The Role of Specific Retinoid Receptors in Sebocyte Growth and Differentiation in Culture[1]

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Retinoic acid derivatives (retinoids) exert their pleiotropic effects on cell development through specific nuclear receptors, the retinoic acid receptors and retinoid X receptors. Despite recent progress in understanding the cellular and molecular mechanisms of retinoid activity, it is unknown which of the retinoid receptor pathways are involved in the specific processes of sebocyte growth and development. In this study, we investigated the roles of specific retinoid receptors in sebocyte growth and differentiation, by testing the effects of selective retinoic acid receptor and retinoid X receptor ligands at concentrations between 10^{-10} M and 10^{-6} M in a primary rat preputial cell monolayer culture system. Cell growth was determined by number of cells and colonies, and cell differentiation by analysis of lipid-forming colonies. All-*trans* retinoic acid and selective retinoic acid receptor agonists (CD271 = adapalene, an RAR-β,γ agonist; CD2043 = retinoic acid receptor pan-agonist; and CD336 = Am580, an RAR-α agonist) caused significant decreases in numbers of cells, colonies, and lipid-forming colonies, but with an exception at high doses of all-*trans* retinoic acid (10^{-6} M), with which only a small number of colonies grew but they became twice as differentiated as controls ($42.2 \pm 4.0\%$ *vs* $22.6 \pm 2.7\%$, mean $\pm$ SEM, lipid-forming colonies, $p < 0.01$). Furthermore, the RAR-β,γ antagonist CD2665 antagonized the suppressive effects of all-*trans* retinoic acid, adapalene, and CD2043 on both cell growth and differentiation. In contrast, the retinoid X receptor agonist CD2809 increased cell growth slightly and lipid-forming colonies dramatically in a clear dose-related manner to a maximum of $73.7\% \pm 6.7\%$ at 10^{-6} M ($p < 0.001$). Our data suggest that retinoic acid receptors and retinoid X receptors differ in their roles in sebocyte growth and differentiation: (i) retinoic acid receptors, especially the β and/or γ subtypes, mediate both the antiproliferative and antidifferentiative effects of retinoids; (ii) retinoid X receptors mediate prominent differentiative and weak proliferative effects; (iii) the antiproliferative and antidifferentiative effects of all-*trans* retinoic acid are probably mediated by retinoic acid receptors, whereas its differentiative effect at high dose may be mediated by retinoid X receptors via all-*trans* retinoic acid metabolism to 9-*cis* retinoic acid, the natural ligand of retinoid X receptors. *Key words: retinoic acid/retinoic acid receptor/retinoid X receptor. J Invest Dermatol 114:349–353, 2000*

Sebocytes are specialized sebaceous gland epithelial cells that differentiate by accumulating neutral fat droplets until they burst and liberate their holocrine secretion, sebum (Wheatley, 1986). Although retinoic acid (RA) and its derivatives (retinoids) in trace amounts promote sebocyte growth and differentiation, larger doses induce atrophy of sebaceous glands and a decrease in lipid production (Landthaler *et al*, 1980; Zouboulis *et al*, 1993). Retinoids have long been used for treatment of acne vulgaris, yet the role of specific retinoid receptors in mediating the diverse retinoid effects is unclear.

The highly pleiotropic effects of retinoids appear to be mediated by specific nuclear receptors that belong to the superfamily of nuclear receptors and act as ligand-dependent transcriptional regulators (Evans, 1988; Green and Chambon, 1988). Two distinct classes of retinoid receptors have been identified: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). Each class of receptor contains three subtypes: α, β, and γ (reviewed by Mangelsdorf *et al*, 1995). The natural ligands of RARs and RXRs have been identified as all-*trans* RA (ATRA) and 9-*cis* RA, respectively (Petkovich *et al*, 1987; Heyman *et al*, 1992; Levin *et al*, 1992). RAR functions only as a heterodimer with RXR (Durand *et al*, 1992; Kliewer *et al*, 1992a; Zhang *et al*, 1992a). In contrast, RXR forms either homodimers or heterodimers with other ligand-regulated receptors including peroxisome proliferator-activated receptors (PPARs) and thus functions as a key regulator of the activity of several nuclear receptors (Zhang *et al*, 1992b; Mangelsdorf and Evans, 1995). The characterization of DNA response elements has revealed a complex pattern of retinoid recognition and activation (Mangelsdorf *et al*, 1995).

Manuscript received April 13, 1999; revised October 22, 1999; accepted for publication November 3, 1999.

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Abbreviations: ATRA, all-*trans* retinoic acid; LFC, lipid-forming colony; PPAR, peroxisome proliferator-activated receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor.

[1]Presented in part at the 1999 Annual Meeting of the Pediatric Academic Societies, San Francisco, CA, May 1–4 (*Pediat Res* 45 (Part 2): 55A [Abst 313] 1999)

As current retinoid research targets the development of receptor-selective retinoids for tailoring and/or improving their therapeutic profiles, it seems important to know which of the retinoid receptor pathways are involved in the different cellular processes. Previous studies have addressed the role of ATRA, 9-*cis* RA and other retinoids in attenuating sebocyte growth and differentiation (reviewed by Geiger, 1995). It has been difficult, however, to identify the distinct role of each retinoid receptor, since ATRA may not only act directly on RAR but may also act on RXR via its metabolism to 9-*cis* RA (Heyman *et al*, 1992; Levin *et al*, 1992). Recently, selective RAR and RXR ligand-activators and their antagonists have become available to dissect the molecular pathways involved in retinoid action (reviewed by Orfanos *et al*, 1997). In this study, using selective RAR and RXR ligands and a primary rat preputial cell monolayer culture system, we have investigated the role of the different classes and subtypes of retinoid receptors in sebocyte growth and differentiation.

MATERIALS AND METHODS

Retinoids ATRA was purchased from Sigma (St. Louis, MO). Other retinoids, characterized in **Table I**, were supplied by Galderma R & D (Sophia Antipolis, France). Retinoids were dissolved in dimethyl sulfoxide to a concentration of 10^{-3} M and stored at –20°C in light-proof containers. The half-maximal activating concentrations obtained in the F9 cell model and the binding properties of these compounds (Martin *et al*, 1992; Charpentier *et al*, 1995; unpublished data) are shown in **Table I**.

Cell culture Single cell suspensions were prepared from the preputial glands of young adult male Sprague-Dawley rats and plated on a mitomycin-C-treated 3T3-J2 fibroblast feeder layer at a density of approximately 75,000 preputial cells and 200,000 3T3 cells per 35 mm well in Dulbecco's modified Eagle's medium with 10% fetal calf serum. On day 3 the medium was switched to a serum-free chemically defined cell culture medium containing 4×10^{-8} M vitamin A (Cellgro Complete, Mediatech, VA), which was supplemented with 10^{-6} M insulin, 10^{-10} M choleratoxin, 10^{-6} M hydrocortisone, and antibiotics as previously reported (Laurent *et al*, 1992; Rosenfield *et al*, 1999). Retinoids were added to the medium under subdued ambient light at concentrations between 10^{-10} M and 10^{-6} M from day 3 to day 9, during which time the medium was changed every other day. In each experiment treatments were added simultaneously in triplicate.

Cell growth Cell growth was assessed by counting the number of cells and colonies on day 9. After 3T3 cells were removed by a brief incubation with 0.02% ethylenediamine tetraacetic acid, cell suspensions were prepared by incubation of cultured cells with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid for 10 min and repeated pipettings. After pelleting at $1000 \times g$, cells from triplicate 35 mm wells were resuspended into phosphate-buffered saline and counted twice in a hemocytometer. The total number of sebocyte colonies per well was counted by light microscopy at 25× magnification after staining (below).

Cell differentiation Cell differentiation was assessed by lipid staining and analysis of lipid-forming colonies (LFCs) on day 9 (Rosenfield *et al*, 1999). Cell cultures were fixed and stained with Oil Red O. Lipid formation in sebocyte colonies was quantified by light microscopy at 25–40× in four groups according to the number of stained cells per colony: 0, 1–5, 6–50, and >50. An LFC was defined as a colony containing more than five Oil Red O-staining cells, i.e., those with fused lipid droplets equivalent to a mid- or late-differentiated stage of sebocyte development.

Statistical analysis One-way analysis of variance followed by Fisher's protected least differences *post hoc* testing was used to compare the various treatments. Statistical analyses were performed using the Statview program, and a p-value (two-tailed) < 0.05 was considered statistically significant.

RESULTS

Effect of ATRA and selective RAR agonists on sebocyte growth As shown in **Fig 1**(***a***, ***b***), ATRA, adapalene (a selective RAR-β,γ agonist), CD2043 (a selective RAR pan-agonist), and CD336 (a selective RAR-α agonist), all of which activate RARs, inhibited cell number and colony growth in a dose-dependent manner. Subtle differences were noted at 10^{-6} M, however. ATRA showed a clear dose-related inhibitory effect to a maximum of approximately 20% of control values at 10^{-6} M, but CD2043 and CD336 at 10^{-6} M showed no more inhibitory effect than at 10^{-8} M and 10^{-7} M, respectively. In contrast, adapalene completely obliterated cell proliferation at 10^{-6} M. Adapalene was then tested in combination with CD336 at 10^{-7} M and 10^{-6} M in a separate set of experiments ($n = 4$, not shown) to determine whether the profound inhibitory effect of the high dose RAR-β,γ agonist would be prevented by the selective RAR-α agonist. The combination showed no evidence of interaction between the RAR-α and RAR-β,γ agonists, neither additive nor inhibitory, and no growth occurred in any incubations with adapalene at 10^{-6} M.

Effect of ATRA and selective RAR agonists on sebocyte differentiation Approximately 20% of untreated colonies were differentiated. At 10^{-8} M, ATRA, adapalene, and CD2043 inhibited the percent LFCs to approximately one-half of control values ($p < 0.01$ *versus* control) (**Fig 1*c***). In a different set of experiments CD336 also significantly inhibited sebocyte differentiation. As with growth, some clear differences were noted among treatments at 10^{-6} M. With ATRA 10^{-6} M, the smaller number of remaining colonies seemed to become significantly more differentiated than controls (42.2% ± 4.0% LFCs, mean ± SEM). Some differentiation also seemed to occur, although to a lesser degree, in response to CD2043 10^{-6} M, at which dose the percent LFCs returned to control levels. In both cases the absolute number of LFCs per well (17.1 ± 2.9 and 13.8 ± 4.1, respectively) remained below the control level (30.4 ± 5.2, $p < 0.01$). Regardless of the degree of differentiation, however, the cells treated with RAR agonists looked distorted and fibroblast-like (**Fig 2**).

Table I. Binding specificity of study retinoids

Retinoids	Action	AC50[a] (nM)	Kd (nM)[b]			
			RAR-α	RAR-β	RAR-γ	RXR-α
ATRA	RAR>RXR agonist	260	16	7	3	730
CD271[c]	RAR-β,γ agonist	~40	1100	34	130	n.b.[d]
CD2043	RAR pan-agonist	~5	31	32	59	n.b.
CD336[e]	RAR-α agonist	20	10	74	450	n.b.
CD2665	RAR-β,γ antagonist	n.a.[f]	>2250	306	110	n.b.
CD2809	RXR agonist	n.a.	6134	1705	5109	538

[a] 50% F9 plasminogen activator activation.
[b] Binding specificity.
[c] Adapalene.
[d] No binding.
[e] Originally known as Am580.
[f] Not active.

RAR-β,γ antagonist abrogates the antiproliferative and antidifferentiative effects of ATRA, adapalene, and CD2043 To further determine the role of RAR subtypes in mediating the antiproliferative and antidifferentiative effect of retinoids, CD2665 (a selective RAR-β,γ antagonist) was added. It was used at 10^{-7} M, the highest concentration at which it alone showed no significant effects on cell growth and differentiation (dose–response data not shown, n = 4). ATRA, adapalene, and CD2043 were tested at one-tenth the concentration of CD2665. As shown in **Fig 3**, CD2665 abrogated the antiproliferative and

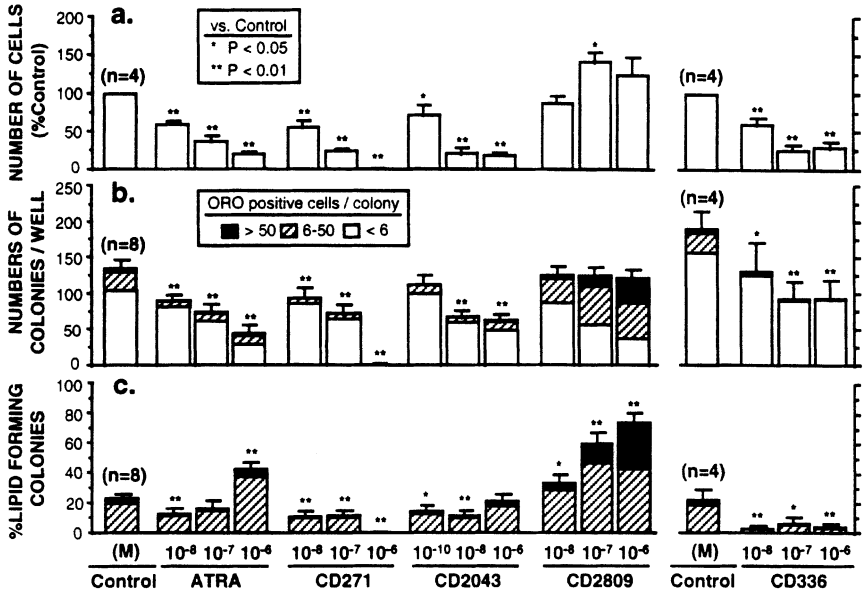

Figure 1. Effect of ATRA and selective retinoid receptor agonists on sebocyte growth and differentiation in a primary rat preputial cell culture system. ATRA (all-*trans* retinoic acid), CD271 (adapalene, an RAR-β,γ agonist), CD2043 (RAR-α,β,γ pan-agonist), CD2809 (RXR agonist), and CD336 (RAR-α agonist). Means ± SEM are shown. ORO, Oil Red O. Effects on cell numbers (*a*), colony numbers (*b*), and differentiation (*c*). ATRA, CD271 (adapalene), and CD2043 decreased numbers of cells and colonies as well as LFCs, although the differences between these retinoids at 10^{-6} M were noteworthy: with ATRA, cell growth was inhibited but colonies became twice as differentiated as controls, whereas the RAR-β,γ agonist adapalene completely obliterated both cell growth and differentiation and the RAR pan-agonist CD2043 showed no more inhibitory effect than at 10^{-8} M. In contrast, the RXR agonist CD2809 increased numbers of cells and LFCs, with no effect on colony numbers. Treatment with the RAR-α agonist CD336, performed in a different set of experiments, significantly decreased both cell numbers and LFCs.

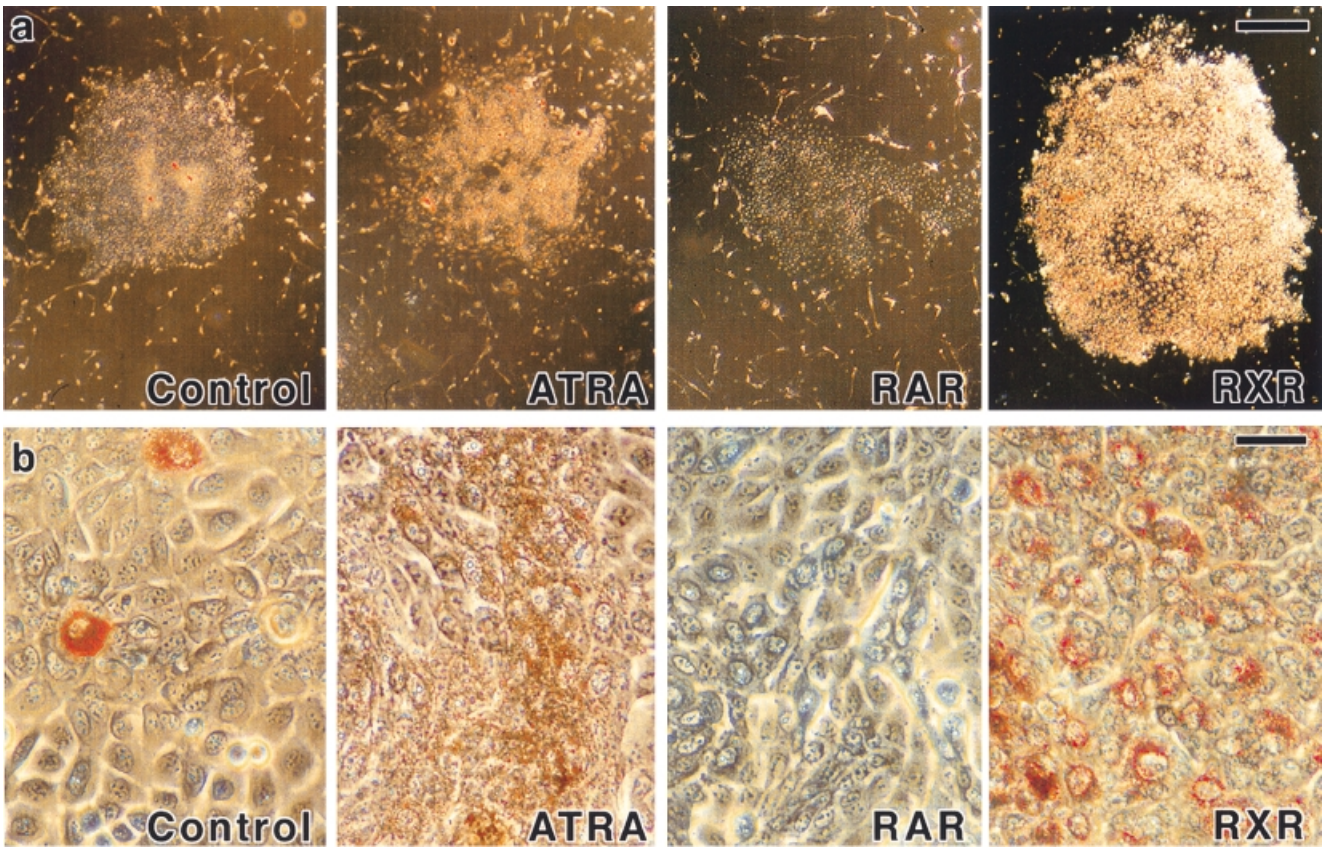

Figure 2. Effect of ATRA and selective retinoid receptor agonists on preputial cell morphology in monolayer culture. Scanning power (*a*; *scale bar*: 400 μm) and high power (*b*; *scale bar*: 50 μm) views of sebocyte colonies on day 9 are shown. Oil Red O stain. Compared with controls, the reduced number of colonies remaining after high dose ATRA (10^{-6} M) were distorted and contained numerous misshapen cells, many of which seemed mid-differentiated or degenerating. Colonies treated with 10^{-7} M RAR-β,γ agonist (CD271, adapalene) were smaller than controls, and many cells were undifferentiated and had become distorted and fibroblast-like in shape. In contrast, colonies treated with 10^{-6} M RXR agonist (CD2809) were bigger and contained numerous mid-differentiated sebocytes.

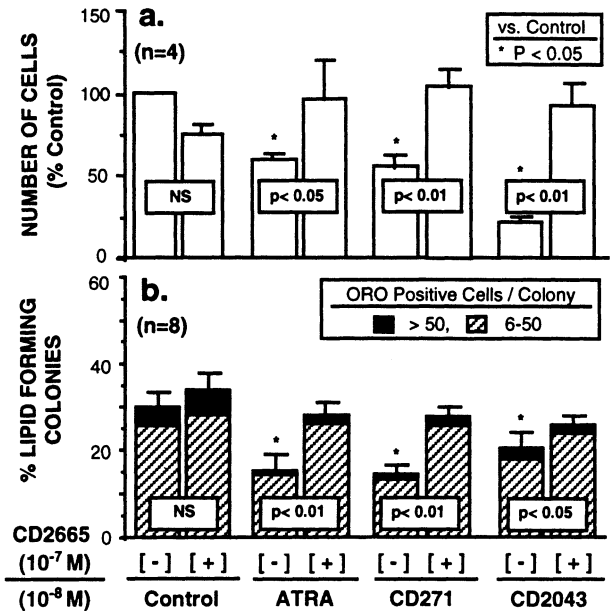

Figure 3. Effect of the RAR-β,γ antagonist on proliferation and differentiation of preputial sebocytes treated with ATRA or selective RAR agonists. (*a*) Proliferation; (*b*) differentiation. Means ± SEM are shown. The RAR-β,γ antagonist CD2665 abrogated both the antiproliferative and antidifferentiative effects of ATRA, CD271 (adapalene, an RAR-β,γ agonist), and CD2043 (RAR-α,β,γ pan-agonist), returning cell numbers and percent LFCs to control levels. CD2665 alone had no significant effects on cell proliferation and differentiation at 10^{-7} M.

antidifferentiative effects of ATRA and these selective RAR agonists, returning the cell number and percent LFCs to the control level.

Effect of RXR agonist on sebocyte growth and differentiation To identify whether the RXR has a distinct role in sebocyte proliferation and differentiation, the RXR agonist CD2809 was tested. As shown in **Fig 1**, CD2809 enhanced both proliferation and differentiation. Colonies were bigger and diffusely lipid-stained, containing mostly mid-differentiated cells (**Fig 2**). Its stimulatory effect was more obvious on differentiation than proliferation, however. It markedly increased LFCs in a dose-related fashion to a maximum of 73.7% ± 6.7% at 10^{-6} M ($p < 0.001$ *versus* control), but the increase in cell number was significant only at 10^{-7} M, with no effects on total number of colonies at any dose (**Fig 1*a*, *b***).

DISCUSSION

We have investigated the role of specific retinoid receptors and their subtypes in sebocyte growth and differentiation using the rat preputial cell monolayer culture system (Rosenfield *et al*, 1999). The expression of retinoid receptors is tissue specific, and sebaceous glands as well as skin express predominantly RAR-γ and RXR-α in mice and humans (Zelent *et al*, 1989; Finzi *et al*, 1992; Reichrath *et al*, 1995). Expression of RAR-α and RAR-β, as well as RAR-γ and RXR-α, have also been reported in cultured human sebocytes at the mRNA level (Doran *et al*, 1991).

First, we looked at the effects of selective agonists for RARs and their subtypes on sebocyte growth and differentiation. We found that all specific RAR agonists including selective RAR-α and RAR-β,γ agonists inhibited both sebocyte growth and differentiation, and their maximum inhibitory effect on differentiation was seen at a lower dose than the dose required for maximum inhibition of proliferation. Furthermore, the inhibitory effects of low doses of the RAR pan-agonist and the RAR-β,γ agonist were normalized by an RAR-β,γ antagonist. These findings suggest that RARs, especially the β and/or γ subtypes, mediate both antiproliferative and antidifferentiative effects of retinoids. Interestingly, unlike other RAR agonists, the RAR-β,γ agonist adapalene completely obliterated cell growth and, consequently, differentiation at high dosage. Since RAR-α agonist itself inhibited both sebocyte growth and differentiation and did not protect against this effect of adapalene, micromolar adapalene may cause apoptosis of sebocytes, as it does in other cells (unpublished data).

In contrast to the selective RAR agonists, the selective RXR agonist enhanced both proliferation and differentiation of sebocytes. The RXR stimulatory effect was more obvious on differentiation than proliferation at 10^{-6} M. It is possible that a proliferative effect of RXR may be masked by its marked differentiative effect at high dose, since cell differentiation is typically not compatible with proliferation (Hu *et al*, 1996; Lacasa *et al*, 1997). On the other hand, sebocytes may be an exception to this rule since differentiating sebocytes seem to retain some capacity to proliferate (Rosenfield, 1989; Zouboulis, 1996). Further work with a selective RXR antagonist is indicated to clarify the role of RXR in sebocyte proliferation and differentiation.

Like selective RAR agonists, the natural RAR ligand ATRA exerted both antiproliferative and antidifferentiative effects at low dose (10^{-8} M), and both of these suppressive effects were also reversed by an RAR-β,γ antagonist. Zouboulis and coworkers demonstrated a similar inhibitory effect of ATRA on proliferation at concentrations between 10^{-8} M and 10^{-5} M and lipid synthesis at 10^{-7} M in cultured human sebocytes (Zouboulis *et al*, 1991). We found that the ATRA effect at high dose was notably different, however, from that of other RAR agonists on differentiation: ATRA 10^{-6} M paradoxically seemed to enhance differentiation of the small number of remaining colonies. It is possible that cell degeneration and autophagocytosis of membrane lipids contributes to this appearance. Alternatively, this effect may be related to the potential for ATRA to be metabolized to an RXR ligand. ATRA has been shown to be capable of stimulating the transcriptional activity of RXRs via its isomerization to 9-*cis* RA *in vivo* and *in vitro* (Heyman *et al*, 1992; Zhang *et al*, 1992b). Taken together, we deduce that RARs probably mediate the antiproliferative and antidifferentiative effects of ATRA at low dose, whereas the differentiative effect of high dose ATRA may be mediated by RXR via its conversion to 9-*cis* RA.

Although RAR- and RXR-selective retinoids have shown distinct biologic effects on different types of cells, including sebocytes as this study shows, the mechanisms underlying the divergent effects of RAR and RXR activation are not entirely clear (Howell *et al*, 1998; Lomo *et al*, 1998). It has been suggested that they activate divergent signaling pathways. The effect of RAR and RXR ligands in other systems depends in part on the direct ligand-dependent stimulation of the RAR:RXR heterodimer and RXR:RXR homodimer signaling pathways, and/or in part on the relative RAR or RXR predominance. RXR predominance not only favors the latter of these two retinoid signaling pathways, but also makes RXR available to other partner receptors, such as PPAR (Kurokawa *et al*, 1994; Hembree *et al*, 1996; Joseph *et al*, 1998). PPARs are of special interest, since we found that PPAR agonists can induce sebocyte differentiation (Rosenfield *et al*, 1999), probably via heterodimerization with RXR as shown to be the case with adipocytes (Wahli *et al*, 1995; Tontonoz *et al*, 1996), and an RXR ligand alone can activate the RXR:PPAR heterodimer (Kliewer *et al*, 1992b).

In summary, we here demonstrate that selective RAR and RXR ligands exert distinct effects on sebocyte growth and differentiation. Our results suggest that RARs, especially the β and/or γ subtypes, mediate both the antiproliferative and antidifferentiative effects of retinoids, whereas RXRs mediate both clear differentiative and minor proliferative effects on sebocytes in culture. These findings have implications for better understanding of the role of specific retinoid receptors in the pathogenesis of acne as well as in regulation of normal sebocyte development, and further indicate

the potential for improved therapies for various skin disorders such as acne vulgaris.

This research was supported in part by USPHS grants HD-06308 (RLR), Lilly Research Laboratories and the Children's Research Foundation (MJK). Editing of the typescript was done by Jean Moore.

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