Effects of 13-Cis-Retinoic Acid, All-Trans-Retinoic Acid, and Acitretin on the Proliferation, Lipid Synthesis and Keratin Expression of Cultured Human Sebocytes In Vitro

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The aim of this study was to determine the effects of 13-cis-RA, all-trans-RA, and acitretin on the proliferation, lipid synthesis, and keratin expression of human sebocytes in vitro and to elucidate possible mechanisms of retinoid action on sebaceous glands at the cellular level.

It was found that 13-cis-RA and all-trans-RA decreased sebocyte proliferation in a dose- and time-dependent manner, with a 13-cis-RA – IC_{50} of 10^{-5} M (after 7 d) and 10^{-6} M (after 14 d) and an all-trans-RA – IC_{50} of 10^{-7} M (after 14 d; no IC_{50} after 7 d). Acitretin inhibited sebocyte proliferation only at 10^{-5} M.

Furthermore, 13-cis-RA was the most potent inhibitor of acetate incorporation into lipids, which indicated lipid synthesis (48.2% reduction), followed by all-trans-RA (−38.6%), and by acitretin (−27.5%). All retinoids tested markedly decreased the synthesis of triglycerides, wax/stearyl esters, and free fatty acids in cultured sebocytes, whereas squalene synthesis remained uninfluenced and cholesterol synthesis slightly increased.

On the other hand, keratin 5 was down-regulated, keratin 17 was up-regulated, and the expression of keratin 13 was virtually unaffected by all retinoids tested. Keratins 6 and 16 were down-regulated by 13-cis-RA and by all-trans-RA, keratin 14 was down-regulated by 13-cis-RA only, and keratin 19 was up-regulated by all-trans-RA.

These investigations indicate that 13-cis-RA and, to a lesser extent, all-trans-RA are potent inhibitors of both cell proliferation and lipid synthesis in human sebocytes in vitro, whereas acitretin only decreases lipogenesis in this model. In addition, retinoids may modify the differentiation of sebocytes in vitro by modulating keratin expression. Models of cultured human sebocytes are useful tools for further investigations on the sebaceous gland and its activity at the cellular level. *J Invest Dermatol 96:792–797, 1991*

Sebum production is generally accepted to be one of the major factors involved in the etiology of acne [1]. Retinoids [2] have been shown both to reduce sebaceous gland size [3,4] and to suppress sebum production [5,6] in rodent models and in humans, possibly due to prolongation of the maturation of basal sebocytes [5,7]. In recent studies, the clinical benefits of 13-cis-retinoic acid (13-cis-RA) in individuals with acne were well documented [8–10].

Despite the wealth of clinical and experimental data, the precise mode of action of the retinoids on sebaceous gland cells remains to be determined. Adequate in vitro models have been recently developed [11–13]. The purpose of this study was to investigate possible mechanisms by which retinoids exert their action on sebocytes. We determined, therefore, the effects of 13-cis-RA, all-trans-retinoic acid (all-trans-RA), and of acitretin on the proliferation, lipid synthesis, and keratin expression of human sebocytes cultured in vitro [13]; in this in vitro model the cultured cells were shown to preserve sebocytic differentiation [14].

MATERIALS AND METHODS

Human Sebocyte Cultures Primary human sebocyte cultures were obtained as previously described [13]. Briefly, full-thickness

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Abbreviations:

^{3}H-AA: ^{3}H-radiolabeled acetic acid, sodium salt (acetate)
^{3}HTdR: ^{3}H-radiolabeled thymidine
DMEM: Dulbecco's modified Eagle's medium
DMSO: dimethyl sulfoxide
DNA: desoxyribonucleic acid
IC_{50}: 50% inhibitory concentration
mRNA: messenger ribonucleic acid
PBS: phosphate-buffered saline
RA: retinoic acid
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC: thin-layer chromatography
Tris: tris-hydroxymethyl aminopeptidase

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human facial skin from young individuals was incubated 20 h in 2.4 U/ml dispase (Boehringer, Mannheim, FRG) at 4°C to separate epidermis from dermis. Epidermis was then maintained 15 min in 0.02% desoxyribonuclease (Sigma, Deisenhofen, FRG) at 37°C. Using microsurgical instruments under microscopical observation of the epidermal underface intact sebaceous glands were isolated and their ducts were removed. The isolated gland lobules were cultivated on mitomycin C (Sigma), inactivated 3T3 cells in Dulbecco's modified Eagle's medium (DMEM) and Ham's F 12 medium (3:1; Gibco, Berlin, FRG) supplemented with 8% fetal calf serum (Seromed, Berlin, FRG), 2% human serum, 10 ng/ml epidermal growth factor (Sigma), 10^{-9} M cholera toxin (Calbiochem, Frankfurt, FRG), 3.4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all Seromed) by 37°C with 5% CO_2. Primary sebocyte cultures resulted as outgrowths from the periphery of the gland lobules. All experiments have been performed using secondary sebocyte cultures.

Treatment with Retinoids 13-cis-RA (isotretinoin, Ro 4-3780), all-trans-RA (tretinoin, Ro1-5488) and acitretin (all-trans-9-[4-methoxy - 2,3,6 - trimethylphenyl] - 3,7 - dimethyl - 2,4,6,8 - nonatetraenoic acid, Ro 10-1670) (all Hoffmann-La Roche, Basel, Switzerland) were dissolved in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, FRG) giving final concentrations from 10^{-8} to 10^{-5} M and 0.2% DMSO, respectively, where 0.2% DMSO alone served as control. Retinoids were handled under dimmed yellow light.

For the proliferation experiments, medium supplemented with retinoids was added 5 d after seeding of 2×10^4 sebocytes per well in 24-well culture plates (Falcon, Jersey City, NJ) on 10^4 3T3 cells/well, inactivated with mitomycin C. Wells seeded only with 3T3 cells served as controls. Medium with or without retinoids was changed every 3 d. The cultures were incubated at 37°C under retinoids up to 7–14 d.

For the experiments on lipid synthesis and keratin expression, medium supplemented with retinoids (10^{-7} M) was added in confluent sebocyte cultures maintained in 60 mm culture dishes (Falcon). Medium with or without retinoids was changed every 3 d. The cultures were incubated at 37°C under retinoids up to 8 d.

Cell Proliferation Cell proliferation was evaluated by cell counting and by determination of ^{3}H-radiolabeled thymidine (^{3}HTdR) incorporation into desoxyribonucleic acid (DNA). Single cell suspensions were counted in a Becton-Dickinson CC-VI blood cell counter. ^{3}HTdR incorporation was assessed by liquid scintigraphy after a terminal pulse-labeling of 6 h with 1 μCi/ml [methyl-^{3}H]-TdR (50–70 Ci/mM; Amersham-Buchler, Braunschweig, FRG) [15].

Labeling and Extraction of Sebocyte Lipids Sebocyte cultures were pulsed with 2 μCi/ml ^{3}H-radiolabeled acetic acid, sodium salt (acetate) (^{3}H-AA, 86.4 mCi/mM; NEN, Dreieich, FRG), maintained in a medium containing ^{3}H-AA with or without retinoids during the whole treatment period. To extract the lipids, the growth medium was removed from the cultures, the cultures were rinsed 3 times with cold phosphate-buffered saline (PBS; Seromed) containing 0.1% fatty acid-free bovine serum albumin (Sigma), twice with PBS, and then manually scraped from the dishes into 10-ml glass vials. The pellets were diluted in a solution of chloroform and methanol (2:1) and mechanically homogenized for 15 min in a Janke & Kunkel homogenator. The solutions were then filtrated and the filtrates were twice evaporated to dryness at 40°C under a stream of nitrogen. The lipid residues were weighted and redissolved in chloroform for analysis. ^{3}H-AA incorporation into lipids was assessed by liquid scintigraphy.

Thin-Layer Chromatography (TLC) Analytical TLC was carried out on 20 × 20 cm glass plates coated with 0.25-mm–thick silica gel 60 (Merck). The plates were treated with hexane and left to dry 24 h and the samples were then applied in 7-mm–wide lanes. Chromatograms of neutral lipids were developed in a hexane–benzene solution (1:1, to 18 cm), left to dry, and redeveloped in a solution of hexane–diethyl ether–acetic acid (80:20:1, to 18 cm). The chromatograms were then heated for 20 min to 150°C, cooled, dipped in 3% H_2SO_4 and reheated for 20 min to 150°C to char the lipids [16]. Lipids were identified by comparison on TLC with standards obtained from Sigma.

Autoradiography of TLC Plates For quantitative analysis, hyperfilm-^{3}H (Amersham–Buchler) was placed over the developed plates 30 d before charring and then processed by a Kodak GBX x-ray film developer. The autoradiograms were scanned on a Shimadzu CS-910 photodensitometer by absorption of 450 nm and the lipids were quantitated according to Downing and Stranieri [17].

Preparation of Keratin-Enriched Protein Fractions Sebocytes were manually scrapped from the dishes, harvested into centifuge tubes, homogenized by ultrasonic disruption in 20 mM tris-hydroxymethyl aminopeptidase (Tris)/HCl (pH 7.4; Merck), containing 1 mM phenylmethylsufonyl fluoride (Serva, Heidelberg, FRG) and 5 g/ml each of pepstatin and antipain (both Sigma), and extracted twice with 600 mM KCl, 5 mM ethylene diamine tetraacetic acid, 5 mM ethylene glycol tetraacetic acid, 50 mM (Tris)/HCl (all Merck), 1% triton X-100 (Serva), and protease inhibitors as described above. Each extraction step included ultrasonic disintegration and centrifugation at 27,000 × g. The resulting pellet, highly enriched in cytoskeletal proteins, was solubilized in sample buffer consisting of 0.5 M Tris/HCl (pH 6.8), 2.1% sodium dodecyl sulfate (Serva), 10% mercaptoethanol (Sigma), 10% glycerol (Merck) and bromphenol blue (Serva), and heated for 10 min at 90°C.

Gel Electrophoresis and Densitometry One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [18]. Gels were stained in 0.2% Serva-Blue R (Serva). For quantitation of protein bands separated by SDS-PAGE, a LKB Ultro-Scan XL laser densitometer connected with an IBM-PC PS 2/50 was used. The measured levels of keratins were referred in ratio to keratin 4 serving as an internal reference, as the expression of keratin 4 remained practically unchanged after retinoid treatment.

Statistical Analysis All experiments were performed in triplicate dishes. Statistical significance of the data was evaluated by the two-sided Student t test. Mean differences were considered to be significant when $p < 0.05$.

RESULTS

Antiproliferative Activity The retinoids tested widely differed in their ability to inhibit proliferation of human sebocytes in vitro (Fig 1). We found that 13-cis-RA exhibited a marked dose-dependent antiproliferative effect after 7 d of treatment at all concentrations tested (10^{-8} M, $p < 0.05$), whereas all-trans-RA inhibited sebocyte proliferation only at high concentrations ($\geq 10^{-6}$ M, $p < 0.05$). After 14 d of treatment, both 13-cis-RA and all-trans-RA significantly inhibited sebocyte proliferation in vitro at all concentrations tested in a dose-dependent manner (10^{-8} M, $p < 0.05$). The 50% inhibitory concentration (IC_{50}) of 13-cis-RA was about 10^{-5} M and 10^{-6} M after 7 and 14 d, respectively. The IC_{50} of all-trans-RA was about 10^{-7} after 14 d of treatment, whereas no IC_{50} was achieved after 7 d at the concentrations tested. Acitretin inhibited sebocyte proliferation only at 10^{-5} M after 7 and 14 d of treatment (10^{-5} M, $p < 0.01$ and < 0.001, respectively). At this concentration, an acitretin-IC_{50} was achieved, however, no inhibitory effect was detected at lower concentrations ($p > 0.1$).

Effects on Lipid Synthesis All retinoids tested decreased the ratio of ^{3}H-AA incorporation into lipids/total lipids' weight indicating an inhibition of lipid synthesis in human sebocytes in vitro, however, quantitative differences were observed (Fig 2). The most potent inhibitor was 13-cis-RA (48.2% reduction of ^{3}H-AA incorporation/μg lipids at 10^{-7} M) followed by all-trans-RA (38.6% reduction) and acitretin (27.5% reduction). On the other hand, analytical TLC of neutral lipids synthesized under retinoid treatment

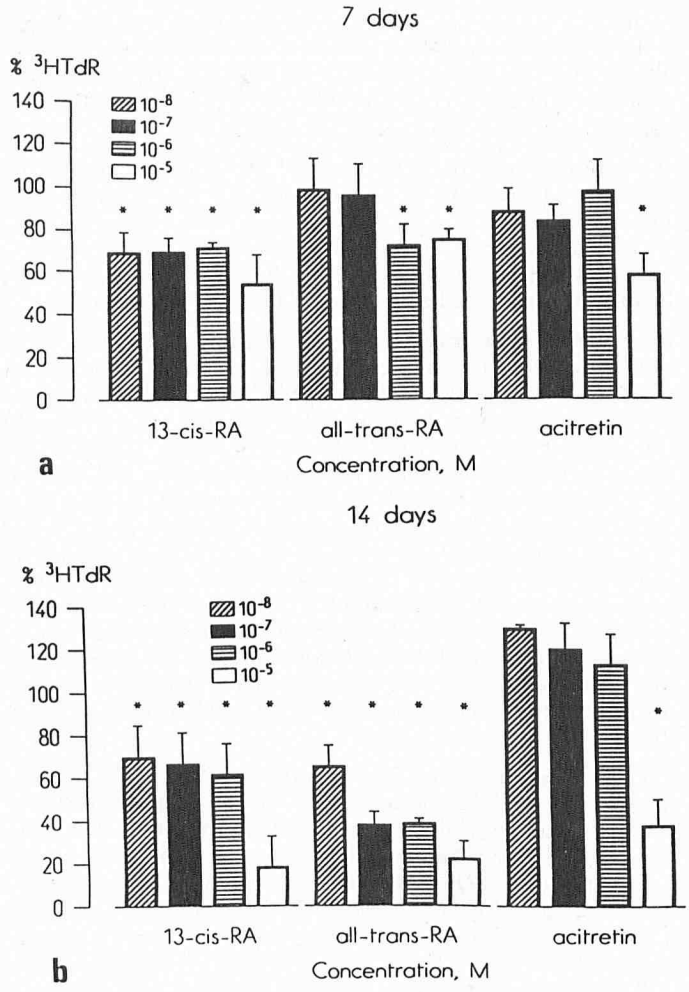

Figure 1. Proliferation of human sebocytes in vitro (*a*) after 7 and (*b*) after 14 d of retinoid treatment, as assessed by ^{3}HTdR incorporation into DNA. The values are means ± SD of triplicate cultures (percent of controls). *Asterisk*, at least $p < 0.05$.

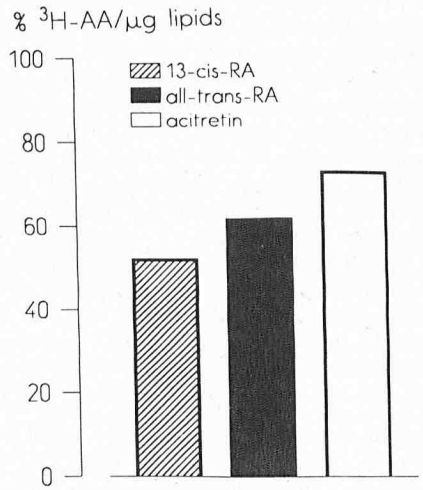

Figure 2. Effects of retinoids on lipid synthesis of human sebocytes in vitro, as assessed by the relationship of the ^{3}H-AA incorporation into lipids to the weight of the lipids extracted. Confluent cultures were treated for 8 d in the presence of ^{3}H-AA. Values resulted from lipids extracted from triplicate cultures (percent of controls).

revealed marked changes of all lipid classes except of squalene (Fig 3). All retinoids tested markedly inhibited synthesis of wax and stearyl esters (90–92% reduction) and of free fatty acids (about 100% reduction), whereas quantitative differences of triglyceride synthesis were obtained among the three retinoid compounds: 13-cis-RA presented the strongest inhibitory effect with 52% reduction followed by all-trans-RA (−37%), and acitretin (−15%). Interestingly enough, cholesterol synthesis increased under retinoid treatment: 69% increase under acitretin and +26–34% under all-trans- and 13-cis-RA. No influence of the retinoids tested on squalene synthesis was identified; it has to be noticed, however, that squalene synthesized in cultured sebocytes represented only 2–3% of the total lipid synthesis (data not shown).

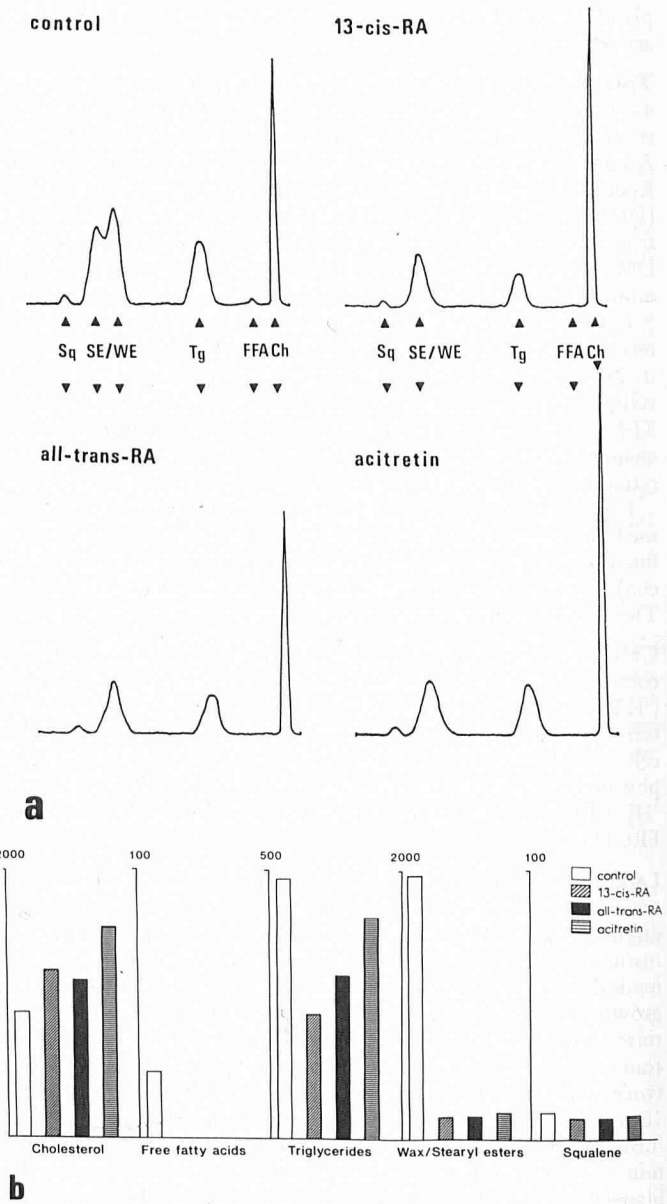

Figure 3. Effects of retinoids on the synthesis of different lipid classes in human sebocytes in vitro, as assessed by photodensitometry of the autoradiograms after TLC of ^{3}H-AA–labeled neutral lipids. Confluent cultures were treated 8 d in presence of ^{3}H-AA. Values resulted from lipids extracted from triplicate cultures. *a*, photodensitometric peak areas obtained on scanning the autoradiograms. Lipid classes were identified by comparison to standards on parallel acid-charred, thin-layer chromatograms. *b*, quantitation of the photodensitometric peak areas. The values on y axes represent the results of calculations according to the formula $H^{1.4} \times W/2$, where H is height and W is width of the in triangles' adjusted peak areas in *a*.

Effects on Keratin Expression The retinoids tested affected the expression of certain keratins in human sebocytes in vitro, however, neither the expression of new keratins nor the disappearance of keratins expressed in untreated cultures were observed (Fig 4). Only minor changes were detected in the expression of keratin 4 under retinoids (data not shown). In ratio to keratin 4, down-regulation of keratin 5 (5a + 5b [19]) by 13-cis-RA, all-trans-RA, and acitretin was detected at rates of 22%, 18%, and 14%, respectively. Keratin 14 was down-regulated by 13-cis-RA (22% reduction), whereas its expression under all-trans-RA and acitretin was practically unchanged (9% and 4% reduction, respectively). Keratins 6 and 16 were down-regulated by 13-cis-RA (19% and 24% reduction, correspondingly) and by all-trans-RA (26% and 30% reduction, respectively). Their expression was only slightly influenced by acitretin (10% reduction). Up-regulation of keratin 17 by 13-cis-RA, all-trans-RA, and acitretin was observed at rates of 17%, 22%, and 26%, respectively. The expression of keratin 13 was not influenced by the retinoids tested (changes $<8\%$). Keratin 19 was up-regulated by all-trans-RA (65% increase) and remained unchanged under 13-cis-RA and acitretin (changes $<6\%$).

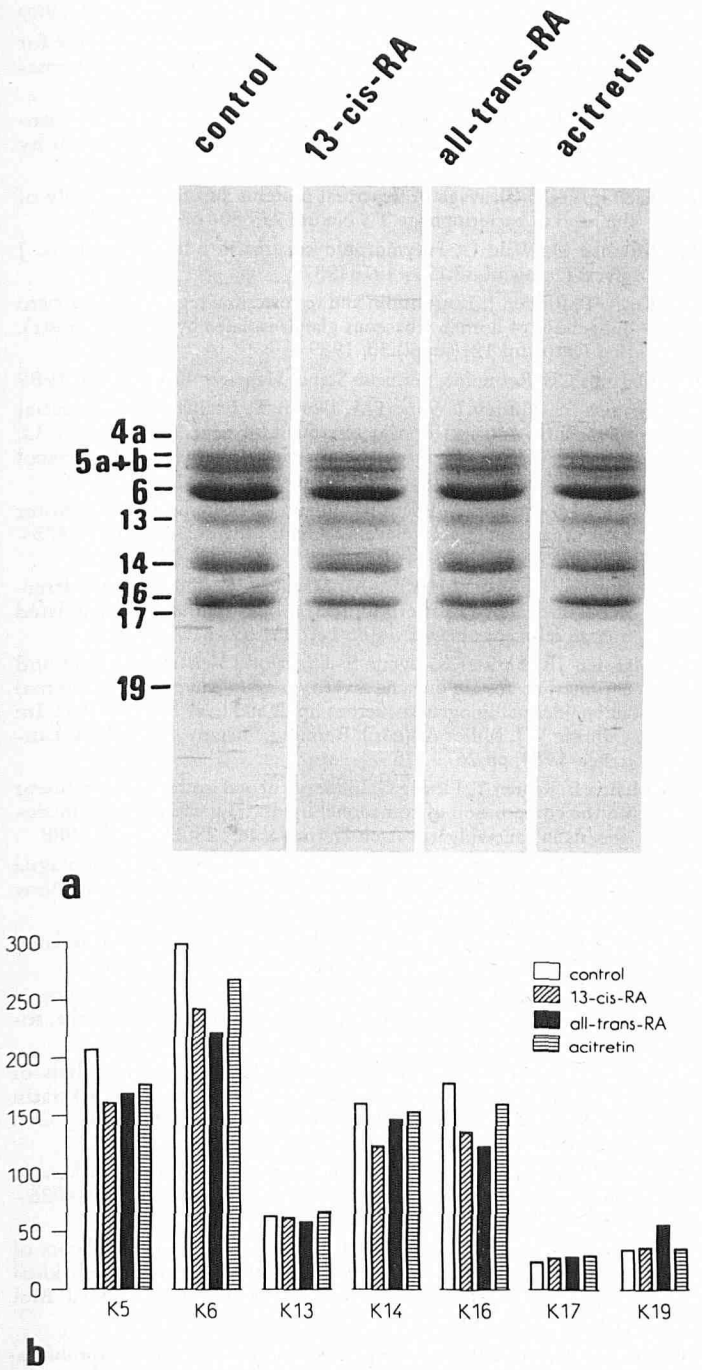

Figure 4. Effects of retinoids on keratin *(K)* expression of human sebocytes in vitro, as assessed by laser densitometry of one-dimensional SDS-PAGE gels. Confluent cultures were treated for 8 d. Values resulted from keratin-enriched protein fractions extracted from triplicate cultures. *a*, bromphenol blue-stained keratin gels after one-dimensional SDS-PAGE. *b*, quantitation of protein bands after laser densitometry of the gels. Values are presented in ratio to keratin 4 (not shown) adjusted to 20.

DISCUSSION

The results obtained in this study clearly demonstrate that retinoic acid inhibits both proliferation and acetate incorporation into lipids, the latter being a marker of lipid synthesis of human sebocytes in vitro. Antisebocytic activities of retinoic acid have also been reported by Doran and Baff [12] using human sebocyte cultures and by Kealy and Ridden [20] using isolated sebaceous glands.

In evaluating the antiproliferative effects of retinoids at concentrations achievable in serum (10^{-9} – 10^{-7} M) [21,22], we found that retinoic acid, especially 13-cis-RA, is a potent inhibitor of sebocyte proliferation in vitro, whereas acitretin can be considered rather inactive in this model. Similar differences between 13-cis-RA and etretinate have been reported by Doran and Baff [12]. Because etretinate has been shown to be less effective in acne than 13-cis-RA [9], models of cultured human sebocytes in vitro may serve as useful tools for screening the anti-acne properties of new retinoids as they are obviously superior to animal models [23].

Although total lipid synthesis is difficult to assess quantitatively, the incorporation of radioactive acetate used as exogenous lipid substrate has been shown to reflect credibly the synthesis of sebaceous lipids [24]. Retinoids may inhibit lipid synthesis in sebocytes either directly by inhibiting enzymes of lipogenesis or indirectly by decreasing the division rate of sebocytes. Because confluent (non-proliferating) cultures were used in our experiments to investigate lipid synthesis, it can be assumed that the inhibitory activity of retinoids on the in vitro lipogenesis of cultured sebocytes is independent from their antiproliferative effect. We conclude, therefore, that retinoids may inhibit lipogenesis in sebocytes in vitro in both direct and indirect manners.

On the other hand, the presence of retinoids caused alterations on the various lipid fractions in vitro, as has already been reported for the action of 13-cis-RA in vivo [25]. The synthesis of triglycerides and of wax esters, two of the three main sebocyte lipid classes, was suppressed under retinoid treatment in vitro as it was detected for 13-cis-RA in vivo [26]. However, squalene synthesis was also reduced after 13-cis-RA treatment in vivo [26], whereas, in the present study the synthesis of squalene remained uninfluenced. This may be due to the low quantity of squalene synthetized by in vitro sebocytes, whose minor changes could not be easily identified. Retinoids enhanced cholesterol synthesis in human sebocytes in vitro, whereas, an enhancement of cholesterol synthesis in sebaceous glands during treatment with 13-cis-RA has been also detected under in vivo conditions [26]. Because cholesterol is the last step in the biosynthetic pathway of sterols [27], it could be suggested that retinoids may accelerate cholesterol biosynthesis. However, further experimental work is needed to elucidate this question.

In our experiments, 13-cis-RA was the most potent inhibitor of both sebocyte proliferation and lipid synthesis among the retinoids tested. Although both 13-cis-RA and all-trans-RA acted similarly on most neutral lipid classes, their different effect on lipogenesis could be well explained by their different capacity to inhibit the synthesis of triglycerides, as triglycerides alone incorporate a high amount (53.8%) of the radiolabeled lipid precursors [28].

The retinoid effect on keratin expression of cultured sebocytes indicates some modification of epithelial differentiation. Down-regulation of the basal keratins 5 and 14 [29] and of the "hyperproliferative" keratins 6 and 16 [30] and up-regulation of keratin 19 were also reported for other epithelial cells, e.g., for keratinocytes [31–

35]. Interestingly, quantitative changes of keratin expression reflected the individual potency of the retinoids tested concerning their effect on sebocytic proliferation and lipogenesis in vitro. However, these changes were minimal in comparison to the alterations detected in keratinocytes, and the expression of keratin 4, which is regarded to be specific for sebocytes [36], was not affected by retinoids. These findings may indicate that sebocytes express cytokeratins like other epithelial cells [37], but changes of this function may not play a prominent role in their differentiation process. A different effect was obtained concerning only keratin 17, which possibly plays a prominent role in cells of the pilosebaceous apparatus [38]; keratin 17 was up-regulated in sebocytes under retinoid treatment, but it was down-regulated or remained unaffected in keratinocytes under the same conditions [31,32]. Keratin 13, which is adversely expressed in relation to cell-to-cell contact [32], was not affected by the retinoids tested similar to other non-keratinizing epithelial cells [31], whereas in epidermal keratinocytes retinoid treatment resulted in up-regulation of keratin 13 [31,32].

In conclusion, 13-cis-RA and, to a lesser extent, all-trans-RA exert marked inhibition of both proliferation and acetate incorporation into lipids, the latter indicating lipid synthesis of human sebocytes in vitro. Acitretin was considered inactive in inhibiting sebocyte proliferation in relevant concentrations and was less potent than 13-cis-RA and all-trans-RA in decreasing lipid synthesis. In addition, retinoids modulate the differentiation of sebocytes in vitro, in a manner reflecting their individual potency to influence this particular cell type.

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