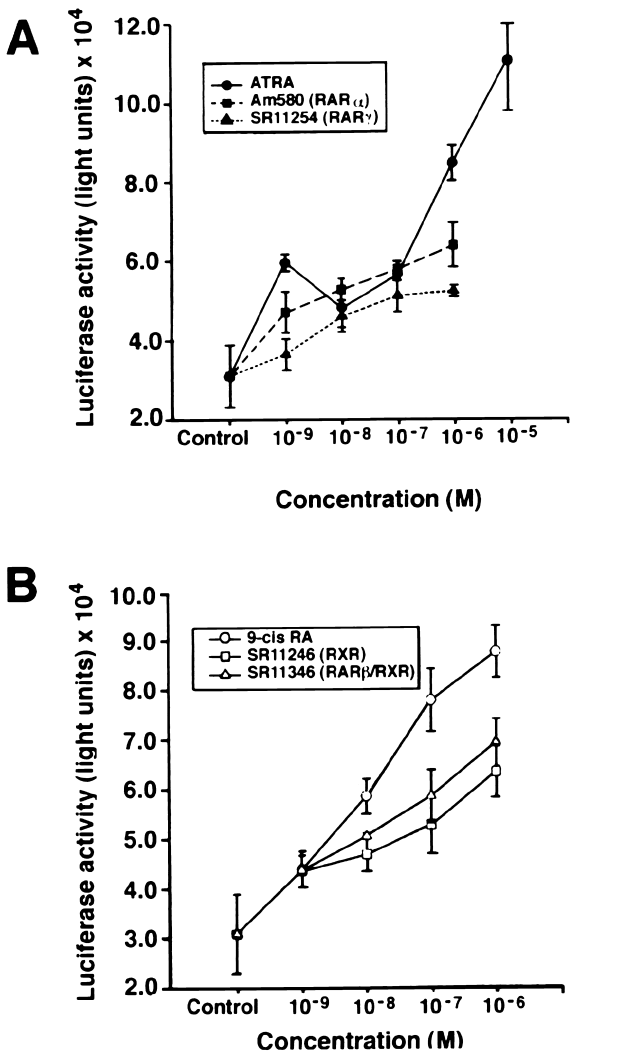

FIG. 5. AP-1 transcriptional activity in B16 cells treated with different retinoids. Cells were co-transfected with an AP-1-luciferase reporter plasmid and a plasmid encoding β-galactosidase (to correct for transfection efficiency) as described in Materials and Methods. After an overnight incubation, the transfection medium was removed, and the cells were refed with growth medium containing different concentrations of retinoids. Cells were harvested 48 hr after transfection and assayed for luciferase and β-galactosidase activities. The data are corrected for β-galactosidase activity and presented as the average luciferase activity ± the SEM (error bars) from triplicate dishes of transfected cells. The entire experiment was repeated three additional times with similar qualitative results. None of the retinoids increased luciferase expression in cells transfected with the luciferase plasmid lacking the AP-1 element (data not shown).

Interaction between RAR and RXR Ligands

It has been reported that RXR is a "silent partner" in the RXR/RAR heterodimer, which activates gene transcription [39]. However, since several recent reports have shown that liganded RXR increases the extent of gene transcription induced by the liganded RAR partner [40–42], we determined whether liganded RXR would increase the retinoid induction of PKCα further. Cells were treated for 24 hr with various retinoids alone or in combination (Fig. 6). Among the receptor-selective retinoids, Am580 (RARα) was most effective in inducing PKCα, verifying our earlier findings (Fig. 4). While the RXR agonist SR11246 did not increase the level of PKCα, it did enhance the induction due to ATRA (column 2 vs 8), Am580 (column 4 vs 9), and SR11254 (RARγ-selective) (column 5 vs 10).

DISCUSSION

B16 cells constitutively express RARα, RARγ, RXRα, and RXRβ. Treatment of the cells with ATRA induces the expression of RARβ [28]. Thus, the question arises as to whether these different receptors mediate different func-

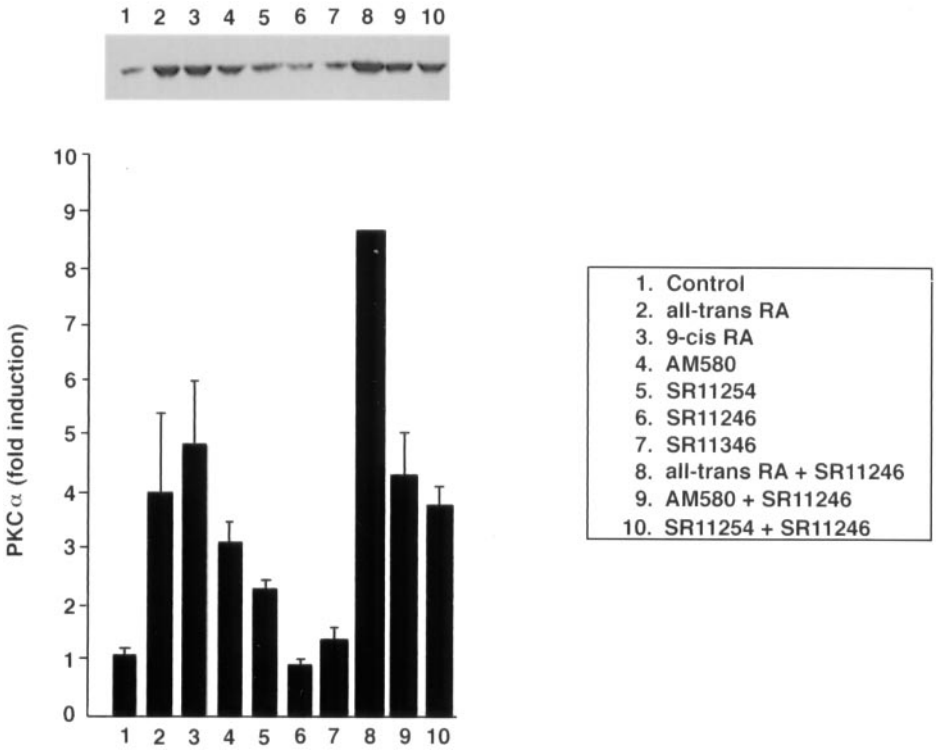

FIG. 6. Effect of retinoids alone and in combination on induction of PKCα protein in B16 cells. Cells were seeded at 2×10^5 in 100-mm tissue culture dishes. After a 24-hr attachment period, cells were refed with growth medium containing the various retinoids alone or the combinations listed. Single retinoids were present at a concentration of 1.0×10^{-7} M; retinoid combinations totaled 2×10^{-7} M (each retinoid component was present at 1.0×10^{-7} M). The data are presented as the mean induction of PKCα as determined by densitometric scans of the autoradiograms from three separate experiments. The error bars represent the SEM of the three experiments. The insert is a western blot of PKCα levels from a representative experiment.

tions during the ATRA-induced growth arrest and differentiation of B16 melanoma cells. The question of receptor function has been addressed using targeted gene disruption in transgenic animals [43–45]. The results of these studies initially led to the concept of total receptor redundancy, since inactivation of any one of the retinoid nuclear receptors did not cause any major defects in the development of the fetuses. However, recent experiments have provided evidence that some functional redundancy between RARs may be an artifactual consequence of gene knockouts [46, 47]. Also, disruption of either RARα or RARγ in F9 teratocarcinoma cells resulted in alterations in ATRA-induced differentiation and ATRA metabolism that were specific to the receptor inactivated [48].

Another approach to investigating nuclear retinoid receptor-specific functions is the use of retinoid analogs that have a greater affinity for binding and activating a specific class or subtype of receptor. Conformationally restricted analogs of ATRA have been synthesized and shown to exhibit receptor-selective activation profiles using the TREpal reporter plasmid [30, 32]. Therefore, we investigated the effect of receptor-selective retinoids on growth and various biochemical changes associated with ATRA-induced differentiation of B16 melanoma cells.

Both ATRA and 9CRA treatment of B16 cells resulted in a concentration-dependent inhibition of anchorage-dependent growth. Interestingly, when receptor-selective retinoids were compared at 0.1 μM concentrations, the RXR-selective ligand SR11246 was the most effective analog for inhibition of growth and also caused the greatest level of inhibition at the maximal concentration used in these experiments. This particular retinoid showed high selectivity for activating RXR in transient transfection assays using the TREpal response element (Table 1), and we have shown recently that it was very effective in activating transcription of a reporter gene containing a DR-1 type RXR response element (Niles RM and Desai SH, unpublished data). RXR-selective retinoids have been shown to be relatively poor inhibitors of cell proliferation in various tumor cell lines [49–51]. However, these retinoids enhance apoptosis in HL-60 cells [52], significantly inhibit the proliferation of three different human prostate cancer cell lines [53], and inhibit the growth of ATRA-resistant human breast cancer cells [54]. It should be noted that we did not observe an increase in apoptosis in B16 cells treated with any retinoid (data not shown). The mechanism by which liganded RXR can inhibit B16 cell growth is not known. RXR agonists alone cannot activate transcription of the RXR:RAR heterodimer due to steric blockage of the ligand binding site by the RAR partner. Ligand binding to RAR removes this block, allowing RXR to bind ligand [55]. In certain RXR heterodimers, binding of ligand to either partner can activate transcriptional activity [56, 57]. Thus, it is possible that other RXR heterodimers are responsible for SR11246-induced growth inhibition in B16 cells.

All retinoids tested were capable of inhibiting anchorage-independent growth (soft agar colony formation). Among the receptor-selective retinoids, SR11254 (RARγ-selective) was somewhat more effective than Am580. The least effective retinoids were SR11246 and SR11346 (RXR- and RXR/RARβ-selective, respectively). Thus, in contrast to anchorage-dependent growth, RXR appears to play a less

important role in suppressing anchorage-independent growth.

One of the early changes in retinoic acid-induced differentiation of B16 cells is a direct stimulation of RARβ gene expression [28]. ATRA was quite potent in inducing the expression of RARβ mRNA (Fig. 3A). 9CRA was also quite effective in inducing RARβ mRNA, but required higher concentrations than ATRA and did not reach the maximal fold induction achieved by ATRA. Among the receptor-selective retinoids, only Am580 was able to induce a major increase in RARβ mRNA levels. These results suggested that in B16 cells the RARα receptor plays the major role in the induction of the RARβ receptor.

We previously reported that ATRA treatment of B16 cells results in a marked increase in PKCα mRNA and protein levels [35] and that this change plays an important role in retinoid-induced growth arrest and differentiation [26]. When receptor-selective retinoids were tested for their ability to induce PKCα protein, Am580, the RARα-selective retinoid, was most potent, being equally effective as ATRA at a 0.1 μM concentration. Other laboratories have reported that RARα appears to play a more prominent role in both growth arrest and specific gene induction in human breast cancer cells [32, 58]. We have shown previously that the induction of PKCα mRNA by ATRA is blocked by inhibitors of protein synthesis [26], indicating that another retinoid-induced protein is required for the increase in PKCα expression. Since Am580 is the only receptor-selective retinoid that significantly induced RARβ, we favor the hypothesis that RARα activates transcription of the RARβ gene, and this receptor (RARβ), in turn, induces expression of the PKCα mRNA and protein.

We have reported recently that, unlike other cell types, treatment of B16 cells with ATRA does not inhibit AP-1 activity, but instead stimulates the activity of this transcription factor complex [27]. Differentiation of melanoma cells has been found to be associated with an increase in AP-1 activity [59]. All of the receptor-selective retinoids stimulated AP-1 activity about 1.7- to 1.9-fold at a 0.1 μM concentration. Thus, it appears that there was no receptor selectivity for stimulating AP-1 activity. The mechanism by which retinoids can increase AP-1 transcriptional activity in B16 cells is at present unknown.

Previous work has suggested that the RXR:RAR heterodimer functions optimally when only the RAR site is bound by ligand [39]. In contrast, other investigators have reported that maximal stimulation of specific gene expression or biologic functions, such as differentiation, requires ligand bound to each partner of the heterodimer [40, 42]. To investigate this question we examined the effects of combinations of receptor-selective retinoids on the induction of PKCα protein. At the concentrations used in these experiments, the RXR agonist SR11246 did not increase PKCα, but when it was combined with an RAR agonist (ATRA, Am580, or SR11254), a further enhancement of PKCα induction was achieved. These results suggest that activation of RAR via ligand binding is obligatory for induction of PKCα, and that ligand binding to RXR is not required. However, ligand binding to RXR can enhance the expression of PKCα further. It has been shown that this heterodimeric subordination of RXR AF-2 activity is due to an allosteric effect of unliganded RAR on the interaction surface for co-activators in the RXR molecule [60].

In summary, our results suggest that different nuclear retinoid receptors have different roles in mediating retinoid-induced growth arrest and differentiation of B16 mouse melanoma cells. Although one could make the argument that differences observed in these experiments could be due to differences in uptake and/or metabolism of the various retinoids, two types of results suggest that this is not the case. First, treatment of cells transfected with a retinoic acid-response element–reporter gene with receptor-selective retinoids results in an *in vivo* activation of RAR transcriptional activity, which correlates with their *in vitro* binding to the different RAR/RXR. Second, the biochemical/cellular changes measured in this study did not all have the same pattern for retinoid selectivity, which would be expected if only different rates of retinoid metabolism were responsible for the observed changes. The RXRs appear to be involved in retinoid-induced inhibition of anchorage-dependent growth, whereas the RARs, especially RARγ, are the primary players in anchorage-independent growth. In terms of induction of specific genes, the RARs, especially RARα, appear to play the dominant role. Retinoid-induced increase in AP-1 transcriptional activity did not appear to depend on any specific retinoid receptor preference, and thus occupancy of either RAR or RXR with ligand may be sufficient to initiate this response. Lastly, it is clear that, at least for induction of PKCα, RXR is not a "silent" partner of the heterodimer, since occupancy of this receptor with ligand maximized the induction of this protein. These results support the concept that retinoid analogs can be designed to target specific cellular functions, thus enhancing the therapeutic usefulness of retinoids.

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