See related commentary on page 2154

13-*cis* Retinoic Acid Induces Apoptosis and Cell Cycle Arrest in Human SEB-1 Sebocytes

Amanda M. Nelson[1], Kathryn L. Gilliland[1], Zhaoyuan Cong[1] and Diane M. Thiboutot[1,2]

Isotretinoin (13-*cis* retinoic acid (13-*cis* RA)) is the most potent inhibitor of sebum production, a key component in the pathophysiology of acne, yet its mechanism of action remains largely unknown. The effects of 13-*cis* RA, 9-*cis* retinoic acid (9-*cis* RA), and all-*trans* retinoic acid (ATRA) on cell proliferation, apoptosis, and cell cycle proteins were examined in SEB-1 sebocytes and keratinocytes. 13-*cis* RA causes significant dose-dependent and time-dependent decreases in viable SEB-1 sebocytes. A portion of this decrease can be attributed to cell cycle arrest as evidenced by decreased DNA synthesis, increased p21 protein expression, and decreased cyclin D1. Although not previously demonstrated in sebocytes, we report that 13-*cis* RA induces apoptosis in SEB-1 sebocytes as shown by increased Annexin V-FITC staining, increased TUNEL staining, and increased cleaved caspase 3 protein. Furthermore, the ability of 13-*cis* RA to induce apoptosis cannot be recapitulated by 9-*cis* RA or ATRA, and it is not inhibited by the presence of a retinoid acid receptor (RAR) pan-antagonist AGN 193109. Taken together these data indicate that 13-*cis* RA causes cell cycle arrest and induces apoptosis in SEB-1 sebocytes by a RAR-independent mechanism, which contributes to its sebosuppressive effect and the resolution of acne.

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INTRODUCTION

Isotretinoin (13-*cis* retinoic acid (13-*cis* RA)) is the most potent inhibitor of sebum production, a key component in the pathophysiology of acne. It is the only retinoid that dramatically reduces the size and secretion of sebaceous glands (Landthaler *et al.*, 1980; Strauss *et al.*, 1980; Goldstein *et al.*, 1982). Despite the fact that isotretinoin is extremely effective against acne, surprisingly little is known regarding its molecular mechanism of action although advances are being made in this area. This unique retinoid has been shown to competitively inhibit the 3α-hydroxysteriod activity of retinol dehydrogenase leading to decreased androgen synthesis *in vitro* as well as inhibit the migration of polymorphonuclear leukocytes into the skin supporting its role in the reduction of inflammation that is associated with acne (Wozel *et al.*, 1991; Karlsson *et al.*, 2003).

Numerous studies indicate that 13-*cis* RA and other retinoids affect cell cycle progression, differentiation, apoptosis, and cell survival in a variety of cell types including human breast cancers, oral squamous cell carcinomas, lymphocytes, and murine neurons (Pomponi *et al.*, 1996; Giannini *et al.*, 1997; Toma *et al.*, 1997; Cariati *et al.*, 2000; Crandall *et al.*, 2004; Sakai *et al.*, 2004). Like previous studies in other cell types, 13-*cis* RA has also been shown to decrease proliferation of sebocytes and inhibit sebocyte differentiation as indicated in histology specimens, primary sebocytes, and SZ95 immortalized human sebocytes (Doran *et al.*, 1980; Jones *et al.*, 1980; Landthaler *et al.*, 1980; Strauss *et al.*, 1980; Ridden *et al.*, 1990; Zouboulis *et al.*, 1991a, b, 1999). Although increased levels of caspase 3 were noted in SZ95 sebocytes 24 hours following treatment with 13-*cis* RA and inhibition of cell growth was evident at 7 days, other markers failed to indicate that SZ95 sebocytes were undergoing apoptosis (Zouboulis *et al.*, 1993; Wrobel *et al.*, 2003). We hypothesized that 13-*cis* RA reduces sebocyte cell counts via cell cycle arrest and/or apoptosis and that these effects might not be apparent within a 24-hour treatment period.

In this paper, we report that after 48 and 72 hours of treatment 13-*cis* RA, but not 9-*cis* retinoic acid (9-*cis* RA) or all-*trans* retinoic acid (ATRA), inhibits growth and induces apoptosis in immortalized human SEB-1 sebocytes but not in HaCaT keratinocytes or normal human epidermal keratinocytes (NHEK). Furthermore, the RAR pan antagonist, AGN 193109, does not inhibit the apoptosis induced by 13-*cis* RA suggesting an RAR-independent apoptotic mechanism. We hypothesize that the ability of 13-*cis* RA to induce cell cycle arrest and apoptosis in sebocytes contributes to the overall effect on suppression of sebum and improvement in acne.

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Abbreviations: ANOVA, analysis of variance; ATRA, all-trans retinoic acid; 9-cis RA, 9-cis retinoic acid; 13-cis RA, 13-cis retinoic acid; NHEK, normal human epidermal keratinocyte; RAR, retinoid acid receptor; RXR, retinoid X receptor; TIG1, tazarotene-induced gene 1

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Results

13-*cis* RA exhibits a more rapid onset of growth inhibition of SEB-1 sebocytes compared to 9-*cis* RA or ATRA

There is a significant dose-dependent decrease in cell count after 48 and 72 hours of treatment with 13-*cis* RA (Figure 1a). At 48 hours, 13-*cis* RA concentrations of 0.1, 0.5, and 1 μM decreased cell count by 19, 22, and 30% respectively, when compared to vehicle ($P<0.05$). After 72 hours cell numbers decreased by 19, 43, and 39% with 13-*cis* RA concentrations of 0.1 μM ($P<0.01$), 0.5 μM ($P<0.0001$), and 1 μM ($P<0.05$) respectively. No significant differences in cell number were noted at 24 hours of treatment.

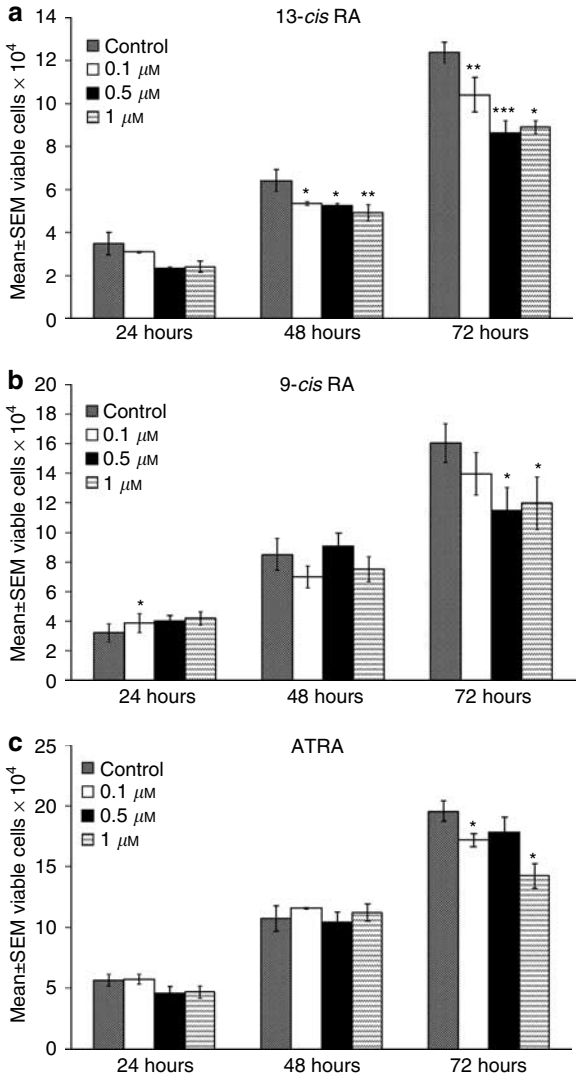

Figure 1. 13-*cis* RA, 9-*cis* RA, and ATRA differentially inhibit SEB-1 sebocyte proliferation. (**a**–**c**) Time-dependent inhibition of SEB-1 cell proliferation with individual retinoid compounds. SEB-1 cells were cultured in the presence of ethanol vehicle alone (0.01% or less; control) 0.1, 0.5, or 1 μM concentrations of 13-*cis* RA, 9-*cis* RA, or ATRA for 24, 48, or 72 hours. Attached cells were collected, stained with Trypan blue, and viable cells counted manually by hemocytometer. Data represent mean±SEM, $n=12$. Statistical analysis was performed with ANOVA Two Factor with Replication. *$P<0.05$, **$P<0.01$, and ***$P<0.0001$.

The effects of 9-*cis* RA and ATRA on SEB-1 sebocyte counts were noted beginning at 72 hours. Decreases of 39 and 43% were noted with 9-*cis* RA (0.5 and 1 μM, respectively) ($P<0.05$) (Figure 1b). ATRA (0.1 and 1 μM) significantly decreased cell number by 14 and 37%, respectively ($P<0.05$) (Figure 1c). Overall, each of these three retinoids decreased SEB-1 sebocyte numbers at 72 hours albeit to varying degrees but effects were noted beginning at 48 hours with 13-*cis* RA.

13-*cis* RA significantly inhibits DNA synthesis in SEB-1 sebocytes

13-*cis* RA (0.1, 0.5, and 1 μM) each significantly decreased thymidine incorporation by approximately 3-fold at 72 hours ($P<0.01$) (Figure 2a). No significant changes were noted at 24 or 48 hours. A 1.85-fold decrease in ^{3}H thymidine

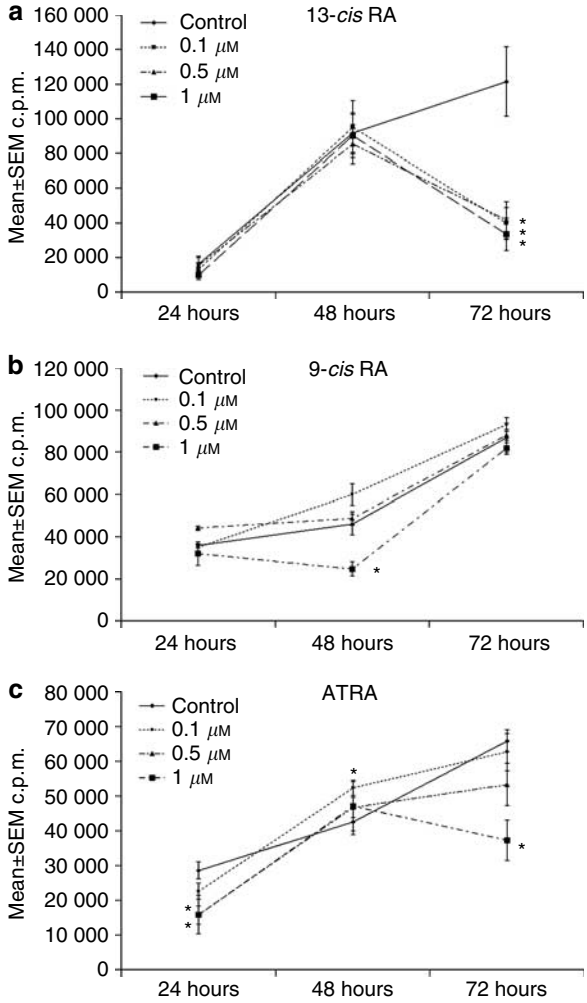

Figure 2. 13-*cis* RA inhibits DNA synthesis to a greater extent than 9-*cis* RA or ATRA. (**a**–**c**) SEB-1 sebocytes were treated with ethanol vehicle (control) or 0.1, 0.5, 1 μM concentrations of 13-*cis* RA, 9-*cis* RA, or ATRA for 24, 48, and 72 hours. 1 μCi ^{3}H thymidine was added to each sample 8 hours before harvesting. Cells were washed and collected for liquid scintillation counting. Data represent mean±SEM, $n\geq12$. Statistical analysis was performed with ANOVA Two Factor with Replication. *$P<0.05$ and **$P<0.01$.

incorporation was noted when cells were treated with 1 μM 9-*cis* RA for 48 hours (Figure 2b). ATRA in concentrations of 0.5 and 1 μM decreased thymidine incorporation by approximately 1.8–fold at 24 and 72 hours, respectively (Figure 2c).

13-*cis* RA, but not 9-*cis* RA or ATRA, increases p21 levels in SEB-1 sebocytes

To further test the hypothesis that 13-*cis* RA changes cell cycle progression, expression of p21, a cell cycle inhibitor, was examined by Western blot. 13-*cis* RA significantly increased p21 expression after 48 and 72 hours of treatment (Figure 3a). Specifically, p21 levels increased on average 2.64-fold and 3.13-fold when cells were treated with 0.1 μM 13-*cis* RA and 1 μM 13-*cis* RA, respectively, for 48 hours ($P=0.008$ and 0.05). After 72 hours of treatment, all concentrations tested increased p21 protein expression. 13-*cis* RA caused 1.47-, 2.27-, and 3.01-fold increases in p21 with 0.1, 1, and 10 μM 13-*cis* RA, respectively. No significant differences in p21 expression were noted at 24 hours (data not shown). When SEB-1 sebocytes were treated with 9-*cis* RA or ATRA in concentrations of 0.1, 0.5, and 1 μM, no significant increases in p21 protein were noted at 48 or 72 hours (Figure 3b and c).

13-*cis* RA, but not 9-*cis* RA or ATRA, decreases cyclin D1 protein in SEB-1 sebocytes

To further explore the possibility that 13-*cis* RA induces a G1 arrest in SEB-1 sebocytes, cyclin D1 protein was examined by Western blot. Cyclin D family members are expressed and function in controlling the progression from G1 to S phase in the cell cycle (Baldin *et al.*, 1993). Overexpression of cyclin D1 shortens the duration of G1 phase and is rate limiting for phase progression (Quelle *et al.*, 1993). Therefore, cyclin D1 is a likely candidate to confirm the actions of 13-*cis* RA in inhibiting cell cycle progression by influencing the G1 to S phase transition. In SEB-1 sebocytes, 13-*cis* RA in concentrations of 0.1, 1, and 10 μM, significantly decrease cyclin D1 protein at 72 hours (Figure 3d). No significant effects of 13-*cis* RA were noted at 24 or 48 hours (24-hour data not shown). 9-*cis* RA or ATRA concentrations of 0.1, 0.5, or 1 μM did not reduce cyclin D1 protein at 72 hours (data not shown).

13-*cis* RA induces apoptosis in SEB-1 sebocytes but not in HaCaT keratinocytes or NHEK

To determine if the effect of 13-*cis* RA on apoptosis is cell-type specific, time course experiments were conducted in SEB-1 sebocytes, HaCaT keratinocytes, and NHEK. In SEB-1

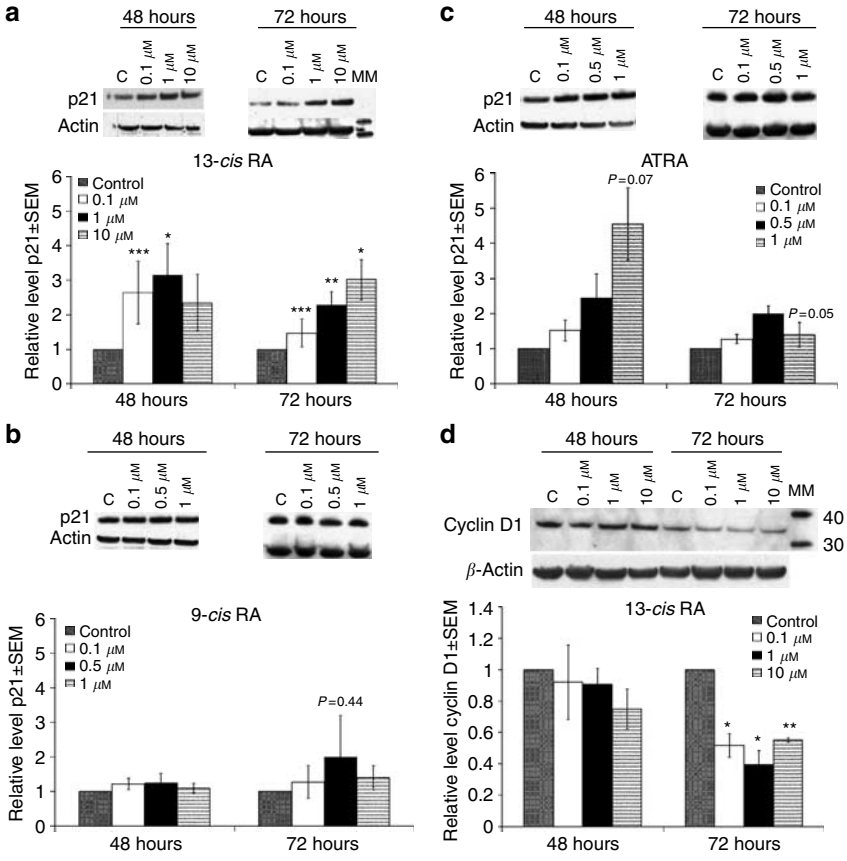

Figure 3. 13-*cis* RA increases p21 and decreases cyclin D1 proteins. (**a**) SEB-1 cells were treated with 0.1, 1, and 10 μM 13-*cis* RA or vehicle. (**b** and **c**) Parallel experiments were performed with 0.1 0.5, or 1 μM concentrations of 9-*cis* RA and ATRA. Blots were incubated with primary antibodies to p21 and β-actin for loading control normalization and analyzed by densitometry. (**d**) SEB-1 cells were treated with 0.1, 1, and 10 μM 13-*cis* RA or vehicle and blots were incubated with primary antibodies to cyclin D1 and β-actin. Magic Mark XP (MM) indicates band size. Blots are representative of minimum of three Western blots. Graphs represent normalized values relative to vehicle (control) expression of a minimum of three independent Western blots. Mean ± SEM. *$P<0.05$ and **$P=0.01$.

sebocytes, no significant differences in apoptosis were noted in cells treated with 13-*cis* RA for 2, 4, 6, or 24 hours. A marginal, yet significant, increase in the percentage of cells in early apoptosis was noted in SEB-1 cells treated with 0.1 μM 13-*cis* RA: 2.03–2.49% at 48 hours and from 2.19 to 2.84% at 72 hours ($P<0.01$ for each time point). Significant increases in the percentage of cells in late apoptosis were noted at 48 and 72 hours with increasing concentrations of 13-*cis* RA (Figure 4a, late apoptosis shown). Specifically, 0.1 μM 13-*cis* increased the percentage of late apoptosis: 4.06–5.22% at 48 hours and 5.31–8.11% at 72 hours. 13-*cis* RA at 1 μM concentration caused increases from 3.64 to 5.08% and from 7.57 to 12.18% at 48 and 72 hours, respectively. Nanomolar concentrations of 13-*cis* RA did not induce apoptosis at any of the time points examined (data not shown).

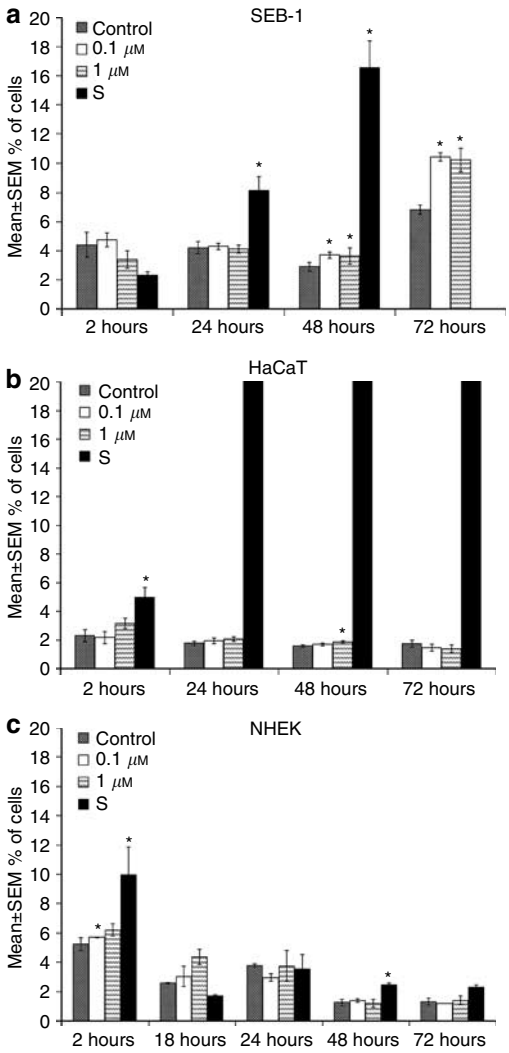

Figure 4. 13-*cis* RA induces late apoptosis in SEB-1 sebocytes but not in HaCaT keratinocytes or NHEK. (**a**) SEB-1 cells were treated with vehicle (negative control), 13-*cis* RA (0.1 or 1 μM), or staurosporine (S) (positive control) for indicated times. (**b**) HaCaT cells were treated with vehicle, 13-*cis* RA (0.1 or 1 μM), or staurosporine (S) for the indicated times. (**c**) NHEK cells were treated with vehicle, 13-*cis* RA (0.1 or 1 μM), or staurosporine (S) for indicated times. In all experiments, cells were prepared according to manufacturer's protocol for Annexin V-FITC / PI staining (BD ApoAlert, BD Biosciences). Data were analyzed with Cell Quest Software and represent mean ± SEM, $n \geq 12$. Statistical analysis was performed with ANOVA Two Factor with Replication. *$P<0.01$ and **$P<0.00001$.

In HaCaT keratinocytes, no significant differences in the percentages of cells in early and late apoptosis or necrosis were noted in cells treated with 0.1 μM 13-*cis* RA at all time points examined. 13-*cis* RA (1 μM) significantly increased the percentage of cells in early and late apoptosis at 24 and 48 hours, respectively. Yet these increases were very minor, with the total percentage of HaCaT cells in apoptosis with 13-*cis* RA being <2% of the cells (Figure 4b, late apoptosis shown). In experiments with NHEK cells, no significant differences in the percentages of cells in early or late apoptosis or necrosis were noted in cells treated with 13-*cis* RA with the exception of an increase from 5.25 to 6.2% in late apoptosis at 2 hours in cells treated with 1 μM 13-*cis* RA (Figure 4c, late apoptosis shown). Apoptosis was significantly induced by staurosporine in SEB-1 sebocytes, HaCaT keratinocytes and NHEK. No significant differences were noted between standard medium and ethanol controls in any cell type at any time point during these studies indicating that the concentrations of ethanol used in these experiments did not induce apoptosis.

13-*cis* RA specifically increases levels of cleaved caspase 3 in SEB-1 sebocytes

SEB-1 sebocytes were treated with 13-*cis* RA and four independent Western blots were run to detect cleaved caspase 3. No cleaved caspase 3 was noted at 24 hours in negative control lanes or in cells treated with 13-*cis* RA. 13-*cis* RA significantly increased cleaved caspase 3 levels at 48 and 72 hours in SEB-1 sebocytes (Figure 5a). Specifically, 0.1 μM 13-*cis* RA and 1 μM 13-*cis* RA increased expression of cleaved caspase 3 on average 3.58-fold ($P<0.01$) and 3.33-fold ($P<0.01$), respectively, at 48 hours. Small fold increases were noted at 72 hours that were not statistically significant. Although the magnitude of the increase in cleaved caspase 3 is greatest with 10 μM 13-*cis* RA at 48 hours, these results were not statistically significant; due to the variability induced by the very limited survival of cells at this higher concentration.

To determine if the induction of apoptosis is a specific to 13-*cis* RA, SEB-1 sebocytes were also treated with 0.1, 0.5, or 1 μM concentrations of 9-*cis* RA and ATRA. Again, no cleaved caspase 3 was detected at 24 hours in negative controls or with any concentration of either retinoid. Furthermore, unlike the case with 13-*cis* RA, no significant increases in cleaved caspase 3 were noted with either 9-*cis* RA or ATRA at 48 and 72 hours (Figure 5b and c).

For additional confirmation that the apoptotic effect of 13-*cis* RA is specific to SEB-1 sebocytes, Western blots for cleaved caspase 3 were performed on NHEK. No cleaved caspase 3 could be detected at any time point examined in NHEK cells treated with 13-*cis* RA. However, cleaved caspase 3 was detected when NHEK were treated with 1 μM staurosporine indicating that these cells are capable of undergoing apoptosis (Figure 6). In summary, 13-*cis* RA specifically induces apoptosis in a dose-dependent manner in

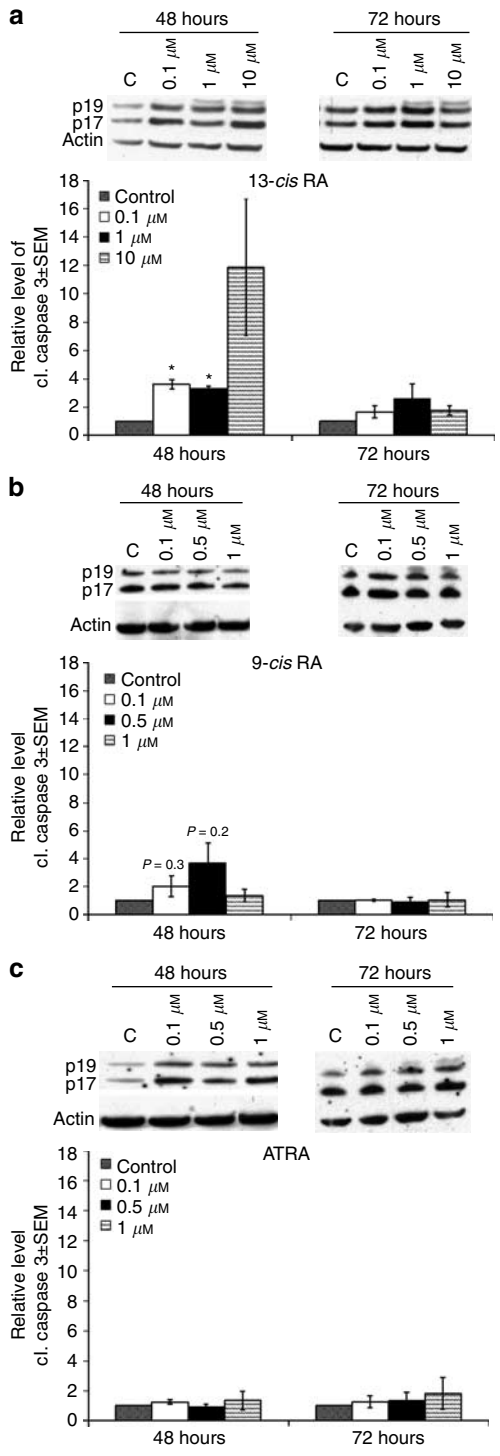

Figure 5. 13-*cis* RA increases cleaved caspase 3 protein in SEB-1 sebocytes. (**a**) SEB-1 sebocytes were treated with vehicle, 0.1, 1, or 10 μM 13-*cis* RA. (**b** and **c**) Parallel experiments were performed with 0.1, 0.5, or 1 μM concentrations of 9-*cis* RA or ATRA. Blots were incubated with primary antibodies to cleaved caspase 3 (1:1,000) and actin (1:1,000) for loading control normalization and analyzed by densitometry. p17 and p19 are cleaved caspase 3 fragments. Blots are representative of a minimum of four independent experiments. Graph represents normalized values relative to vehicle (control) expression for four independent Western blots. Data represent mean ± SEM *$P<0.01$.

SEB-1 sebocytes but not in NHEK. Furthermore, 9-*cis* RA or ATRA does not induce apoptosis in SEB-1 sebocytes.

13-*cis* RA, but not 9-*cis* or ATRA, increases TUNEL staining in SEB-1 sebocytes

To further test the hypothesis that 13-*cis* RA induces apoptosis and to confirm the results from the Annexin V-FITC experiments in SEB-1 sebocytes, we examined the effects of 13-*cis* RA on SEB-1 cells by TUNEL assay. 13-*cis* RA (0.1 and 1 μM) increased the percentage of TUNEL-positive cells by 3.5- and 5.67-fold, respectively ($P \leqslant 0.01$) at 48 hours, while each concentration of 13-*cis* RA increased the percentage of TUNEL-positive cells by approximately 13-fold at 72 hours ($P \leqslant 0.01$) (Figure 7b). No differences were noted at 24 hours (data not shown). To compare the actions of 13-*cis* RA to its isomerization products, SEB-1 sebocytes were also treated with the same concentrations of 9-*cis* RA and ATRA and no significant increases in TUNEL-stained cells were noted at any time point examined (Figure 7a and b). Both 9-*cis* RA and ATRA had 1–3% TUNEL-positive cells at time points examined. Fenretinide treatment of SEB-1 sebocytes significantly increased the percentage of TUNEL-positive cells in a dose-dependent fashion at 48 and 72 hours (ranging from 15 to 85% positive cells) (Figure 7a and b). No significant increase in the percentage of TUNEL-positive cells was noted with retinoid X receptor (RXR) pan agonist, CD 3254 at 48 hours. However, 50 nM CD 3254 significantly increased TUNEL-positive cells from 3 to 48% at 72 hours ($P<0.01$) (data not shown).

Apoptosis induction by 13-*cis* RA in SEB-1 sebocytes is not blocked by RAR antagonist AGN 193109

To determine if the effects of 13-*cis* RA on apoptosis are mediated by RA receptors, SEB-1 sebocytes were treated with 1 μM 13-*cis* RA in the presence of 10 μM AGN 193109, an RAR pan antagonist, and the TUNEL assay was performed. 13-*cis* RA alone significantly increased the percentage of cells in apoptosis over vehicle control by approximately 5-fold at 48 and 72 hours ($P<0.05$). These increases were not inhibited in the presence of AGN 193109 at 48 and 72 hours (Figure 7c and d) (48-hour data not shown). To verify the activity of AGN 193109 within our cells at the time points examined in the TUNEL assay, we performed quantitative PCR for RAR responsive gene, tazarotene-induced gene 1 (*TIG1*). RAR activation induces the expression of *TIG1* (Nagpal *et al.*, 1996). In the presence of 1 μM 13-*cis* RA alone, *TIG1* expression was approximately 13- and 17-fold higher than controls at 48 and 72 hours, respectively. With the addition of AGN 193109, *TIG1* gene expression dramatically decreases at 48 and 72 hours and is lower than vehicle-treated controls (Figure 7e).

13-*cis* RA is isomerized to ATRA over time in SEB-1 sebocytes

To study the kinetics of 13-*cis* RA uptake in SEB-1 sebocytes and its possible isomerization to ATRA or 9-*cis* RA, SEB-1 sebocytes were treated with 13-*cis* RA and subjected to HPLC analysis. 13-*cis* RA remains relatively stable in standard culture medium for approximately 24 hours (Figure 8a). The

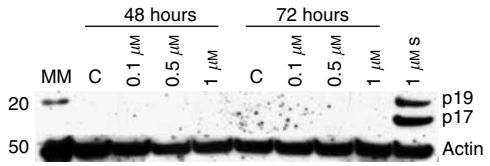

Figure 6. 13-*cis* RA does not increase cleaved caspase 3 in NHEK. NHEK were treated with vehicle, 0.1, 0.5, or 1 μM concentrations of 13-*cis* RA or 1 μM staurosporine (positive control). Blots were incubated with primary antibodies to cleaved caspase 3 (1:1,000) and β-actin (1:1,000) for loading control normalization and analyzed by densitometry. Data are a representative blot.

concentration of 13-*cis* RA in standard culture medium alone is similar to the concentration in medium from SEB-1 sebocyte-containing plates (Figure 8a and b). The concentrations of 13-*cis* RA within SEB-1 sebocytes increases to a maximum of 350 ng/ml at 12 hours, at which point the concentration declines for the duration of the experiment (Figure 8c). The concentration of ATRA in the medium alone and from plates containing SEB-1 sebocytes was much lower than 13-*cis* RA concentrations at the corresponding time points. The concentration of ATRA within SEB-1 sebocytes begins to rise at 12 hours and continues through the remaining time periods. 9-*cis* RA concentrations are minimal at best in medium alone and medium from SEB-1 plates during the time course. Within SEB-1 sebocytes, 9-*cis* RA concentrations range from 1.4 ng/ml at 0 hour to a maximum of 12 ng/ml at 72 hours, magnitudes lower than either 13-*cis* RA or ATRA at the same time periods (Figure 8).

DISCUSSION

Determining the actions of isotretinoin on the sebaceous gland is essential in advancing our understanding of the molecular mechanism of action of this drug and in our search for safer therapeutic alternatives. Several studies indicate that the effects of retinoids on cell proliferation, cell cycle, and apoptosis are retinoid specific or cell-type specific. For example, growth inhibition with 13-*cis* RA has been reported in human breast cancer cell lines, primary glioblastoma cells, Epstein–Barr virus-immortalized B lymphocytes, and oral squamous cell carcinoma cell lines (Pomponi *et al.*, 1996; Giannini *et al.*, 1997; Toma *et al.*, 1997; Bouterfa *et al.*, 2000). In some cases the effects noted with 13-*cis* RA or 9-*cis* RA were not duplicated by ATRA (Bouterfa *et al.*, 2000). Most studies in other cell types suggest that retinoids cause a block in the G1/S phase of the cell cycle triggering decreased S phase and increased the percentage of cells in G0/G1 (Giannini *et al.*, 1997; Toma *et al.*, 1997; Crandall *et al.*, 2004). It is also well established that retinoids induce apoptosis in numerous cell types, both normal cells and tumor cell lines, although not previously demonstrated in sebocytes. For example, in doses comparable to those given for the treatment of acne in humans, 13-*cis* RA reduces the survival and genesis of murine hippocampal neurons *in vivo* (Crandall *et al.*, 2004; Sakai *et al.*, 2004). ATRA has been shown to induce apoptosis in primary and metastatic melanoma cells (Zhang and Rosdahl, 2004) as well as inducing growth arrest followed by apoptosis in orbital fibroblasts from patients with Graves' disease (Pasquali *et al.*, 2003). In *OCI/AML-2* retinoid-sensitive cell line subclones derived from leukemia cells, 9-*cis* RA inhibited cell growth and induced apoptosis to a greater extent than 13-*cis* RA or ATRA (Koistinen *et al.*, 2002). These studies demonstrate that the actions of retinoids are unique and specific to the model used.

The exact mechanism of action of 13-*cis* RA in the treatment of acne remains largely unknown. 13-*cis* RA has little to no ability to bind to cellular retinol-binding proteins or the RA nuclear receptors (RARs and RXRs) (Levin *et al.*, 1992; Allenby *et al.*, 1993; Fogh *et al.*, 1993). It has been suggested 13-*cis* RA may, in fact, act as a pro-drug that is isomerized intracellularly to ATRA, an agonist for RAR nuclear receptors, and 9-*cis* RA, which is a non-specific agonist for both RAR and RXR nuclear receptors (Allenby *et al.*, 1993; Ott *et al.*, 1996). Studies of immortalized human sebocyte line SZ95 showed that 13-*cis* RA is preferentially metabolized to ATRA, which can bind to and activate RAR, which leads to the overall inhibition of sebocyte proliferation (Tsukada *et al.*, 2000). Our studies confirm that 13-*cis* RA is primarily metabolized to ATRA in SEB-1 sebocytes beginning at 24 hours. It is well established, however, that 13-*cis* RA is superior to either 9-*cis* RA or ATRA for sebosuppression (Geiger *et al.*, 1996; Hommel *et al.*, 1996; Ott *et al.*, 1996). Alternatively, 13-*cis* RA may act in a receptor independent manner by influencing cellular signaling pathways by direct protein interactions as demonstrated with other retinoids or by enzyme inhibition (Zorn and Sauro, 1995; Hoyos *et al.*, 2000; Imam *et al.*, 2001; Karlsson *et al.*, 2003).

Previous studies have examined the actions of 13-*cis* RA, 9-*cis* RA, and ATRA on cultured human sebocytes, SZ95 immortalized sebocytes, and rat preputial cells (Zouboulis *et al.*, 1991a, 1993; Tsukada *et al.*, 2000; Wrobel *et al.*, 2003). 13-*cis* RA at concentrations greater than 10^{-7} M and ATRA (10^{-5} to 10^{-6} M) significantly decreased human sebocyte proliferation after 7 or 14 days (Zouboulis *et al.*, 1991a, 1993). Studies of immortalized human sebocytes SZ95, showed that 13-*cis* RA, 9-*cis* RA, and ATRA at concentrations of 10^{-7} M significantly reduced proliferation by approximately 50% after 9 days (Tsukada *et al.*, 2000). In primary rat preputial cells, ATRA and other RAR-selective agonists significantly decreased cell numbers after 9 days (Kim *et al.*, 2000).

Processes such as cell cycle arrest or apoptosis may explain the histological data in human skin biopsies that demonstrates a drastic decrease in the size, shape, and lipid content of sebaceous glands after 16 weeks of isotretinoin (Goldstein *et al.*, 1982) Since proliferation studies in SZ95 sebocytes suggested that the effects of 13-*cis* RA and other retinoids may be noted after 7–9 days, we designed experiments to look at the effects of 13-*cis* RA, 9-*cis* RA, and ATRA on proliferation, cell cycle progression, and apoptosis in SEB-1 sebocytes at time points later than 24 hours but prior to 7 days. Our proliferation studies show that 13-*cis* RA causes a dose-dependent decrease in cell count after 48 and 72 hours whereas 9-*cis* RA and ATRA show significant decreases beginning at 72 hours. We would expect

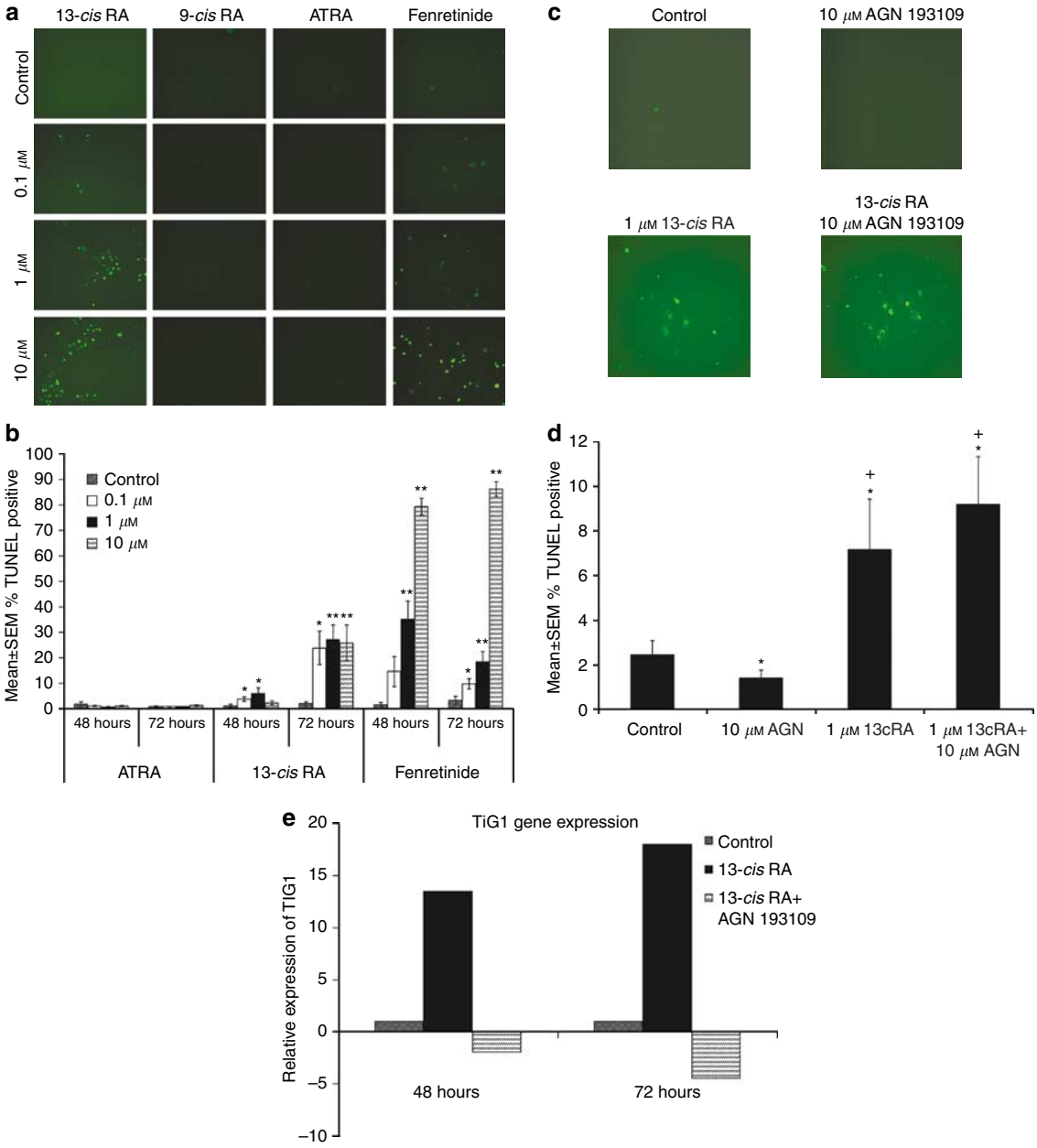

Figure 7. The increase in TUNEL staining with 13-*cis* RA is not inhibited in the presence of RAR pan antagonist AGN 193109. (**a**) Representative images of control, 0.1, 1, and 10 μM 13-*cis* RA, 9-*cis* RA, ATRA, and fenretinide treatments at 72 hours (48 hours data not shown).(**b**) Quantification of the percentage of TUNEL-positive-stained cells per treatment at 48 and 72 hours (9-*cis* RA not shown). Data represent mean ± SEM, $n = 6$–12. Statistical analyses were performed with ANOVA Two Factor with Replication. *$P \leqslant 0.01$ and **$P < 0.001$. (**c**) Representative images of negative control, 1 μM 13-*cis* RA, AGN 193109, and 13-*cis* RA combined with 10 AGN 193109 at 72 hours (48 hours data not shown). (**d**) Quantification of the percentage of TUNEL-positive cells at 72 hours. Data represent mean ± SEM, $n = 12$. Statistical analyses were performed with ANOVA Two Factor with Replication. *$P \leqslant 0.05$ when compared to control; + not statistically different. (**e**) Quantitative PCR verification of RAR antagonist AGN 193109 activity in SEB-1 sebocytes. Bars represent the efficiency corrected normalized average fold change of TIG1 under the experimental conditions as determined by REST-XL software, $n = 4$.

that if our experiments were extended, the magnitude of this decrease would be greater as previously reported in SZ95 sebocytes after 9 days (Tsukada *et al.*, 2000). Overall, 13-*cis* RA at the concentrations tested in our study acts sooner in inhibiting proliferation than either 9-*cis* RA or ATRA. These data are supported by studies demonstrating an approximate 3-fold decrease in ^{3}H thymidine incorporation in SEB-1 sebocytes that were treated with 13-*cis* RA for 72 hours. This decrease is nearly 2-fold greater than the decreases produced by 9-*cis* RA or ATRA. This experiment suggests that 13-*cis* RA is more potent at growth inhibition than either 9-*cis* RA or ATRA in SEB-1 sebocytes.

Further supporting the hypothesis that 13-*cis* RA causes a block in the G1/S phase as demonstrated in other cell types,

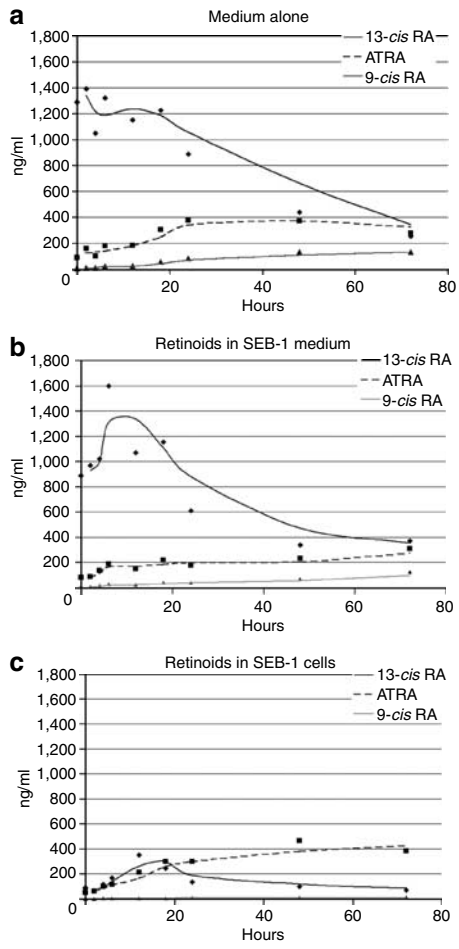

Figure 8. 13-*cis* RA is isomerized to ATRA within SEB-1 sebocytes. HPLC analysis of (**a**) SEB-1medium alone, (**b**) medium removed from SEB-1 sebocyte-containing plates, and (**c**) SEB-1 sebocytes after 5 μM 13-*cis* RA treatment for the indicated times. Points are the average of duplicate samples.

we show that 13-*cis* RA increases p21 protein and decreases cyclin D1 protein at 48 and 72 hours. Cyclin D1 protein expression decreases by approximately 50% at 72 hours, which coincides with our ^{3}H thymidine studies where 13-*cis* RA had the most striking effect at 72 hours. Furthermore, cyclin D1 was not decreased with 9-*cis* RA or ATRA at 72 hours, which is also consistent with our ^{3}H thymidine studies. No significant increases in p21 protein were noted with 9-*cis* RA or ATRA although increasing trends were noted. Taken together, these experiment show that in SEB-1 sebocytes, 13-*cis* RA is much more effective than 9-*cis* RA or ATRA in both decreasing the proportion of cells synthesizing DNA and inducing a G1/S phase cell cycle block by increasing p21 and decreasing cyclin D1 expression.

Studies in SZ95 sebocytes did not demonstrate apoptosis in cells treated up to 24 hours with 13-*cis* RA (10^{-5} to 10^{-8} M) and assayed by DNA fragmentation and lactate dehydrogenase release (Wrobel *et al.*, 2003). At 24 hours, no changes in apoptosis were noted when SZ95 sebocytes were treated with 10^{-7} M 13-*cis* RA as assessed by annexin V staining, cell death assays, or FACS analysis and reverse transcriptase-PCR for the apoptotic proteins, bcl-2 and bax. Interestingly, in SZ95 sebocytes, 13-*cis* RA increased levels of caspase 3 as detected by FACS analysis at 24 hours. Accordingly, in our studies of SEB-1 sebocytes, no increase in apoptosis was noted 24 hours after treatment with 13-*cis* RA as assayed by Annexin V-FITC FACS. However, increases in early and late apoptosis were noted at 48 and 72 hours with concentrations of 13-*cis* RA similar to those used in SZ95 sebocytes, although the magnitude of the number of cells in apoptosis is small compared to the positive control, staurosporine. In contrast, the magnitude of the changes in apoptosis induced by 13-*cis* RA was much greater in the TUNEL assay. By extending the treatment times in our assay we were able to detect the induction of apoptosis by 13-*cis* RA, which was verified by increased expression of cleaved caspase 3. Furthermore, the increase in apoptosis was limited to 13-*cis* RA as no significant increases in apoptosis were noted when SEB-1 sebocytes were treated with 9-*cis* RA or ATRA.

The effects of 13-*cis* RA on apoptosis and growth inhibition may or may not be mediated by retinoid receptors. It is possible that the effects of 13-*cis* RA on apoptosis and growth inhibition may be mediated by other isomerization products such as 4-oxo-isotretinoin or 4-hydroxy-isotretinoin (Orfanos and Zouboulis, 1998). The 4-oxo metabolites of retinoids have been shown to be functionally active in human keratinocytes and fibroblasts by their ability to induce changes in gene expression (Baron *et al.*, 2005). Our data show that RAR pan antagonist AGN 193109 sufficiently blocks RAR activation in the presence of 13-*cis* RA as measured by a significant decrease in *TIG1* gene expression, yet does not block apoptosis induced by 13-*cis* RA in SEB-1 sebocytes, thus supporting the hypothesis that apoptosis induction by 13-*cis* RA is independent of RAR activation. Alternatively, apoptosis may be mediated through RXR nuclear receptor activation (Zhao *et al.*, 2004). Using the RXR pan agonist CD 3254 (50 nM), a significant increase in the percentage of TUNEL-positive SEB-1 sebocytes was noted at 72 hours. Although our HPLC data indicate very low levels of 9-*cis* RA (a maximum of 12 ng/ml at 72 hours), RXR activation by 9-*cis* RA is possible (Allenby *et al.*, 1993) or 13-*cis* RA may be metabolized to another as yet unidentified metabolite that is capable of RXR activation.

Alternatively, 13-*cis* RA may have effects that are independent of retinoid receptors. Interestingly, we showed that fenretinide, a retinoid known to induce apoptosis primarily by RAR- and RXR-independent means is able to induce significant apoptosis in our SEB-1 sebocytes. In fact, the degree of apoptosis induced by fenretinide at 48 hours is very similar to that observed with 13-*cis* RA treatment at 72 hours. Fenretinide induces apoptosis by elevating reactive oxygen species and increases in activation of ceramide and caspases (Wu *et al.*, 2001). In addition, a retinoid-related molecule, AGN 193198 induces apoptosis without activation of the classical retinoid receptors (Keedwell *et al.*, 2004; Balasubramanian *et al.*, 2005). It may be possible that 13-*cis* RA acts similarly to fenretinide or AGN 193198 via

receptor-independent mechanisms; although additional experiments are required to test this hypothesis.

Since the actions of retinoids differ in various cell types and the effects of 13-*cis* RA are most profound on sebaceous glands *in vivo*, it is possible that the induction of apoptosis and cell cycle arrest may be specific to sebocytes since 13-*cis* RA failed to induce apoptosis in HaCaT keratinocytes or NHEK. It is possible that with higher concentrations of 13-*cis* RA or longer treatment times that apoptosis may be induced in keratinocytes. Although there is no evidence in the literature of 13-*cis* RA-induced apoptosis in keratinocytes, ATRA, and tazarotene, an RARβ/γ-selective agonist, have been shown to induce apoptosis in HaCaTs (Louafi *et al.*, 2003; Papoutsaki *et al.*, 2004). Taken together, these experiments support the hypothesis that 13-*cis* RA specifically induces apoptosis in SEB-1 sebocytes and not keratinocytes.

In conclusion, our data indicate that 13-*cis* RA inhibits growth and induces apoptosis in SEB-1 sebocytes and not keratinocytes at concentrations that are therapeutically achievable in human plasma (Rollman and Vahlquist, 1986; Adamson, 1994; Almond-Roesler *et al.*, 1998). Previous studies in human sebocytes and immortalized sebocytes have also documented growth inhibition with 13-*cis* RA, however, we have extended these studies to show that this growth inhibition is most likely due to influencing the G1/S phase of the cell cycle as evidenced by decreased DNA synthesis, increased p21 protein, and decreased cyclin D1 protein. In addition, we report for the first time, that 13-*cis* RA also induces apoptosis in SEB-1 sebaceous cells. The ability of 13-*cis* RA to induce apoptosis is specific to sebocytes, not keratinocytes, and is distinct from effects observed with 9-*cis* RA and ATRA that may account, in part, for the superior efficacy of 13-*cis* RA in reducing sebum production. Furthermore, the induction of apoptosis by 13-*cis* RA does not appear to involve RAR nuclear receptors. Elucidating the cellular processes that are affected by 13-*cis* RA in sebocytes is a step toward understanding the overall molecular mechanism of action of this drug, which may lead to the identification of alternative strategies for the treatment of acne.

MATERIALS AND METHODS

Cell culture

The SEB-1 human sebocyte cell line was generated by transfection of secondary sebocytes by SV40 Large T antigen as previously described (Thiboutot *et al.*, 2003). SEB-1 cells were cultured and maintained in standard culture medium containing: 5.5 mM low glucose DMEM 3:1 Ham's F12, 2.5% fetal bovine serum, hydrocortisone 0.4 μg/ml, adenine 1.8×10^{-4} M, insulin 10 ng/ml, epidermal growth factor 3 ng/ml, cholera toxin 1.2×10^{-10} M, and antibiotics.

HaCaT keratinocytes were cultured and maintained in 5.5 mM low glucose DMEM, 5% fetal bovine serum, and antibiotics and served as a control cell line in Annexin V-FITC FACS apoptosis assays. NHEK-neonatal (pooled) (NHEK-neo, Clonetics Keratinocyte System, Cambrex Bioscience, Walkersville, MD) were cultured in keratinocyte growth medium-2 (Cambrex Bioscience, Walkersville, MD). NHEK-neo cells served as control cells in Annexin V-FITC FACS apoptosis assays and Western blots for cleaved caspase 3.

Effects of retinoids on SEB-1 proliferation

Retinoid compounds were purchased through SIGMA (St Louis, MO): 13-*cis* RA (R 3255), 9-*cis* RA (R 4653) and ATRA (R 2625). Stock solutions of retinoids were handled under dimmed yellow light, dissolved in 100% ethanol at a concentration of 10 mM, and stored under N_2 gas at -20°C until use. The RAR pan antagonist AGN 193109 was obtained from Allergan (gift, Dr Rosh Chandraratna) and dissolved in DMSO at a concentration of 10 mM, and stored at -70°C until use. Treatments were made from retinoid stocks diluted to the appropriate concentration in standard culture mediums solutions under dimmed yellow light. Staurosporine (S 5921, Sigma, St Louis, MO) was solubilized in 100% ethanol at a concentration of 10 mM, stored at -20°C, and diluted to desired final concentration in appropriate cell culture medium for a positive control for apoptosis.

SEB-1 sebocytes (passages 20–23) were seeded at 4×10^4 cells per 35-mm plate and grown until approximately 40% confluent. Plates were each treated with 0.1, 0.5, 1 μM concentrations of 13-*cis* RA, 9-*cis* RA, ATRA, or ethanol vehicle alone (0.01% or less) in triplicate for 24, 48, and 72 hours. Cells were detached using trypsin (0.05%), collected, and diluted in standard culture medium for manual cell counts using a hemocytometer. Cell viability was assessed using Trypan blue dye exclusion. Each proliferation assay was performed three independent times. Analysis of variance (ANOVA) Two Factor with Replication was used for analysis. Results were considered significant if $P<0.05$.

^{3}H thymidine incorporation assay

SEB-1 sebocytes (passages 21–26) were at 2.5×10^4 cells per well in 12-well plates and grown until 30–40% confluent. Wells were rinsed with phosphate-buffered saline (PBS) prior to the addition of 0.1, 0.5, or 1 μM concentrations of 13-*cis* RA, 9-*cis* RA, ATRA, or ethanol vehicle alone (0.01% or less) in triplicate wells in standard culture medium. ^{3}H thymidine (1 μCi/well) was added a minimum of 8 hours prior to the end of the treatment period. At the end of the treatment period, medium was removed and cells were rinsed twice with PBS, detached using trypsin (0.05%), and collected for liquid scintillation counting. Each assay was performed a minimum of three independent times. Statistical significance was determined with ANOVA Two Factor with Replication. Results were considered significant if $P<0.05$.

Western blot analysis for p21, cyclin D1, and cleaved caspase 3

To confirm results from cell proliferation and apoptosis assays, protein levels of p21, cyclin D1, and cleaved caspase 3 were examined using Western blot in our various cell lines. p21, a cyclin-dependent kinase inhibitor, blocks progression through the G1/S phase of the cell cycle. Cyclin D1 is specifically required for progression into S phase. Caspase 3, the key executioner caspase, is synthesized in the cell as a pro-caspase, which then becomes cleaved and activated when cells undergo apoptosis. Primary antibodies for p21 Waf/Cip1 (DCS60), cyclin D1 (DCS6), cleaved caspase 3 (Asp175), and β-actin and as well as secondary anti-rabbit IgG horseradish peroxidase antibody were obtained from Cell Signaling Technology (Beverly, MA). Actin primary antibody and

anti-mouse horseradish peroxidase-linked secondary antibody were obtained from Santa Cruz Biotechnology INC (Santa Cruz, CA).

SEB-1 sebocytes (passages 20–26) were grown in 100-mm plates in standard culture medium until 50–75% confluent. Plates were rinsed with PBS and then treated with: 13-*cis* RA (0.1, 1, and 10 μM); 9-*cis* RA (0.1, 0.5, and 1 μM); ATRA (0.1, 0.5, and 1 μM); ethanol vehicle (0.01% or less) as a negative control; or 1 μM staurosporine dissolved in ethanol as a positive control. Cells were treated for 24, 48, or 72 hours. NHEK cells (passage 3) were grown in 100-mm plates in standard culture medium until approximately 50–75% confluent. Plates were rinsed with PBS and then treated with: 13-*cis* RA (0.1, 0.5, and 1 μM); ethanol vehicle (0.01% or less); or 1 μM staurosporine for 2, 4, 6, 18, 24, 48, or 72 hours. Total cell protein lysates from adherent and floating cells of SEB-1 sebocytes, or NHEK were collected, flash frozen in liquid nitrogen, and stored at −80°C until needed. Protein concentration of each sample was determined by the BCA Protein Assay (Pierce, Rockford, IL). Equal amounts of protein were run on NuPage 10% or 4–12% Bis-Tris Gels with MES Running Buffer (Invitrogen Life Technologies, Carlsbad, CA). Gels were *trans*ferred to polyvinylidene difluoride membrane, blocked for 1 hour at room temperature in 5% non-fat dry milk, and incubated with a 1:1,000 dilution of Cleaved Caspase 3 (Asp 175) (5A1) rabbit monoclonal antibody, 1:1,000 dilution of cyclin D1, or a 1:8,000–15,000 dilution of p21 Waf1/Cip1 (DCS60) mouse monoclonal antibody. Secondary anti-rabbit IgG horseradish peroxidase-linked antibody and anti-mouse horseradish peroxidase-linked antibody were used to detect primary antibodies. Supersignal West Pico Chemiluminescent Substrate (Cat no. 34077, Pierce, Rockford, IL) was used for protein detection. Blots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford IL) and re-probed with β-actin (no. 4967 Cell Signaling Technologies) or actin (H300 cat no. sc10731) for a loading control. Films of blots were analyzed and quantified by densitometry with QuantityOne Software (Bio-Rad, Hercules, CA) after background subtraction. Western blots were repeated a minimum of three times. Data was analyzed using a Student's *t*-test and results were considered significant if $P<0.05$.

Annexin V-FITC/propidium iodide FACS apoptosis assay

To determine if 13-*cis* RA induces apoptosis in SEB-1 sebocytes and the time course of this effect, the annexin V-FITC FACS assay was chosen (Martin *et al.*, 1995). Apoptosis assays were performed in SEB-1 sebocytes, HaCaT keratinocytes, and NHEK that were treated with 13-*cis* RA. SEB-1 sebocytes (passages 22–26) and HaCaT keratinocytes (passages 23–29) were seeded at 8×10^4 cells per 35-mm plate in their standard culture mediums and allowed to grow for 3 days, feeding once before treatment. Treatments consisted of: standard culture medium or ethanol vehicle (0.01% or less) as negative controls, 1 μM staurosporine as a positive control, and 13-*cis* RA at a final concentrations of 0.1 or 1 μM in SEB-1 sebocytes and HaCaT keratinocytes for the initial studies. For follow-up studies examining a possible 13-*cis* RA dose–response, SEB-1 sebocytes were subjected to treatments of: 0.1, 1, 10 nM, 0.1, 1, and 10 μM and previously mentioned controls. All samples were run in triplicate and treatments were carried out for 2, 4, 6, 24, 48, and 72 hours. In parallel experiments, NHEK cells (passage 3) were grown in keratinocyte growth medium-2 until 70% confluent. Treatments consisted of ethanol vehicle, 1 μM staurosporine, or 13-*cis* RA (0.1, 0.5, and 1 μM). Samples were run in triplicate and assayed at 2, 4, 6, 18, 24, 48, or 72 hours. Each sample was prepared according to BD ApoAlert Annexin V Protocol (Cat no. K2025-1, BD Biosciences Clontech, Palo Alto, CA). Ten thousand events were collected per sample using flow cytometry and debris was excluded by scatter gating. Single Annexin V-FITC and propidium iodide-stained samples as well as no-dye-negative control samples determined quadrants for data analysis. Data analysis was by Cell Quest software (Becton Dickinson, Canada) and percentage of cells in early apoptosis, late apoptosis, necrosis, and viable (unaffected) quadrants were calculated and compared by ANOVA Two Factor with Replication. Assay was performed three independent times. Results were considered significant if $P<0.05$.

TUNEL staining

SEB-1 sebocytes (passages 22–28) were cultured in 12-well plates in standard medium until approximately 30–40% confluent. Wells were rinsed with PBS and were treated in triplicate with ethanol (0.01% or less) as a vehicle control, 13-*cis* RA, 9-*cis* RA, or ATRA each in concentrations of 0.1, 1, or 10 μM. Retinoids were diluted in standard culture medium and treatments were carried out for 24, 48, or 72 hours. In parallel experiments, SEB-1 sebocytes (passages 22–24) were cultured in 12-well plates in standard medium until approximately 30–40% confluent. Wells were rinsed with PBS and were treated in triplicate with: ethanol (0.01% or less), DMSO (0.01% or less), or ethanol and DMSO together as vehicle controls, 1 μM 13-*cis* RA alone, 10 μM AGN 193109 alone, or 1 μM 13-*cis* RA with 10 μM AGN 193109. Additional experiments were performed with fenretinide, a synthetic retinoid known to induce apoptosis and act via a retinoid receptor-independent mechanism (Wu *et al.*, 2001). Fenretinide (4-hydroxyphenyl-retinamide) was handled under dimmed yellow light and dissolved in 100% ethanol to create a 10 mM stock solution stored at −20°C (H 7779 Sigma, St Louis, MO). SEB-1 sebocytes were treated in triplicate with 0.1, 1, and 10 μM concentrations. Furthermore, experiments were performed with the RXR pan agonist CD 3254 (Galderma R&D, Sophia Antipolis, France). CD 3254 was handled under normal light conditions and dissolved in DMSO to create a 10 mM stock solution stored at −20°C. SEB-1 sebocytes were treated in triplicate with 1 and 50 nM concentrations. Compounds were diluted in standard culture medium and applied for 48 or 72 hours. Each well was considered one sample. Samples were prepared by manufacturer's instructions for *in situ* Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Additional assay controls included negative controls of labeling solution only and DNase I-treated wells as positive controls. Results were analyzed and quantified by counting positive staining cells/total cells in three representative fields per well for each of the treatments carried out in triplicate. Each assay was performed three independent times; fenretinide and CD 3254 experiments were repeated twice. Data analysis was performed using ANOVA Two Factor with Replication and considered significant if $P<0.05$.

Quantitative PCR

To verify RAR antagonist activity in TUNEL experiments, quantitative PCR was used to document downregulation of the RAR target gene, tazarotene-induced gene 1 (*TIG1*; retinoic acid receptor responder 1). SEB-1 sebocytes were handled, maintained, and treated with 13-*cis* RA and RAR antagonist AGN 193109 under experimental

conditions that were identical to those used in the TUNEL assays. Total RNA was isolated and quantitative PCR performed as previously described (Trivedi *et al.*, 2006) Primer – probe sets for TATA-binding protein (TBP; reference gene), and retinoic acid receptor responder 1 (*TIG1*), were purchased from Applied Biosystems (Foster City, CA) ''no template'' and ''no amplification'' controls were included. The Relative Expression Software Tool (REST-XL) was used for data analysis.

HPLC

13-*cis* RA is reported to isomerize mainly to ATRA in other cell types including SZ95 sebocytes (Tsukada *et al.*, 2000). To eliminate the possibility of an alternative pattern of isomerization and to study the kinetics of 13-*cis* RA uptake into SEB-1 sebocytes, we utilized liquid–liquid extraction, reversed phase HPLC with UV detection. SEB-1 sebocytes (passage 22) were grown to 80% confluence in 100-mm plates. For ''medium only'' controls, SEB-1 medium alone was placed in 100-mm plates. 5 μM 13-*cis* RA was applied to SEB-1 sebocytes and ''medium only'' control plates in duplicate for 0, 2, 4, 6, 12, 18, 24, 48, and 72 hours. Experimental samples included medium collected from ''medium only'' control plates, medium from SEB-1 sebocyte plates, and SEB-1 sebocyte cell pellet. Sample preparation was by liquid–liquid extraction with ethyl acetate. Ethyl acetate was evaporated and the residue was re-dissolved in a mixture of acetonitrile and purified water (80/20, vol/vol) before injection. Internal standard (acitretin), 13-*cis* RA, 9-*cis* RA, and ATRA standards and quality controls solutions were made and analyzed to generate calibration curve. Samples were injected into Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA) using Nucleosil® 100-5 C18 ($250 \times 4\,mm^2$) HPLC columns (Macherey-Nagel Inc., Düren, Germany). Samples were eluted in a gradient solution composed of purified water and acetonitrile containing 0.2% acetic acid. Retinoid compounds were detected by UV detection at 350 nm.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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