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TRAIL contributes to the apoptotic effect of 13-*cis* retinoic acid in human sebaceous gland cells

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Abstract

Background—Isotretinoin's (13-*cis* RA) full mechanism of action in treating acne is unknown. 13-*cis* RA induces key genes in sebocytes that are involved in apoptosis. In this study, we report that 13-*cis* RA up-regulates expression of Tumor necrosis factor Related Apoptosis Inducing Ligand (TRAIL) within SEB-1 sebocytes.

Methods/Results—Treatment with recombinant human TRAIL (rhTRAIL) protein increases TUNEL positive staining in SEB-1 sebocytes. TRAIL siRNA significantly decreases the percentage of TUNEL positive SEB-1 sebocytes in response to 13-*cis* RA treatment. Furthermore, TRAIL expression increases in the skin of acne patients after one week of isotretinoin therapy compared to baseline. TRAIL expression localized within sebaceous glands. Unlike sebocytes, TRAIL protein expression is not increased in normal human epidermal keratinocytes in response to 13-*cis* RA, nor does rhTRAIL induce apoptosis in keratinocytes, suggesting that TRAIL is key in the sebocyte-specific apoptotic effects of 13-*cis* RA.

Conclusions—Taken together, our data suggests that TRAIL, like the neutrophil gelatinase-associated lipocalin is involved in mediating 13-*cis* RA apoptosis of sebocytes.

Keywords

isotretinoin; sebaceous gland; acne; TRAIL; apoptosis

INTRODUCTION

The full mechanism by which isotretinoin (13-*cis* retinoic acid (13-*cis* RA)) reduces the size and secretion of human sebaceous glands is unknown¹. Recent studies indicate that isotretinoin induces apoptosis in human sebaceous glands and in sebocytes in part by increasing expression of the neutrophil gelatinase-associated lipocalin (NGAL)^{2,3}.

13-*cis* RA induces key genes involved in apoptosis and cell cycle control including *TIG1*, *CDKN1A*, *LCN2* (encodes NGAL), *TNFSF10* (encodes TRAIL) and *Fas*³. Since siRNA to *LCN2* partially blocked the apoptotic response of sebocytes to 13-*cis* RA, and the Tumor necrosis factor Related Apoptosis Inducing Ligand (TRAIL) induces apoptosis, we

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hypothesize that TRAIL contributes to the apoptotic effect of 13-*cis* RA, possibly through changes in *LCN2* expression in sebocytes³.

We report that 13-*cis* RA increases expression of TRAIL within SEB-1 sebocytes and that rhTRAIL induces apoptosis in these cells. siRNA to TRAIL significantly decreased the apoptotic response of sebocytes to 13-*cis* RA; yet had no effect on levels of *LCN2* mRNA or NGAL protein. In contrast, although 13-*cis* RA increases TRAIL mRNA in normal human epidermal keratinocytes, TRAIL protein was not increased; nor did rhTRAIL induce apoptosis in keratinocytes. Taken together these data indicate that TRAIL, like NGAL, may play a role in the cell-specific apoptotic effects of 13-*cis* RA.

MATERIALS AND METHODS

Reagents

13-*cis* RA (SIGMA, St. Louis, MO) stock solutions (10mM in ethanol) were handled under yellow light and stored under N₂ gas at -20°C. Ethanol was used as control for 13-*cis* RA experiments. Recombinant human TRAIL (rhTRAIL) protein (R&D Systems; Minneapolis, MN) was reconstituted in 0.1% BSA/PBS and stored at -80° C. TRAIL monoclonal antibody, GAPDH monoclonal antibody, β -actin antibody and anti-rabbit HRP linked secondary antibody were obtained from Cell Signaling Technology (Beverly, MA). TRAIL-R1 (DR4) mouse monoclonal antibody (Abcam, Cambridge, MA) and TRAIL-R2 (DR5) goat polyclonal (R&D Systems, Minneapolis, MN) were purchased. Mouse IgG₁ and rabbit IgG (Dako, Denmark), or goat IgG (BD Pharmingen, San Jose, CA) isotype antibodies were used as negative controls.

Cell Culture

SEB-1 sebocytes were cultured as previously described^{3,4}. SEB-1 sebocytes are an immortalized cell line established from SV40 Large T antigen transformation of cultured sebocytes from a 55-year old male. Commercially available NHEK keratinocytes (neonatal-pooled) were cultured in Keratinocyte Growth Medium-2 (KGM-2) (Cambrex Bioscience, Walkersville, MD). SV40 immortalized KH-SV keratinocytes (gift, Dr. C. Gaggioli and Dr. G. Meneguzzi) were cultured in KGM-2 medium. Stock solutions of 13-*cis* RA and rhTRAIL were diluted in standard culture medium.

Immunohistochemistry

Staining was performed on methanol-fixed SEB-1 sebocytes or formalin-fixed paraffin-embedded human skin sections using the avidin-biotin complex method and AEC development (ABC and AEC Substrate Kit for Peroxidase, Vector Laboratories, Inc.; Burlingame, CA). Samples were incubated with TRAIL antibody, TRAIL-R1 (DR4) or TRAIL-R2 (DR5) antibody. Samples were counter-stained with hematoxylin using standard procedures. Representative images were captured under 10X (skin sections) or 20X (cells) objectives (100–200X total magnification).

Immunoblotting

Cells were treated with control (ethanol), 0.1 μ M or 1 μ M 13-*cis* RA for 24, 48 and 72 hours. siRNA nucleofected SEB-1 sebocytes (1.5×10^6) were plated in 100-mm plates and treated with 0.1 μ M 13-*cis* RA 24 hours after nucleofection for 12, 24, or 48 hours. Total cell protein lysates were harvested and prepared for SDS-PAGE and immunoblotting as previously described². Each experiment included 2–3 replicates of samples and was repeated a minimum of 3 independent times. Data was analyzed using a paired *t*-test and considered significant if $p < 0.05$.

QPCR

Quantitative real-time PCR was performed using Applied Biosystems' Taqman Universal PCR Master Mix and primer probe sets with ABI's 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Cells were treated with vehicle or 0.1 μ M 13-*cis* RA in triplicate for 12, 24, 48 and 72 hours. Total RNA was isolated (RNeasy kit Qiagen Inc., Valencia, CA) and its integrity verified by agarose gel electrophoresis followed by cDNA conversion (Superscript First-Strand Synthesis System Invitrogen, Carlsbad, CA). Diluted cDNA samples were run for the reference gene *TATA* binding protein (TBP), TNFSF10 (TRAIL) and *LCN2* (siRNA experiments only) with appropriate assay controls. Data were analyzed using the REST-XL^c program with efficiency corrected values and considered significant if $p < 0.05$.

siRNA

Nucleofection optimization and efficiency in SEB-1 sebocytes was previously described³. ON-TARGET plus human TNFSF10 (TRAIL), GAPDH and siCONTROL siRNA duplex oligonucleotides were purchased from Dharmacon Research (Lafayette, CO). SEB-1 sebocytes were nucleofected with 1 μ g siCONTROL, 1 μ g GAPDH or 1 μ g TNFSF10 (TRAIL) siRNA. Nucleofection was performed in triplicate samples. 13-*cis* RA (0.1 μ M) was added 24 hours post-nucleofection to induce TRAIL gene expression. Expression of TRAIL and extent of siRNA knockdown was verified by QPCR and immunoblotting for TRAIL at 12, 24 or 48 hours of 13-*cis* RA treatment. *LCN2* mRNA and NGAL protein expression were also verified in these samples to evaluate whether or not TRAIL acts upstream of *LCN2* in the apoptotic pathway in sebocytes.

TUNEL

rhTRAIL protein or non-muscle actin protein was applied in duplicate samples at concentrations of 1 ng/mL, 12 ng/mL, 20 ng/mL, 100 ng/mL rhTRAIL or vehicle control in culture medium for 72 hours. Samples were prepared with appropriate assay controls per manufacturer's instructions (*In Situ* Cell Death Detection Kit Roche Applied Science). Results were analyzed and quantified by counting the number positive staining cells/total cells in 4 representative fields per well for three independent experiments. Actin experiments were repeated twice. Analysis was performed using ANOVA Two-Factor with Replication and significant if $p < 0.05$.

RESULTS

13-*cis* RA increases TRAIL expression in SEB-1 sebocytes

Microarray analysis indicated that TRAIL is up-regulated 4.18-fold with 13-*cis* RA compared to control in SEB-1 sebocytes³. The time course of TRAIL expression was examined using QPCR. TRAIL mRNA increased by 5.92-, 4.56-, 3.68- and 2.53-fold after 12, 24, 48 and 72 hours of treatment with 0.1 μ M 13-*cis* RA, respectively. TRAIL mRNA expression was maximal at 12 hours of 13-*cis* RA treatment in SEB-1 (Figure 1a). TRAIL mRNA expression in response to 13-*cis* RA was also assessed in normal human epidermal keratinocytes (NHEK) with a marginal (~2-fold) increase compared to control at 24, 48 or 72 hours ($p < 0.05$) (Figure 1a).

Immunocytochemistry demonstrated that TRAIL predominantly localized to the cytoplasm in SEB-1 with increased expression following treatment with 13-*cis* RA (Figure 1b). To examine the possibility that TRAIL expression in SEB-1 sebocytes was influenced by the presence of SV40 Large T antigen, parallel experiments were performed in the SV-40 large T antigen immortalized keratinocyte cell line, KH-SV, in addition to normal human epidermal keratinocytes (NHEK). Baseline (control) levels of TRAIL protein were minimal

in all cells. 13-*cis* RA induced a dose-dependent increase in TRAIL protein expression in SEB-1 with immunoblotting (Figure 1c). TRAIL expression was increased ~3-fold (300%) and ~6–8 fold (600–800%) in SEB-1 in the presence of 0.1 μ M and 1 μ M 13-*cis* RA respectively at 24 and 48 hours (Figure 1c). Increased TRAIL expression persisted at 72 hours with the higher dose. The overall level of TRAIL protein within NHEKs was markedly less than SEB-1 sebocytes as assessed by western blotting using similar quantities of protein. Despite the increase in TRAIL mRNA in 13-*cis* RA treated NHEK; protein levels of TRAIL were barely detectable at each time point (Figure 1c). In KHSV, 13-*cis* RA increased TRAIL protein levels at 24 hours by ~2-fold over control (data not significant). Unlike sebocytes, TRAIL protein was barely detectable after 24 hours of treatment (Figure 1c). These data imply that cell-specific differences in TRAIL protein expression exist in response to 13-*cis* RA.

Sebocytes are more sensitive to rhTRAIL- induced apoptosis compared to keratinocytes

To determine if the TRAIL apoptotic pathway is intact within sebocytes and keratinocytes, rhTRAIL protein was added to cell cultures for 72 hours and followed by a TUNEL assay (Figure 2). In SEB-1, rhTRAIL (12 ng/mL) significantly increased the percentage of cells undergoing apoptosis from ~4% in control to ~16% (4-fold increase). Higher concentrations of rhTRAIL (20 ng/mL and 100 ng/mL) increased the percentage of cells in apoptosis to ~23% and 35% (~6- and 9-fold increase), respectively (Figure 2a, 2b).

TRAIL induces apoptosis in cancer cells and immortalized cell lines. Recent studies demonstrated that TRAIL also induces apoptosis in normal cells and in primary cell lines^{5–10}. To rule out the possibility that induction of apoptosis by rhTRAIL in SEB-1 sebocytes was a function of immortalization by the SV40 large T antigen, parallel experiments were run in the KHSV keratinocytes. In contrast to SEB-1 sebocytes, rhTRAIL induced apoptosis in KH-SV keratinocytes only at the highest concentration tested with 17% of cells undergoing apoptosis compared to 35% in SEB-1 sebocytes (Figure 2b). Previous studies in our laboratory demonstrated that NHEK did not undergo apoptosis with 13-*cis* RA². As shown above, 13-*cis* RA does not induce TRAIL protein expression in NHEK, nor does treatment of NHEK with rhTRAIL significantly increase apoptosis (data not shown). These data suggest that NHEK is resistant to rhTRAIL induced apoptosis.

Parallel TUNEL experiments were performed with increasing concentrations of non-muscle actin protein (1, 12, 20, and 100 ng/mL) in all cell lines. No increase in apoptosis was noted compared to control, indicating that apoptosis in response to rhTRAIL is not due to effects of exogenous protein.

siRNA knockdown of TRAIL inhibits TUNEL positive staining in SEB-1 sebocytes

RNA interference methods were utilized to examine the role of TRAIL in mediating the apoptotic actions of 13-*cis* RA in SEB-1 sebocytes. Twenty-four hours after nucleofection with siRNA to TRAIL, sebocytes were treated with 0.1 μ M 13-*cis* RA to induce TRAIL expression. TRAIL mRNA and protein levels were assessed by QPCR and immunoblotting.

Messenger RNA for *TNFSF10* (TRAIL) mRNA was successfully decreased ~3.2-fold (70%) by the TRAIL-specific siRNA compared to siCONTROL in the presence of 13-*cis* RA for 12 hours (Figure 3a). The specificity of siRNA knockdown was verified using siRNA to *GAPDH* in parallel samples. *GAPDH* and *TNFSF10* gene expression were successfully inhibited in their respective samples. At 24 hours of 13-*cis* RA treatment, TRAIL protein levels decreased 2.6-fold (62%) compared to the siCONTROL with a slight recovery after 48 hours of 13-*cis* RA treatment that was still 37% less than siCONTROL (Figure 3b).

Decreasing TRAIL by siRNA in the presence of 13-*cis* RA for 24 hours significantly decreased the percentage of TUNEL positive cells from ~ 25% in siCONTROL and GAPDH samples to ~8% (2.85-fold reduction) with TRAIL-specific siRNA (Figure 3c). The percentage of cells undergoing apoptosis with 13-*cis* RA did not differ between siCONTROL and GAPDH knockdown samples and is similar to previous published studies²

TRAIL does not influence expression of *LCN2* in the presence of 13-*cis* RA in SEB-1 sebocytes

Since our prior reports indicate that 13-*cis* RA induces apoptosis in sebocytes via increases in *LCN2* and its protein product, NGAL, we sought to determine whether TRAIL affects expression of *LCN2*. *LCN2* mRNA and NGAL protein expression were assessed in TRAIL-specific siRNA samples compared to siCONTROL in the presence of 13-*cis* RA. As above, TRAIL expression decreased 3.2-fold ($p < 0.05$) in the presence of TRAIL-specific siRNA, however, *LCN2* mRNA was decreased 1-fold (not statistically significant, $p = 0.983$). In addition, NGAL protein levels were not influenced by the knockdown of TRAIL (data not shown).

TRAIL-R1 is abundantly expressed in SEB-1 sebocytes

Differences between TRAIL-induced apoptosis in SEB-1 sebocytes and NHEK could result from differential expression of TRAIL receptors. Apoptosis induction by TRAIL occurs via binding to its cell surface death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5)¹¹. The expression of both receptors was examined by immunocytochemistry and immunoblotting in the cell lines. Western analysis indicated the presence of similar levels of DR4 in SEB-1 and NHEK under baseline conditions (Figure 4a). DR5 however was expressed only in NHEK (Figure 4a). Alternative splicing of DR5 mRNA generates two isoforms of the receptor with molecular weights of 44kD and 47kD¹². Both isoforms are present in HepG2 (positive control) and the larger isoform is present in NHEK. Immunocytochemistry confirmed the presence of DR4 and the absence of DR5 in SEB-1 sebocytes (Figure 4b). Treatment of SEB-1 sebocytes with 13-*cis* RA for 48 hours did not influence the localization of DR4 or DR5 as assessed by immunohistochemistry (data not shown).

TRAIL expression is increased in sebaceous glands from acne patients after 1 week of isotretinoin therapy

To assess the clinical relevance of our findings, we examined TRAIL expression in skin and sebaceous glands of patients undergoing isotretinoin therapy. Using patient skin biopsies obtained from the **upper back section** of patients, immunohistochemistry using an antibody to TRAIL was performed on 7 acne patients before isotretinoin and at 1 week of treatment. Patient biopsies were previously gathered under an IRB-approved protocol and patient demographics are previously published³. Each patient's baseline and 1 week sections were processed together and comparisons in TRAIL expression were made. Representative images are shown for 5 different patients, illustrating the range of TRAIL expression among patients (Figure 5). TRAIL expression increased at 1 week of isotretinoin treatment in 3/5 patients. Of note, many acne patients expressed TRAIL at baseline within their sebaceous glands. This was not observed in normal skin from subjects without acne (data not shown). Of the 7 acne patients examined: four patients had more TRAIL expression within the basal layers of the sebaceous glands at 1 week of isotretinoin compared to baseline (Pts. 9–12), two patients had increased TRAIL expression within the cells of the sebaceous duct at one week of treatment (Pts. 13, 14) one patient had no expression of TRAIL either at baseline or 1 week (Pt. 11) and no comparison was possible for the final patient (Pt. 15) because no sebaceous glands were contained within the baseline biopsy.

DISCUSSION

Elucidating the cellular processes affected by 13-*cis* RA in sebocytes is key to understanding its overall molecular mechanism of action in the treatment of acne. To this end, our studies focused on understanding how 13-*cis* RA leads to apoptosis in SEB-1 sebocytes. Gene expression analysis revealed significant up-regulation of many genes involved in apoptosis including lipocalin 2 (*LCN2*), TRAIL (*TNFSF10*) and Fas (*CD95*)³. *LCN2* encodes neutrophil gelatinase associated lipocalin (NGAL) whose lipocalin family members are involved in innate immune defense, retinal transport, prostaglandin synthesis, renal tube morphogenesis and cellular growth, metabolism and apoptosis^{13–17}. Previous studies in our lab demonstrate that NGAL mediates, in part, the apoptotic effect of isotretinoin³. Successful inhibition of mRNA to *LCN2*, however, does not completely block apoptosis suggesting involvement of additional molecule(s) such as TRAIL.

TRAIL is a type II transmembrane protein expressed on the surface of cells. Its extracellular C-terminal domain can be proteolytically cleaved to form soluble TRAIL. Both the membrane and soluble forms of TRAIL induce apoptosis in a variety of primary cells and tumor cell lines via activation of cell-surface death receptors^{5,6,9,18–20}. TRAIL expression has been reported in human epidermis, notably in the basal layers, infundibulum and the outer root sheath of hair follicles²¹. Our studies indicate that TRAIL is also expressed in the sebaceous gland and that expression is increased by 13-*cis* RA.

TRAIL-R1 (DR4) and TRAIL-R2 (DR5) death receptors detect extracellular stimuli and, upon binding of TRAIL ligand, rapidly activate an intracellular caspase signaling cascade leading to apoptosis¹¹. DR4 and DR5 receptors are expressed in human skin, NHEK and HaCaT keratinocytes^{10,18,21,22}. We confirmed the presence of DR4 in NHEK and demonstrated for the first time, its presence in SEB-1 sebocytes. DR5 was detected in NHEK but not SEB-1 cells.

Our study is the first to demonstrate up-regulation of TRAIL by 13-*cis* RA both *in vitro* and *in vivo* in human sebocytes and patient skin, respectively. All-*trans* retinoic acid (ATRA) and other RAR α specific ligands increase TRAIL expression, leading to apoptosis in acute promyelocytic leukemia (APL)²³. In addition, retinoids including fenretinide, synthetic retinoid CD437, and ATRA increase sensitivity to TRAIL-induced apoptosis by increasing expression of TRAIL-R1 and -R2 receptors in colon, prostate, and lung cancer cell lines^{24–26}. 13-*cis* RA alone or in combination with interferon is reported as beneficial in the treatment of some leukemias through induction of apoptosis by an undefined mechanism^{27,28}. Our data indicate that 13-*cis* RA induces TRAIL expression in sebocytes. Although not tested in this study, it is possible that the induction of TRAIL by 13-*cis* RA may play a role in the apoptotic effect of this drug on leukemic cells.

Interferon regulatory factor 1 (*IRF1*) is a critical factor in mediating TRAIL induction by retinoic acid in NB4 APL leukemia cells and SK-BR-3 breast cancer cells²⁹. Interestingly, 13-*cis* RA significantly increases *IRF1* gene expression in SEB-1 sebocytes which in turn may induce TRAIL expression³.

Sebocytes are more sensitive to rhTRAIL-induced apoptosis than keratinocytes. Free fatty acids (FFA) can trigger 'lipo-apoptosis' in cells. Primary rat hepatocytes and hepatocyte cell lines become sensitive to TRAIL-induced apoptosis in the presence of physiological concentrations of oleic or stearic acid³⁰. Fatty acids are abundant within sebaceous glands and sebocytes. Although the majority of fatty acids are complexed within triglycerides, diglycerides, cholesterol esters and wax esters, free fatty acids such as oleic acid are found in sebaceous glands³¹. It would be interesting to determine if the presence of greater levels

of fatty acids within sebocytes increases their susceptibility to rhTRAIL mediated apoptosis compared to keratinocytes.

13-*cis* RA specifically induces apoptosis in sebocytes but not in keratinocytes via induction of NGAL³. Similar findings were made in this study with regard to the induction of TRAIL expression by 13-*cis* RA, raising the question as to the relative contributions of NGAL and TRAIL in the apoptotic effects of 13-*cis* RA in sebaceous glands. In this study, a maximal reduction in TRAIL protein expression of ~65% was obtained with siRNA which reduced apoptosis by 65% in response to 13-*cis* RA. In contrast, siRNA knockdown of *LCN2* (encodes NGAL) within SEB-1 sebocytes completely abolishes NGAL protein expression, yet reduces apoptosis in response to 13-*cis* RA by ~80%, suggesting that an additional protein such as TRAIL may be involved in the apoptotic response of sebaceous glands to 13-*cis* RA. Our data shows that siRNA knockdown of TRAIL also decreases 13-*cis* RA induced apoptosis in sebocytes, however, knockdown of TRAIL does not influence 13-*cis* RA induction of *LCN2*/NGAL in sebocytes, thus suggesting two independent pathways of apoptosis induction by 13-*cis* RA.

In summary, 13-*cis* RA-induced apoptosis is mediated, in part, by increased expression of TRAIL in SEB-1 sebocytes. As in the case of the NGAL protein, the up-regulation of TRAIL protein is minimal in NHEK and KH-SV, and rhTRAIL (or NGAL) does not induce apoptosis in keratinocytes. These data suggest that TRAIL and NGAL are key proteins in the sebocyte-specific apoptotic response to 13-*cis* RA, which in part may be due to the lipid-rich environment of the sebocyte. Further elucidation of the mechanisms by which 13-*cis* RA induces apoptosis may have therapeutic implications both in dermatology and in cancer treatment.

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What is already known?

- Isotretinoin decreases size and secretion of human sebaceous glands
- Isotretinoin induces apoptosis within sebaceous glands
- Neutrophil gelatinase-associated lipocalin mediates isotretinoin-induced apoptosis

What this study adds?

- Isotretinoin increases TRAIL protein expression in sebocytes but not in normal human epidermal keratinocytes
- Sebocytes are more sensitive to TRAIL- induced apoptosis compared to keratinocytes
- Data suggests that TRAIL plays a key role in the cell-specific effects of isotretinoin

Figure 1. 13-cis RA increases TRAIL expression in SEB-1 sebocytes

SEB-1 sebocytes, NHEK, and KH-SV keratinocytes were treated with vehicle (C) or 13-cis RA (0.1 μM or 1 μM) for 12, 24, 48 and 72 hours. (a) QPCR verification of *TNFSF10* (*TRAIL*) mRNA expression after 0.1 μM 13-cis RA treatment. Mean ± SE; n=3 samples with REST-XL statistical analysis; * p < 0.05. (b) Immunocytochemistry of SEB-1 sebocytes treated for 48 hours with 13-cis RA using a rabbit monoclonal antibody to TRAIL. Representative images are shown. (c) Representative TRAIL immunoblots are shown after 13-cis RA treatment for 24, 48 and 72 hours. Graph represents the fold-change relative to control. Band intensity was normalized to β-actin. Data represent the (mean ± SE) of 3

independent sample sets and immunoblots. Statistical analysis was performed with paired t -test, * $p < 0.05$.

Figure 2. rhTRAIL increases apoptosis in SEB-1 sebocytes with effects on keratinocytes only at the highest dose

SEB-1 sebocytes and KH-SV keratinocytes were treated with vehicle (control), 1 ng/mL, 12 ng/mL, 20 ng/mL, or 100 ng/mL of purified recombinant human TRAIL protein (R&D Systems). **(a)** Representative images of the SEB-1 TUNEL assay using rhTRAIL are shown. Original magnification: 200X. **(b)** Quantification of the percentage of TUNEL positive stained cells per treatment at 72 hours. Data represent mean $\pm$ SE, $n = 4-6$. Statistical analyses were performed between control and each treatment concentration of rhTRAIL using ANOVA Two Factor with Replication; * $p < 0.05$.

Figure 3. Specific siRNA to TRAIL “knocksdown” TRAIL expression and decreases apoptosis in the presence of 13-cis RA in SEB-1 sebocytes

(a) Decrease in *TNFSF10* (*TRAIL*) mRNA expression was verified by QPCR after 12 and 24 hours of 0.1 μM 13-*cis* RA treatment following nucleofection with siRNA to TRAIL. Data represent the mean ± SE of the fold-change of 3 independent nucleofection samples. **(b)** Total protein lysates from parallel samples were subjected to immunoblotting for TRAIL and GAPDH after 24 and 48 hours of 0.1 μM 13-*cis* RA treatment following nucleofection with siRNA to TRAIL. Representative blots are shown. **(c)** TUNEL assay on SEB-1 sebocytes nucleofected with siRNA to TRAIL and treated 13-*cis* RA for 24 hr (representative images). Graph shows quantification of the percentage of TUNEL positive stained cells (mean ± SE, n = 6 independent samples); * p < 0.05.

Figure 4. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) expression and localization in SEB-1 sebocytes

(a) Total protein lysates from SEB-1 sebocytes, NHEK, and positive controls (HeLa and HepG2 cells) were cultured under standard conditions and assessed by western blotting for TRAIL-R1 (DR4) and TRAIL-R2 (DR5). Equal protein loading was verified by β -actin. Representative blots are shown. (b) SEB-1 sebocytes were subjected to immunocytochemistry with TRAIL-R1 and TRAIL-R2 antibodies. IgG isotype antibodies were used as negative controls. Representative images are shown. Total magnification: 200X.

Figure 5. TRAIL expression is increased in acne patients and after 1 week of isotretinoin therapy Biospy sections of skin at baseline and 1 week after starting isotretinoin were available from a previous study³. Immunohistochemistry was performed with TRAIL monoclonal or rabbit IgG control antibodies (data not shown) according to standard procedures. Representative images are shown for 5 different patients at baseline and at 1 week of isotretinoin therapy to illustrate the location and range of TRAIL expression. Patient 11 had no TRAIL expression at baseline and did not increase at 1 week of isotretinoin. Patients 13 and 14 had TRAIL expression at 1 week with no expression at baseline, and Patients 9 and 12 had minimal TRAIL expression at baseline that was increased after 1 week isotretinoin.