5-15-2000

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Recommended Citation

Desai SH, Boskovic G, Eastham L, Dawson M, Niles RM (2000) Effect of receptor-selective retinoids on growth and differentiation pathways in mouse melanoma cells. Biochem Pharmacol 59(10):1265-1275.

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

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Running Title: Receptor-Selective Retinoids

Classification: Hormones and Growth Factors

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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; PKCα, protein kinase Cα

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 RARβ expression, an increase in protein kinase Cα (PKCα) expression and enhanced activator protein-1 (AP-1) transcriptional activity. These cells constitutively express nuclear retinoic acid receptors (RAR) α and γ and nuclear retinoid X receptors (RXR) α and β. We tested the ability of receptor-selective retinoids to induce the biochemical changes found in ATRA-treated melanoma cells and also their effectiveness in decreasing anchorage-dependent and independent growth. The RXR-selective ligand SR11246 was most effective at inhibiting anchorage-dependent growth, while the RARγ-selective ligand SR11254 was most potent at inhibiting anchorage-independent growth. In contrast, 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 dose-dependent increase in AP-1 transcriptional activity with little difference in the 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 preferentially activate different growth and differentiation pathways in B16 melanoma cells, most likely due to their ability to activate a different subset of receptors.

KEY WORDS: receptor-selective retinoids, melanoma, proliferation, differentiation

Retinoids have been found to inhibit growth and induce differentiation in a variety of cancer cell lines [1-5]. These effects are thought to be mediated by nuclear receptors which belong to the super-family of steroid/thryoid hormone receptors. There are two major classes of retinoid nuclear receptors, the RARs and the RXRs. The RARs bind ATRA and 9CRA, while the RXR 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 consists of a direct repeat of PuG(G,T)TCA with a 1 to 5 base pair spacing between the repeats [13-15]. Both RARs and RXRs can form homo (RXR:RXR) or hetero (RXR:RAR) dimers, 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/thryoid 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 G1 phase of the cell cycle [25], loss of anchorage-independent growth, and acquisition of cell surface NGF receptors [24]. The pathway that leads to this differentiated phenotype is not known, but induction of PKCα plays a significant role [26]. Retinoic acid treatment also leads to a 4-fold increase in AP-1 transcriptional activity, which in contrast to TPA-induced AP-1 activity, is sustained for at least 36 h [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 β 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 SR11246 was most potent in inhibiting anchorage-dependent growth, while SR11254 (RARγ-selective) was most potent in inhibiting anchorage-independent growth. In contrast, 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 with ligand.

MATERIAL AND METHODS

Retinoids

All-*trans* -retinoic acid was purchased from Sigma Chemical Co. St. Louis, MO. 9-*cis* -retinoic acid, Am580 (RARα-selective), SR11254 (RARγ-selective), SR11246 (RXR-selective) and SR11346 (RARβ/RXR-selective) were synthesized as previously described [31, 32]. All retinoids were dissolved in DMSO and kept under nitrogen gas at -80^{o}C. 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 (DMEM) supplemented with 10% heat-inactivated calf serum (Sterile Systems, Logan, UT), 2 mM L-glutamine, 2 mM sodium pyruvate, 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate. Cells were incubated at 37°C in a 5% CO_2/95% air, humidified atmosphere. New cultures were initiated from frozen stock every 6 weeks in order 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 x 10^4 /well in 6 well tissue culture plates . One day after seeding cells were refed with tissue culture media containing different concentrations of retinoids. Cells were incubated with the retinoids for 48 h and then harvested, and cell number 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, 5,000 cells were mixed with 0.35% agarose in tissue culture media, and this mixture overlaid onto a 1% agarose base in 60 mm tissue culture dishes. Lastly, a small amount of tissue culture media was added to cover the top layer. All three layers contained retinoids at the concentrations indicated in the figures. Every two days, the top layer of media with or without the appropriate concentration of retinoid was replenished and after ten days of incubation the number of colonies containing greater 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 h. Control cell received the solublization agent DMSO. Total RNA was isolated from cells using the TRI regent (Sigma, St. Louis, MO, according to the manufacturer's protocol. 30-40 μg of total RNA was separated on 1% formaldehyde agarose gels and transfered to

Hybond NX membranes (Amersham, Arlington Heights, IL). Following prehybrdization, blots were probed simultaneously for RARβ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Full length GAPDH cDNA and a 615 bp EcoRI fragment from mouse RARβ cDNA were used as probes. These DNAs were labeled using the Prime-a-Gene labeling system (Promega, Madison, WI) according to the manufacturer's protocol. Unincorporated nucelotides were removed using QIAquick Nucleotide Removal kit (Qiagen, Chatsworth, CA). Hybridization of the probes was performed at 68^{o} C for 1 h using Express Hyb solution (Clontech, Palo Alto, CA). 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^{o} C in 15 mM NaCl, 1.5 mM sodium citrate (pH 7.0), 0.1% SDS. Blots were then exposed to X-ray film at -80^{o} C for various times in order to generate a signal in the linear range for densitometry (Molecular Dynamics, Sunnyvale, CA). Intensity of the RARβ bands was normalized to the corresponding GAPDH band and the results expressed as a fold induction relative to the RARβ mRNA signal in control cells.

Western Blot Analysis of PKCα

Following a 24 h 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 x 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 Na HPO_4, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml aprotinin, 87 μg/ml PMSF). 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 x g. Protein was determined by the BCA method (Pierce Chemical Co, Rockford, IL) 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, Chicago, IL) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA). The nylon membrane was incubated with 5% non-fat dry milk overnight at room temperature. The membrane was then washed 3x with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 5 m each, at room temperature. The membrane was then incubated with a 1:500 dilution of antibody to PKCα (UBI, Saranac Lake, NY) for 1 h at room temperature. Subsequently, the membrane was washed as described above and further incubated with horseradish peroxidase conjugated goat-anti-mouse IgG at room temperature for 1 h. The membrane was washed three times with TBST and developed using the ECL kit (Amersham, Chicago, IL).

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 incubated with different concentrations of the various retinoids as indicated in the results. Cells were harvested 48 h after transfection and assayed for luciferase and β-galactosidase activity using kits from Promega (Madison, WI). Luciferase activity in each individual sample was normalized by the amount of β-galactosidase activity in the same sample, in order to correct for the transfection efficiency between dishes of cells. All transfections were performed in triplicate dishes and the experiments were repeated 3-5 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 activates RARα , however, at higher concentrations, it also activates RARβ. SR11254 has greater selectivity for activating RARγ vs all the other receptors, while SR11246 is the most specific retinoid with activity mostly limited to activating RXR. SR11346 is an interesting retinoid, since it activates 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, induction of RARβ, and PKCα and increasing AP-1 transcriptional activity at 10^{-7}M concentration. The choice of this concentration was a comprimise between inducing a measureable change in the parameter under investigation and retaining receptor selectivity.

Regulation of anchorage-dependent growth by receptor-selective retinoids.
We have previously found that ATRA inhibited the growth of B16 mouse melanoma cells in a concentration-dependent manner [24]. Using both 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. 1 panel A). Among the receptor-selective

retinoids, SR11246 (RXR-selective) gave the greatest inhibition of growth (60% of control) at 1 μM concentration (Fig. 1 panels B and C). When all of the retinoids were compared at 10^{-7}M concentration, only ATRA, 9CRA, and SR11246 significantly inhibited cell proliferation (Fig. 1, panel D).

Effect of retinoids on anchorage-independent growth

Normal somatic cells require substrate attachment in order to proliferate, whereas tumor cells acquire the ability to replicate without attachment to a substrate. This property of transformed 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 greater 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 significantly inhibited colony formation in soft agarose (Fig. 2, panel D). Overall, ATRA was the most potent compound at this concentration (65% inhibition of colony formation), while 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 h of treatment. RARβ mRNA was induced at least 15-fold by the lowest concentration of ATRA tested (1 nM, Fig. 3A). This particular response was

already maximal at 10 nM of 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 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, while down-regulation of PKC by phorbol esters antagonizes the ATRA-induced differentiation [26]. To determine the role of RAR and RXR in PKCα induction, cells were treated for 24 h with different concentrations of the various retinoids and PKCα protein levels were determined by Western blots. The most effective retinoid was ATRA, which induced an 6-fold increase in PKCα at a concentration of 1 nM (Fig. 4, panel A). 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. 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 3-4 fold increase in PKCα (Fig. 4A).

Increase in AP-1 activity 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

RA inhibits AP-1 DNA binding and transcriptional activity [36-38]. To examine which receptors might mediate this response we transfected B16 cells with an AP-1 reporter plasmid and then treated the transfected cells for 24 h with different concentrations of the various retinoid analogs. 9CRA was the most effective compound, inducing an 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. 5A and B).

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 further increase the retinoid induction of PKCα. Cells were treated for 24 h 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 RAR α, γ and RXRα, β. Treatment of the cells with ATRA induces the expression of RARβ [28]. Thus the question arises as to whether these different receptors mediate different functions 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 lead 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 knock-outs [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 which was inactivated[48].

Another approach to investigating nuclear retinoid receptor-specific functions is the use of retinoid analogs which have a greater affinity for binding and activating a specific class or subtype of receptor. Conformationally restricted analogs of ATRA were 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 shows high selectivity for activating RXR in transient transfection assays using the TREpal response element (Table 1), and we have recently shown that it is very effective in activating transcription of a reporter gene containing a DR-1 type RXRE (Niles and Desai, 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 enhanced apoptosis in HL-60 cells [52], significantly inhibited the proliferation of three different human prostate cancer cell lines [53] and inhibited 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 can not 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 RA-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 suggest 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 and that this change plays an important role in retinoid-induced growth arrest and differentiation [35, 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 0.1 μM concentration. Other laboratories have reported that RARα appears to play a more prominant role in both growth arrest and specific

gene induction in human breast cancer cells [32, 58]. We have previously shown that the induction of PKCα mRNA by ATRA is blocked by inhibitors of protein synthesis, indicating that another retinoid-induced protein is required for the increase in PKCα expression. Since Am580 is the only receptor-selective retinoid which 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 recently reported 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- slective retinoids stimulated AP-1 activity about 1.7 - 1.9 fold at 0.1 μM concentration. Thus it appears that there is 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, require ligand bound to each partner of the heterodimer [40, 42]. To investigate this question we examined 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 ligand binding to RXR is not required. However, ligand binding to RXR can further enhance the expression of PKCα. It has been shown that this heterodimeric subordination of RXR AF-2 acitivity is due to an allosteric

effect of unliganded RAR on the interaction surface for co-activators in the RXR molecular [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 altered metabolic rates of the various retinoids, two types of results suggest that this is not the case. First, treatment of cells transfected with an RARE –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 altered rates of retinoid metabolism were responsible for the observed changes. The RXR appear to be involved in retinoid-induced inhibition of anchorage-dependent growth while the RAR, especially RARγ , are the primary players in anchorage-independent growth. In terms of induction of specific genes, the RAR, 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 occupany 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.

Acknowledgements

We would like to thank Margaret McFarland for constructing all the illustrations in this manuscript and Dr. Beverly Delidow for critical review and

discussion of this work. This work was supported in part by Public Health Service Grant CA 59530 from the National Cancer Institute (R.M.N.)

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FIGURE LEGENDS

Fig. 1. The effect of various retinoids on B16 mouse melanoma anchorage-dependent growth.

B16 cells were seeded at 5 x 10^4 /60 mm tissue culture dish. After a 24 h attachment period, cells were refed with growth medium containing the indicated concentrations of retinoids. After an additional 48 h of incubation, cells were harvested and their number determined with a hemocytometer. A, B, and C are the concentration -response curves for growth inhibition plotted as a % 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. (*) : means were 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

Fig. 2. Inhibition of B16 anchorage-independent growth by retinoids.

B16 cells (5,000) were mixed with 0.35% agarose in growth media 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 ten days of incubation the number of colonies containing greater 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 less than the symbol. Panel D compares the inhibitory

effect of all retinoids tested at 10^{-7}M concentration. Notice that all retinoids are significantly different from control (*) at $p < 0.05$ using ANOVA followed by Neuman-Keuls multiple comparisons. This experiments was repeated two additional times with similar results.

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 h. Cells were then harvested and processed for RNA purification and Northern blotting as described in 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 is 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 is presented as the mean +/- SEM (error bars) from three separate experiments.

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 h. They were then refed with growth medium containing the retinoids at the concentrations indicated in the figure. After a 24 h incubation with these compounds, cells were harvested and assayed for the relative level of PKCα by Western blotting as described in Methods. Autoradiograms were

quantitated using a Molecular Dynamics laser densitometer and plotted as OD 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 Methods. After an overnight incubation, the transfection medium was removed and the cells refed with growth medium containing different concentrations of retinoids. Cells were harvested 48 h 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)

Fig. 6. The 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 h 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 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.