Altered Proliferation, Synthetic Activity, and Differentiation of Cultured Human Sebocytes in the Absence of Vitamin A and Their Modulation by Synthetic Retinoids

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Human sebocytes maintained in medium containing delipidized serum were studied for ultrastructural characteristics, cell proliferation, lipid synthesis, immunophenotype, and keratin expression before and after the addition of the synthetic retinoids isotretinoin and acitretin (10^{-8}–10^{-5} M).

Compared to the properties of sebocytes cultured in normal sebocyte medium ($1-2 \times 10^{-7}$ M vitamin A), the use of delipidized serum (undetectable amounts of vitamin A) resulted in prominent decrease of i) proliferation; ii) number of intracellular lipid droplets and synthesis of total lipids, especially triglycerides, squalene, and wax esters; and iii) labeling with monoclonal antibodies identifying progressive and late-stage sebocyte differentiation. Intercellular spaces narrowed and cell-to-cell contacts were established by abundant desmosomes. Lanosterol was induced. Keratins 14, 16, 17, and 18 were upregulated and the keratin 16 : keratin 4 ratio, negatively correlating with sebocyte differentiation, increased.

Addition of isotretinoin and acitretin exerted a biphasic effect. At concentrations $\leq 10^{-7}$ M, both compounds enhanced sebocyte proliferation and synthesis of total lipids, especially triglycerides and cholesterol, and decreased lanosterol, keratin 16, and the keratin 16 : keratin 4 ratio. In contrast, retinoid concentrations $> 10^{-7}$ M inhibited sebocyte proliferation in a dose-dependent manner.

Our findings indicate that vitamin A is essential for proliferation, synthetic activity, and differentiation of human sebocytes *in vitro*. Synthetic retinoids partially reinstate the altered functions of sebocytes maintained in medium containing delipidized serum. In contrast to the previously shown isotretinoin-specific response of cultured sebocytes in the presence of vitamin A, similar effects of isotretinoin and acitretin were obtained in its absence. This suggests different interactions of synthetic retinoids with vitamin A, possibly influencing their efficacy on the sebaceous gland. Key words: lipids/keratins/isotretinoin/acitretin. *J Invest Dermatol 101: 628–633, 1993*

Evidence for control of epithelial differentiation by vitamin A dates back to the 1920s [1,2]. These original observations that vitamin A deficiency causes hyperkeratinization of the skin and induces squamous metaplasia and keratinization in many internal epithelia were followed by an equally important observation that excess of vitamin A inhibits keratinization of chick embryo skin and transforms it into a mucous-secreting epithelium [3]. Numerous *in vitro* studies showed that vitamin A and synthetic retinoids enhance proliferation and desquamation of cultured epidermal cells and suppress their differentiation, confirming the clinical observations [4–6].

Despite the abundant data concerning the epidermis, there is little information on the changes of the sebaceous gland under vitamin A deficiency or excess. Marked reduction of the size of the sebaceous gland and fragmentation of cell nuclei [7,8], small atrophic glands [9], and missing sebaceous structures in histologic sections of hair follicles [8] have been reported in hypovitaminosis A. Studies of the sebaceous gland in hypervitaminosis A are lacking.

Having established the proliferation, synthetic activity, and differentiation of cultured sebocytes in a vitamin A–containing medium [10–13] and determined the effects of synthetic retinoids [13,14], we were interested in investigating the functions of sebocytes maintained in a vitamin A–depleted medium and their modulation by retinoids.

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Abbreviations: DL medium, medium containing delipidized serum; ^{3}H-acetate, [^{3}H]-acetic acid, sodium salt; N control, culture(s) maintained in normal sebocyte medium; TLC, thin-layer chromatography; Tris/HCl/NaCl, tris-hydroxymethyl aminomethan 10 mM/HCl, pH 7.4/NaCl 140 mM.

MATERIALS AND METHODS

Human Sebocyte Cultures Primary human sebocyte cultures were obtained according to Xia *et al* [10]. Epidermis and the appendages were separated from dermis after incubation of full-thickness skin in 2.4 U/ml dispase (Boehringer, Mannheim, Germany) for 20 h at 4°C and then maintained in 0.02% desoxyribonuclease (Sigma, Deisenhofen, Germany) for 15 min at 37°C. Intact sebaceous glands were isolated by microdissection and cultivated on mitomycin C (Sigma)–inactivated 3T3 cells with 5% CO_2 at 37°C. The normal sebocyte medium consisted of Dulbecco's modified Eagle's medium and Ham's F12 medium (3 : 1; Gibco, Berlin, Germany), 8% fetal calf serum (Seromed, Berlin, Germany), 2% human serum, 10 ng/ml epidermal growth factor (Sigma), 10^{-9} M cholera toxin (Calbiochem, Frankfurt, Germany), 3.4 mM L-glutamine, 100 IU/ml penicillin, and

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100 μg/ml streptomycin (all Seromed). Primary sebocyte cultures resulted as outgrowths from the periphery of the gland lobules and were grown to confluence before subcultivation. All experiments were performed using secondary sebocyte cultures, which have been demonstrated to consist of proliferating cells that consequently undergo sebocyte differentiation [10–14].

Delipidized Serum Delipidized serum was prepared as described [15]. Fetal calf serum : human serum, 4 : 1, were slowly added to precooled acetone : ethanol, 1 : 1 (both Merck, Darmstadt, Germany), while gently swirling. The final 10% v/v mixture was allowed to stand in an ice bath for 4 h with occasional swirling and then filtered by suction. Precooled ethyl ether (25% v/v of the acetone : ethanol solution; Merck) was added to the delipidized protein collected on the filter and suction was continued for 10–20 min until the solvent odor had dissipated. An amount of dry protein corresponding to serum in a 10% v/v serum/medium mixture was added to serum-depleted sebocyte medium to form the medium containing delipidized serum (DL medium).

Vitamin A concentrations of normal sebocyte medium and DL medium were examined by reverse-phase high-performance liquid chromatography, using standard techniques for retinoids (sensitivity levels 10^{-10}–10^{-9} M) [16,17].

Electron Microscopy Sebocyte cultures grown to confluence on plastic Lux Thermanox coverslips (Miles Laboratories, Naperville, IL) in DL medium were processed for transmission electron microscopic examination, using standard techniques [18].

Treatment with Retinoids Isotretinoin (13-cis-retinoic acid) and acitretin (all-trans-9-[4-methoxy-2,3,6-trimethylphenyl]-3,7-dimethyl-2,4,6,8-nonatetraenoic acid; both Hoffmann-La Roche, Basel, Switzerland) were dissolved in dimethyl sulfoxide (Merck). The concentration of dimethyl sulfoxide in medium without and with retinoids was 0.2%. Retinoids were handled under dimmed yellow light. For proliferation experiments, 2×10^4 sebocytes/well were seeded in 24-well culture plates (Falcon, Jersey, NJ) on 10^4 inactivated 3T3 cells/well and maintained in sebocyte medium for 5 d. After rinsing twice with phosphate-buffered saline without Ca^{++} and Mg^{++}, pH 7.4 (PBS, Seromed), DL medium without or with retinoids (10^{-8}–10^{-5} M) was added to the cultures. For experiments on lipid synthesis and protein expression DL medium without and with retinoids (10^{-7} M) was added in confluent sebocyte cultures maintained in 60-mm culture dishes (Falcon). Medium with and without retinoids was changed every 3 d. The cultures were incubated at 37°C with retinoids up to 7 d (proliferation) and 8 d (lipid synthesis and protein expression). In all experiments, sebocytes maintained in normal sebocyte medium without retinoids served as additional controls (N control).

Cell Proliferation Cell proliferation was evaluated by determining the ratio of ^{3}H-thymidine uptake to 10^4 cells. ^{3}H-thymidine incorporation into DNA was assessed as counts per minute by liquid scintigraphy after a terminal pulse-labeling of 6 h with 1 mCi/ml [methyl-^{3}H]-thymidine (50–70 Ci/mM; Amersham-Buchler, Braunschweig, Germany) [19]. Single-cell suspensions were counted in a Becton-Dickinson CC-VI blood cell counter.

Lipid Synthesis Lipid synthesis was assessed by liquid scintigraphy of the incorporation of [^{3}H]-acetic acid, sodium salt (^{3}H-acetate; 2 mCi/ml, 86.4 mCi/mM; NEN, Dreieich, Germany; counts per minute) into lipids during the whole treatment period. To extract lipids, the cultures were rinsed three times with cold PBS containing 0.1% fatty acid-free bovine serum albumin (Sigma) and twice with PBS and then scraped from the dishes into 10-ml glass vials. The pellets were diluted in chloroform : methanol 2 : 1 and homogenized for 15 min. The solutions were then filtered and the filtrates were twice evaporated to dryness at 40°C under a stream of nitrogen. The lipid residues were weighed and redissolved in chloroform for analysis.

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) pretreated with hexane. Chromatograms of neutral lipids were developed in hexane : benzene : (1 : 1, to 18 cm), left to dry, and redeveloped in hexane : diethyl ether : acetic acid (80 : 20 : 1, to 18 cm). They 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 [20]. The lipid fractions were identified by comparison with standards (Sigma) and quantitated according to Downing and Stranieri [21] after scanning the chromatograms on a Shimadzu CS-910 photodensitometer by absorption of 450 nm.

Immunocytochemistry Dispersed cells of subconfluent sebocyte cultures were attached to glass slides by cytocentrifugation, air dried, fixed in acetone for 10 min at 20°C, and stained with monoclonal antibodies (MoAbs) using the alkaline phosphatase–anti-alkaline phosphatase technique [22]. A 30-min incubation with the primary MoAb was followed by 10-min incubations with a rabbit anti-mouse IgG (1 : 100) and an antibody labeled with alkaline phosphatase (1 : 75; both Dakopatts, Copenhagen, Denmark). The preparations were then stained for 30 min in buffered solution (pH 8.8) with new fuchsin and a naphthol salt (both Sigma) and counterstained with Mayer's hemalum (Merck). A series of MoAbs shown to identify progressive or late-stage sebocyte differentiation‡ and the MoAb RPN1160 (Amersham) identifying keratin (K) 18 [23] were used in this study (Table I). All MoAbs have been previously described [23–28].

Gel Electrophoresis of Cytoskeletal Proteins and Densitometry Sebocytes were scraped from the dishes, harvested into centrifuge tubes, homogenized, and extracted as described [30]. The resulting pellet, highly enriched in cytoskeletal proteins, was solubilized in sample buffer. One-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis according to Laemmli [31] and two-dimensional analyses by non-equilibrium pH gradient gel electrophoresis according to O'Farrell *et al* [32] were performed as described [30]. Gels were stained in 0.2% Serva-Blue R (Serva, Heidelberg, Germany). Protein bands of one-dimensional gels were quantitated by a LKB Ultro-Scan XL laser densitometer. To determine sebocyte differentiation, the K 16 : K 4 ratios were evaluated, because K16 is apparently proliferation related [29] and K4 is only expressed in fully differentiated and mature sebocytes.‡

Immunoblot Analysis Proteins from unstained one- and two-dimensional gels were transferred by semi-dry blotting onto nitrocellulose sheets as previously described [33]. Unspecific protein binding sites were blocked in 10 mM tris-hydroxymethyl aminomethan/HCl, pH 7.4, and 140 mM NaCl (Tris/HCl/NaCl; Merck) supplemented with 0.05% Tween 20 (Serva) and 15% horse serum. The filters of one-dimensional gels were incubated overnight with a mixture of the rabbit polyclonal antisera 8-2/4, specific for the acidic K subfamily, and 10-2/2, identifying the neutral-to-basic K subfamily [29,30], washed twice with Tris/HCl/NaCl/0.05% Tween 20, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit (BIO-RAD, Munich, Germany) and the enzyme-conjugated antibody. After washing twice with Tris/HCl/NaCl/0.05% Tween 20 and three times with Tris/HCl/NaCl, the peroxidase reaction was developed using 0.02% 4-chloro-1-naphthol (freshly prepared as a 0.3% solution in methanol) and 0.015% hydrogen peroxide (both Sigma) in Tris/HCl/NaCl.

Statistical Analysis In proliferation experiments each value represents the mean of three cultures obtained from different individuals ± one standard deviation. The values of sebocyte labeling with MoAbs represent the mean of random evaluation of 200 cells from each of the three cultures. Statistical significance was assessed by Student t-test. Mean differences were considered to be significant when $p < 0.05$. For determining lipid synthesis and K expression, lipid and cytoskeletal protein extracts were pooled from three different cultures.

RESULTS

Activity and Differentiation of Sebocytes Maintained in DL Medium Vitamin A concentration in normal sebocyte medium was found to be $1–2 \times 10^{-7}$ M. No vitamin A was detected in DL medium.

At the ultrastructural level human sebocyte cultures in DL medium consisted of 1–2 layers of polygonal cells with large nuclei and regularly preserved cell organelles such as mitochondria, rough endoplasmic reticulum, and Golgi apparatus. Only a few cytoplasmic lipid droplets and concentric membrane lamellae, probably representing neutral lipids and phospholipids, respectively, were present in the cells. Intercellular spaces were narrow and cell-to-cell contacts were established by abundant complete desmosomes.

Sebocyte proliferation and synthesis of total lipids were markedly reduced in DL medium compared with the rates of N control, representing 14.5 ± 3.1% and 7.8% of the latter, respectively (Figs 1,2*A*). Analytical TLC of neutral lipids revealed markedly decreased triglycerides (8.2%) and wax/sterol esters (4.6%) in sebocytes maintained in DL medium compared with N control (Fig 2*B,C*). No peak area for squalene was identified in lipid extracts from sebocytes maintained in DL medium, whereas a peak area for

‡ Zouboulis, ChC, Krieter A, Gollnick H, Mischke D, Orfanos CE: Progressive differentiation of human sebocytes *in vitro* is characterized by increased cell size and altered antigenic expression and is regulated by culture duration and retinoids (submitted).

Table I. Labeling of Sebocyte Cultures with MoAbs Identifying Progressive or Late-Stage Sebocyte Differentiation

MoAb Identifying Sebocyte Differentiation	Commercial Source	Dilution	Specificity For	% of Strongly Positively Labeled Cells: N Medium	DL Medium	Isotretinoin (10^{-7} M)	Acitretin (10^{-7} M)
Progressive							
CK8.12	Bio Makor	1:50	K13, 15[c], 16	27	14	20	17
				$p < 0.01$			
34D11[a]		1:100	82-kD protein	55	65	60	65
Late-stage							
RPN1162	Amersham	1:50	K7	12	7	15	10
CK13	Camon	Pure	K13	10	10	15	10
CK8.60	Bio Makor	1:200	K1, 4[c], 10	32	37	35	38
OM-1[b]		1:150	Sebaceous cell antigen	14	6	12	9
				$p < 0.05$			
24F10[a]		1:100	Basic polypeptides	25	15	10	20
				$p < 0.05$			

[a] Gift from Prof. H.P. Baden, Boston, MA.
[b] Gift from Dr. T.A. de Kretser, Melbourne, Australia.
[c] Additional reactivity on immunoblots (M. Mischke, unpublished [29]).

lanosterol was found, which was missing in N control. Free fatty acids were decreased in DL medium; the cholesterol levels were identical to N control.

Confluent sebocyte cultures in DL medium presented lower rates of cells strongly labeled with the MoAb CK8.12, which recognizes progressive sebocyte differentiation, and the MoAbs OM-1 and 24F10, which identify late-stage sebocyte differentiation, compared with the labeling rates of N control (Table I). Upregulated expression of K 14, 16, and 17 (Fig 3) and an increased K16:K4 ratio (340%; Fig 4) were found by gel electrophoresis in confluent sebocyte cultures maintained in DL medium in comparison to N control. In addition, 32 ± 6% of sebocytes in DL medium, but no cells in normal medium, were labeled with the MoAb RPN1160, specific for K18.

Retinoids and Sebocyte Proliferation Isotretinoin and acitretin exerted a similar biphasic effect on the proliferation of human sebocytes maintained in DL medium (Fig 1). Both compounds stimulated sebocyte proliferation at the lower concentrations tested (10^{-8} and 10^{-7} M) with a pronounced effect at 10^{-7} M (isotretinoin, 218.1 ± 59.6%, $p < 0.05$; acitretin, 191.6 ± 9.6%, $p < 0.01$). By increasing retinoid concentrations the proliferation of sebocytes decreased in a dose-dependent manner. Sebocyte proliferation with retinoids did not exceed 50% of the N control.

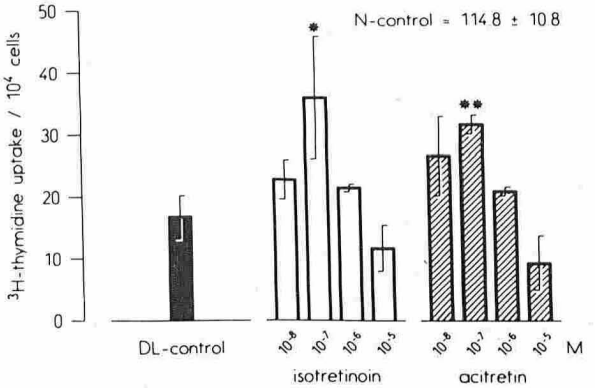


Figure 1. Proliferation of human sebocytes *in vitro* maintained in DL medium without *(black bar)* and with retinoids *(white and shaded bars)* for 7 d. The values are means ± standard deviations of three cultures derived from different individuals and are presented as ^{3}H-thymidine uptake (cpm)/10^4 cells. Data obtained by sebocytes maintained in normal sebocyte medium without retinoids (N control) are presented for comparison. * $p < 0.05$, ** $p < 0.01$.

Retinoids and Lipid Synthesis Both retinoids increased the incorporation of ^{3}H-acetate into lipids (isotretinoin 256%, acitretin 356%), indicating a stimulation of sebocyte lipid synthesis in DL medium by the retinoids tested (Fig 2*A*). However, the ^{3}H-acetate incorporation into lipids with retinoids (10^{-7} M) did not exceed 30% of the N control. Analytical TLC of neutral lipids revealed qualitative and quantitative changes of certain lipid classes when lipid extracts of retinoid-treated cells were compared with untreated cells in DL medium (Fig 2*B,C*). Lanosterol was not detected and the peak areas for triglycerides and cholesterol markedly increased under retinoids (173–213% and 246–354%, respectively). When compared with the N control, triglycerides only increased up to 30% with retinoids in DL medium; cholesterol exceeded 200%. In contrast, the wax/sterol esters fraction remained unchanged and the free fatty acids unexpectedly decreased.

Retinoids and Antigenic Expression An increasing number of strongly labeled cells with the MoAbs CK8.12, OM-1, and 24F10, which recognize progressive and late-stage sebocyte differentiation,‡ were found in cultures treated with retinoids compared with cells in DL medium (Table I). Isotretinoin decreased the amount of cells strongly reacting with the K 18-specific MoAb RPN1160 (19 ± 5%; $p < 0.01$). There were no significant differences of labeling between sebocytes in N control and retinoid-supplemented DL medium with these MoAbs. As detected by gel electrophoresis, both retinoids downregulated K16 expression (Fig 3) and reduced the K16:K4 ratio by 25–30% (Fig 4).

DISCUSSION

The results obtained in this study demonstrate that human sebocytes cultured in medium containing delipidized serum depart from their normal functions and pathways of differentiation [11–14]. The only partial reinstatement of normal sebocyte functions and differentiation by retinoids either reflects an inability of retinoids to fully substitute vitamin A or indicates that other essential factors are missing. The procedure of delipidization removes vitamin A from the serum and has been used to obtain vitamin A–depleted serum-containing media [4,34], but it also substracts other fat-soluble agents that may be biologically active on sebocytes.

The narrowing of the intercellular spaces and the abundant formation of complete desmosomes observed in sebocytes maintained in DL medium compared with N control [11,13] were probably mainly induced by vitamin A depletion. The presence of vitamin A was shown to inhibit desmosome formation and increase intercellular spaces in other epithelial cells, like in explants of guinea pig skin and mouse epidermal cell cultures [35,36]. In epidermal cells, however, vitamin A also induced changes in their rough endoplasmic reticulum and Golgi complexes and in the number of plasma mem-

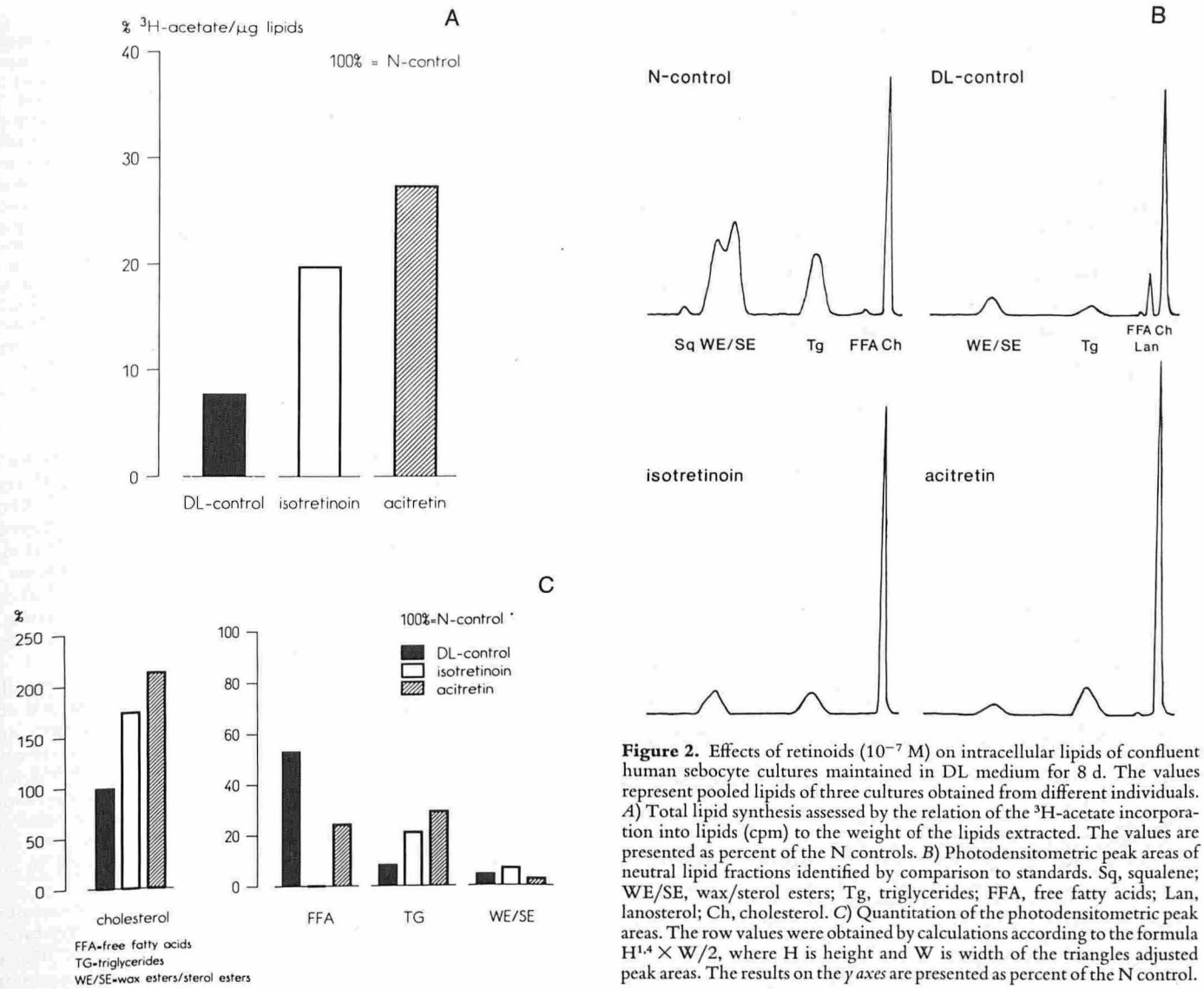


Figure 2. Effects of retinoids (10^{-7} M) on intracellular lipids of confluent human sebocyte cultures maintained in DL medium for 8 d. The values represent pooled lipids of three cultures obtained from different individuals. *A*) Total lipid synthesis assessed by the relation of the ^{3}H-acetate incorporation into lipids (cpm) to the weight of the lipids extracted. The values are presented as percent of the N controls. *B*) Photodensitometric peak areas of neutral lipid fractions identified by comparison to standards. Sq, squalene; WE/SE, wax/sterol esters; Tg, triglycerides; FFA, free fatty acids; Lan, lanosterol; Ch, cholesterol. *C*) Quantitation of the photodensitometric peak areas. The row values were obtained by calculations according to the formula $H^{1,4} \times W/2$, where H is height and W is width of the triangles adjusted peak areas. The results on the *y axes* are presented as percent of the N control.

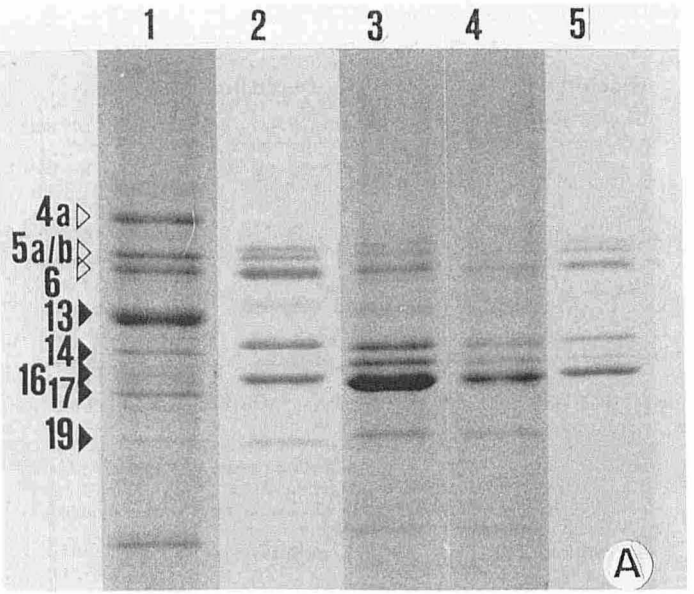
brane microvilli, which we did not observe in cultured human sebocytes.

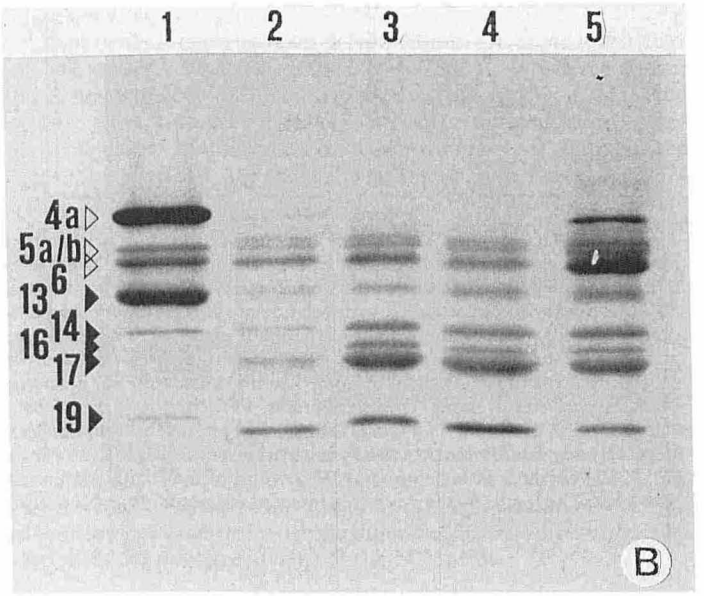
The marked reduction of sebocyte proliferation in DL medium is possibly related with the clinical observations of atrophic or missing human sebaceous glands in hypovitaminosis A [7–9]. The stimulatory effect of retinoids on sebocyte proliferation at concentrations lower or equal to the vitamin A concentration in serum-supplemented media ([4,34], this study) could be explained as a vitamin A-substituting function of synthetic retinoids. In a recent study rat sebaceous glands started to degenerate afater a 10-d maintenance in vitamin A–depleted medium but they remained intact after 14 d in medium containing 2×10^{-6} M arotinoid [37]. Higher retinoid concentrations inhibited sebocyte proliferation in our study, a retinoid effect which was also observed in sebocytes maintained in normal sebocyte media [14,38,39].

Confluent sebocyte cultures were used to investigate affection of lipid synthesis by retinoids to avoid an association with alterations of cell proliferation. In addition to vitamin A, other fat-soluble agents may be responsible for lipid synthesis in cultured sebocytes: i) retinoids added in DL medium only partially reinstated total lipid synthesis and the amount of triglycerides, which is the largest lipid fraction in sebocytes [40]; and ii) the sebum-specific lipids squalene and wax esters [41], which indicate sebocyte differentiation *in vitro* [11,12,14], were markedly reduced in DL medium but not induced by retinoids. Increased cholesterol synthesis seems to be consistently related with retinoid treatment of human sebocytes *in vivo* and *in vitro* under all conditions tested ([14,42], this study).

Sebocytes cultured in DL medium presented a lower grade of sebocyte differentiation compared with sebocytes maintained in normal medium: i) significantly fewer cells were positively labeled with MoAbs identifying progressive and late-stage sebocyte differentiation,‡ and ii) the pattern of keratins expressed was indicative of a more simple epithelial cell type [23,43,44]. K14, especially, is expressed in basal cells of stratified epithelia; K17 and K18 are found in simple epithelial cells. In contrast to sebocytes, keratinocytes start to synthesize K1 in DL medium, which indicates increased terminal differentiation, and abandon this function in the presence of retinoids [4]. The low-grade sebocyte differentiation in DL medium was combined with a prolonged expression of the "hyperproliferative" K16 [29], probably leading to the increased K16:K4 ratio detected in confluent sebocyte cultures maintained in DL medium. An upregulation of K16 was also reported in conjuctival, but not in epidermal, keratinocytes cultured in DL medium [4].

The absence of vitamin A was probably mainly responsible for the





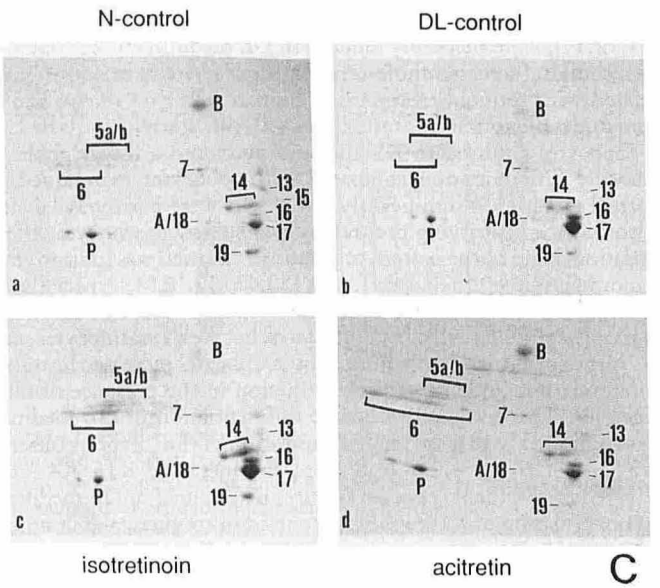


Figure 3. Keratin expression of confluent human sebocyte cultures maintained in DL medium without and with reinoids for 8 d. The values represent pooled cytoskeletal protein extracts from three different individuals. *A*) One-dimensional gel electrophoresis with Serva-Blue R-stained gel. *B*) Corresponding immunoblot reacted with a mixture of the rabbit polyclonal antisera 8-2/4 and 10-2/2 to detect all keratins. *Lane 1*, human tonsillar epithelium (control); *lane 2*, sebocytes maintained in normal sebocyte medium (N control); *lane 3*, sebocytes maintained in DL medium (DL control); *lane 4*, sebocytes treated with isotretinoin 10^{-7} M; *lane 5*, sebocytes treated with acitretin 10^{-7} M. Members of the neutral-basic keratin subfamily are indicated by *open arrowheads*, those of the acidic subfamily by *closed arrowheads*. *C*) Serva-Blue R-stained two-dimensional gel electrophoresis of human keratins expressed in N control *(a)*, sebocytes in DL medium *(b)*, sebocytes treated with isotretinoin 10^{-7} M *(c)*, and sebocytes treated with acitretin 10^{-7} M *(d)*. B, bovine serum albumin (67 kD/pI 6.35); A, bovine muscle actin (approximately 42 kD/pI 5.4); and P, yeast phosphoglycerate kinase (43 kD/pI 7.4) are enclosed as references for better orientation.

effects of delipidized serum on sebocyte differentation. Addition of both retinoids (10^{-7} M) to DL medium induced sebocyte differentiation. Cultured sebocytes,‡ like keratinocytes, express both keratins and vimentin, which is a common property of cells being sensitive to regulation of keratin expression by retinoids [45,46]. It is also likely that vitamin A and retinoids hinder lanosterol synthesis in human sebocytes *in vitro*. Lanosterol, a lipid marker of epithelial cell stratification [43], was also found in cultured keratinocytes grown in a "reconstructed epidermal model" and drastically reduced after treatment with all-trans-retinoic acid and acitretin [43].

Interestingly, whereas isotretinoin and acitretin exerted similar effects on human sebocytes in this study, isotretinoin, but not acitretin (10^{-7} M), resulted in inhibition of cell proliferation and downregulation of sebocyte differentiation in the presence of normal concentrations of vitamin A [14,38].‡ On the other hand, isotretinoin, but not aromatic retinoids, interferes with vitamin A metabolism [47,48]. These findings indicate separate modes of interaction of synthetic retinoids with their natural precursor and may explain their different efficacy on the sebaceous gland *in vivo* [49].

In conclusion, vitamin A at normal concentrations appears essential for sebocyte proliferation, synthetic activity, and differentation *in vitro*. Synthetic retinoids partially reinstate normal functions of sebocytes in the absence of vitamin A; however, high retinoid concentrations again inhibit sebocyte activities. In contrast to the previously shown isotretinoin-specific response of cultured sebocytes in

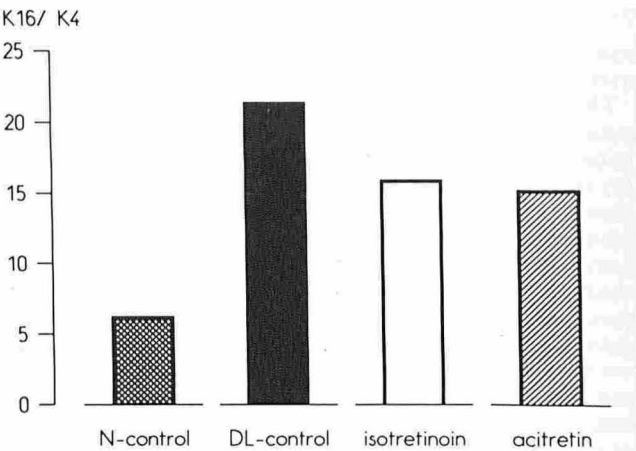


Figure 4. Quantitation of protein bands by laser densitometry. One-dimensional Serva-Blue R-stained gels of pooled cytoskeletal proteins from sebocyte cultures obtained from three different individuals were evaluated. Sebocytes were maintained in normal medium and DL medium without and with retinoids (10^{-7} M) for 8 d and values are presented as K16 : K4 ratios, which correlate negatively with sebocyte differentiation.

the presence of vitamin A, isotretinoin and acitretin seem to exert similar effects in its absence.

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REFERENCES

1. Mori S: The changes in the para-ocular glands which follow the administration of diets low in fat-soluble vitamin A; with notes of the effect of the same diets on salivary glands and the mucus of the larynx and trachea. *Bull Johns Hopkins Hosp* 33:357–359, 1922
2. Wolbach SB, Howe PR: Tissue changes following deprivation of fat soluble A vitamin. *J Exp Med* 42:753–777, 1925
3. Fell HB, Mellanby E: Metaplasia produced in cultures of chick ectoderm by vitamin A. *J Physiol* 119:470–488, 1953
4. Fuchs E, Green H: Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. *Cell* 25:617–625, 1981
5. Eichner R: Epidermal effects of retinoids: in vitro studies. *J Am Acad Dermatol* 15:789–797, 1986
6. Darmon M: Retinoic acid in skin and epithelia. *Semin Dev Biol* 2:219–228, 1991
7. Fasal P: Clinical manifestations of vitamin deficiencies as observed in the Federated Malay States. *Arch Dermatol Syph* 50:160–166, 1944
8. Hume EM, Krebs HA: Vitamin A requirements of human adults: experimental study of vitamin A deprivation in man: report of a vitamin A subcommittee of accessory food factors committee. *Medical Research Council Special Report Series, #264.* Her Majesty's Stationery Office, London, 1949
9. Frazier CN, Hu CK: Cutaneous lesions associated with a deficiency in vitamin A in man. *Arch Int Med* 48:507–514, 1931
10. Xia L, Zouboulis Ch, Detmar M, Mayer-da-Silva A, Stadler R, Orfanos CE: Isolation of human sebaceous glands and cultivation of sebaceous gland-derived cells as an in vitro model. *J Invest Dermatol* 93:315–321, 1989
11. Zouboulis ChC, Xia L, Detmar M, *et al:* Cultivation of human sebocytes and markers of sebocytic differentiation in vitro. *Skin Pharmacol* 4:74–83, 1991
12. Zouboulis ChC: Sebaceous cells in monolayer culture. *In Vitro Cell Dev Biol* 28A:699, 1992
13. Zouboulis ChC, Xia L, Korge B, Gollnick H, Orfanos CE: Cultivation of human sebocytes in vitro: cell characterization and influence of synthetic retinoids. In: Saurat J-H (ed.). *Retinoids: 10 years on.* Karger, Basel, 1991, pp 254–273
14. Zouboulis ChC, Korge B, Akamatsu H, Xia L, Schiller S, Gollnick H, Orfanos CE: 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. *J Invest Dermatol* 96:792–797, 1991
15. Rothblat GH, Arbogast LY, Ouellette L, Howard BV: Preparation of delipidized serum protein for use in cell culture systems. *In Vitro* 12:554–557, 1976
16. Bugge CJL, Rodriguez LC, Vane FM: Determination of isotretinoin or etretinate and their major metabolites in human blood by reverse-phase high-performance liquid chromatography. *J Pharm Biomed Anal* 3:269–277, 1985
17. Gollnick H, Ehlert R, Rinck G, Orfanos CE: Retinoids: an overview of pharmacokinetics and therapeutic value. In: Packer L (ed.). *Retinoids. Part B. Cell differentiation and clinical applications. Methods in Enzymology, Vol 190.* Academic Press, San Diego, 1990, pp 291–303
18. Detmar M, Mayer-da-Silva A, Stadler R, Orfanos CE: Effects of azelaic acid on proliferation and ultrastructure of mouse keratinocytes in vitro. *J Invest Dermatol* 93:70–74, 1989
19. Zouboulis Ch, Garbe C, Orfanos CE: Factors influencing the in vitro growth of human melanoma cell lines. *Skin Cancer* 4:63–72, 1989
20. Saint-Leger D, Bague A: A simple and accurate routine procedure for qualitative analysis of skin surface lipids (SSL) in man. *Arch Dermatol Res* 271:215–222, 1981
21. Downing DT, Stranieri AM: Correction for deviation from the Lambert-Beer law in the quantitation of thin-layer chromatograms by photodensitometry. *J Chromatogr* 192:208–211, 1980
22. Cordell JL, Falini B, Erber WN, *et al:* Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J Histochem Cytochem* 32:219–229, 1984
23. Lane EB: Monoclonal antibodies provide specific intramolecular markers for the study of epithelial tonofilament organization. *J Cell Biol* 92:665–673, 1982
24. Tölle H-G, Weber K, Osborn M: Microinjection of monoclonal antibodies specific for one intermediate filament protein in cells containing multiple keratins allow insight into the composition of particular 10 nm filaments. *Eur J Cell Biol* 38:234–244, 1985
25. Debus E, Moll R, Franke WW, Weber K, Osborn M: Immunohistochemical distinction of human carcinomas by cytokeratin typing with monoclonal antibodies. *Am J Pathol* 114:121–130, 1984
26. Huszar M, Gigi-Leitner O, Moll R, *et al:* Monoclonal antibodies to various acidic (type I) cytokeratins of stratified epithelia. Selective markers for stratification and squamous cell carcinomas. *Differentiation* 31:141–153, 1986
27. de Kretser TA, Thorne HJ, Picone D, Jose DG: Biochemical characterization of the monoclonal antibody-defined ovarian carcinoma-associated antigen SGA. *Int J Cancer* 37:705–712, 1986
28. Baden HP, Kubilus J, Phillips SB: Characterization of monoclonal antibodies generated to the cornified envelope of human cultured keratinocytes. *J Invest Dermatol* 89:454–459, 1987
29. Korge B, Stadler R, Mischke D: Effect of retinoids on hyperproliferation-associated keratins K6 and K16 in cultured human keratinocytes: a quantitative analysis. *J Invest Dermatol* 95:450–455, 1990
30. Mischke D, Wild G: Polymorphic keratins in human epidermis. *J Invest Dermatol* 88:191–197, 1987
31. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 27:680–685, 1970
32. O'Farrell PZ, Goodmann HM, O'Farrell PH: High resolution two-dimensional electrophoresis of basic as well acidic proteins. *Cell* 12:1133–1142, 1977
33. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc Natl Acad Sci USA* 76:4350–4354, 1979
34. Kim KH, Schwartz F, Fuchs E: Differences in keratin synthesis between normal epithelial cells and squamous cell carcinomas are mediated by vitamin A. *Proc Natl Acad Sci USA* 84:4280–4284, 1984
35. Barnett ML, Szabo G: Effect of vitamin A on epithelial morphogenesis in vitro. *Exp Cell Res* 76:118–126, 1973
36. Yuspa SH, Harris CC: Altered differentiation of mouse epidermal cells treated with retinyl acetate in vitro. *Exp Cell Res* 86:95–105, 1974
37. Liauw L, Lim DJ, Bakaletz LO, van Blitterswijk C: Effect of vitamin A on the growth and differentiation of rat external auditory canal epithelium in organ culture. *Am J Otolaryngol* 12:67–75, 1991
38. Doran TI, Shapiro SS: Retinoid effects on sebocyte proliferation. In: Packer L (ed.). *Retinoids. Part B. Cell Differentiation and Clinical Applications. Methods in Enzymology, Vol 190.* Academic Press, San Diego-New York-Boston, 1990, pp 334–338
39. Ridden J, Ferguson D, Kealy T: Organ maintenance of human sebaceous glands: in vitro effects of 13-cis-retinoic acid and testosterone. *J Cell Sci* 95:125–136, 1990
40. Cassidy DM, Lee CM, Laker MF, Kealy T: Lipogenesis in isolated human sebaceous glands. *FEBS Lett* 200:173–176, 1986
41. Downing DT, Stewart ME: Analysis of sebaceous lipids. In: Skerrow D, Skerrow CJ (eds.). *Methods in skin research.* John Wiley & Sons, Chichester, 1985, pp 349–379
42. Melnik B, Kinner T, Plewig G: Influence of oral isotretinoin treatment on the composition of comedonal lipids: implications for comedogenesis in acne vulgaris. *Arch Dermatol Res* 280:97–102, 1988
43. Ponec M: Retinoid-induced modulation of differentiation, keratin and lipid composition in reconstructed epidermis in vitro. In: Reichert U, Shroot B (eds.). *Pharmacology of retinoids in the skin, Vol 3.* Karger, Basel, 1989, pp 45–51
44. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R: The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 31:11–24, 1982
45. Kim KH, Stellmach V, Javors J, Fuchs E: Regulation of human mesothelial cell differentiation: opposing roles of retinoids and epidermal growth factor in the expression of intermediate filament proteins. *J Cell Biol* 105:3039–3052, 1987
46. Glass C, Fuchs E: Isolation, sequence, and differential expression of a human K7 gene in simple epithelial cells. *J Cell Biol* 107:1337–1350, 1988
47. Vahlquist A, Rollman O, Holland DB, Cunliffe WJ: Isotretinoin treatment of severe acne affects the endogenous concentration of vitamin A in sebaceous glands. *J Invest Dermatol* 94:496–498, 1990
48. Grønhøj Larsen F, Vahlquist C, Andersson E, Törma H, Kragballe K, Vahlquist A: Oral acitretin in psoriasis: drug and vitamin A concentrations in plasma, skin and adipose tissue. *Acta Dermatol Venereol* 72:84–88, 1992
49. Zouboulis ChC: Sebocyte cultures: how they can improve our understanding of diseases of the sebaceous glands. *Retinoids Today and Tomorrow* 26 (suppl):6–12, 1992