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Influence of oral isotretinoin treatment on the composition of comedonal lipids. Implications for comedogenesis in acne vulgaris

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Summary. One of the primary events in the pathogenesis of acne vulgaris is abnormal follicular keratinization. Since oral isotretinoin therapy reduces follicular hyperkeratinization in acne, our study has been designed to determine whether epidermal lipid composition of the epithelium of sebaceous follicles is affected by isotretinoin treatment.

Noninflamed early comedones obtained from ten patients with nodulocystic acne before and after the 6th week of isotretinoin therapy (mean daily dose 0.7 mg/kg b.wt.) were used as probes of the hyperkeratinizing follicular epithelium. Comedonal lipids were analyzed by high-performance thin-layer chromatography. Oral isotretinoin caused a decrease of the comedonal glyceride fraction by 36% ($P < 0.01$), whereas free sterols and total ceramides increased by 34% ($P < 0.10$) and 19%, respectively. The changes of comedonal lipids were associated with a significant elevation of the free sterols/cholesterol sulfate ratio of 86% from pretreatment levels ($P < 0.05$). The isotretinoin-induced changes of the comedonal lipid composition in direction to a pattern of epidermal lipids of normal desquamating stratum corneum are discussed as a possible comedolytic mechanism of oral isotretinoin treatment.

Key words: Acne – Isotretinoin – Comedonal lipids – Follicular hyperkeratinization – Epidermal cholesterol sulfate – Desquamation – Comedogenesis

The beneficial clinical effect of oral isotretinoin treatment on severe nodulocystic acne has been established by numerous clinical and experimental studies [11, 15, 16, 18, 20, 25, 27–29]. There is a chronological relationship between an increased sebum secretion rate in acne and the onset of comedogenesis [35–37].

One of the primary events in the pathogenesis of acne vulgaris is cohesive hyperkeratinization of the infrainfundibular portion of the epithelium of sebaceous follicles leading to microcomedo formation [17, 26]. Histopathologically, a microcomedo can be regarded as a follicular retention hyperkeratosis. Besides sebum suppression, isotretinoin reduces these follicular hyperkeratoses by still unknown mechanisms [28, 29].

Recent advances in our understanding of lipid biochemical abnormalities in several retention hyperkeratoses, like x-linked recessive ichthyosis, resulted in the introduction of a two-compartment model of the stratum corneum that can be compared as a wall of corneocytes (or "bricks") that are primarily proteinaceous, and intercellular material (or "mortar") that are predominantly lipids [4, 6]. Evidence is growing that intercorneocyte lipids are implicated in the cohesion-dishesion properties of epidermal horny cells [4, 6, 7, 21, 31, 32, 40, 41]. In this regard, the balance between free cholesterol and cholesterol sulfate seems to be of critical importance for the regulation of normal corneocyte desquamation. Free cholesterol is sulfated in the viable epidermis and desulfated in the outer stratum corneum by the enzyme steroid sulfatase [7, 10, 31, 41]. A decrease of the mass ratio of free cholesterol and cholesterol sulfate due to an increase in cholesterol sulfate and a concomitant decrease of free cholesterol is associated with scale formation in patients with x-linked recessive ichthyosis, a retention hyperkeratosis with steroid sulfatase deficiency [7, 40]. Further support for the cohesive properties of cholesterol sulfate is derived from organ culture studies of mouse ear skin [30] as well as lipid analyses of intact cohesive versus desquamated human stratum corneum [21]. Another link between epidermal choles-

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terol metabolism and the induction of scaling skin disorders is derived from side-effects of certain hypocholesterolemic drugs [41]. By oral administration of one of these agents, *20,25*-diazacholesterol, it has been possible to produce an animal analogue of a retention hyperkeratosis in hairless mice associated with a marked decrease of free cholesterol in the stratum corneum [8]. Scaling could be corrected by systemic coadministration of isotretinoin [13]. Furthermore, in hairless mice scale formation without erythema could be directly induced by topical application of cholesterol sulfate [22].

With respect to these new insights into cellular lipid biochemical dishesion-cohesion phenomena, this study was designed to characterize the effect of isotretinoin on the composition of comedonal lipids in order to confirm our hypothesis that isotretinoin accelerates shedding of comedonal corneocytes by restoration of epidermal lipid composition of the follicular epithelium.

Materials and methods

Patients

Equal masses of noninflamed open and closed comedones as well as plantar stratum corneum were obtained from ten patients with nodulocystic acne (eight males and two females; mean age 23.2 years) under casual conditions before and after the 6th week of oral isotretinoin treatment (mean daily dose 0.69 ± 0.23 mg/kg b.wt.). Facial comedones before and during treatment were collected from contralateral sites. Simultaneously, plantar stratum corneum shave biopsy specimens were taken from contralateral corresponding areas of the soles.

Lipid analyses

Reagents and lipid standards. Precoated silica gel 60 high-performance thin-layer chromatography plates, 10 × 20 cm, without concentration zone (Merck, Darmstadt, FRG) were used for the separation of lipids. Acetic acid, diethyl ether, petroleum benzine (boiling range 40°–60°C), phosphoric acid and cupric sulfate hydrate were laboratory grade (Merck). Methanol, chloroform, n-hexane, and water were LiChrosolv quality (Merck). Pure lipid standards were purchased from Sigma (Munich, FRG): cholesterol-3-sulfate (C-9523), cerebroside, type II (C-1516), ceramide, type III (C-2137), ceramide, type IV (C-2512), cholesterol, oleic acid, triolein, and cholesterol oleate in lipid standard stock (178-4), squalene (S–3626), and n-pentacosane (P-7260).

Lipid extraction. Lipids of comedones and plantar shave biopsy specimens were extracted using a modified Folch method [12]. Instead of water, 300 m*M* sodium chloride was added prior to phase separation to ensure complete recovery of cholesterol sulfate in the organic phase [40].

Sample application. 5 µl of chloroform-methanol (2:1) diluted lipid samples (6–8 µg) were spotted parallel to the bottom edge of the HPTLC plates at a constant distance of 1.0 cm using the CAMAG Nanomat II (CAMAG, Muttenz, Switzerland) with disposable 5.0-µl Microcap pipettes. Lipid standard mixtures containing 25 ng to 2.0 µg of each lipid were applied for the preparation of standard curves.

Table 1. Composition of comedo lipids before and after the 6th week of oral isotretinoin treatment ($n = 10$)

Lipid fraction	Lipid weight (% ± SD)		Change (%)
	Before Rx	During Rx	
Polar lipids	1.9 ± 2.0	2.1 ± 1.9	+10.5
Cholesterol sulfate	0.9 ± 1.0	0.8 ± 1.0	−11.1
Total ceramides	14.1 ± 9.2	16.8 ± 11.1	+19.1
Free sterols	6.8 ± 5.3	9.1 ± 5.0	+33.8[a]
Free fatty acids	45.4 ± 9.5	41.4 ± 11.3	− 8.8
Triglycerides and diglycerides	7.2 ± 3.1	4.6 ± 1.9	−36.1[b]
Wax esters and sterol esters	16.1 ± 4.3	16.8 ± 4.9	+ 4.3
Squalene	8.2 ± 2.6	7.7 ± 2.9	− 6.1

[a] $p < 0.10$
[b] $p < 0.0125$

Chromatographic separation. All major comedo and stratum corneum lipids were analyzed by sequential high-performance thin-layer chromatography [23, 42]. Separations were carried out in developing chambers (Desaga, Heidelberg, FRG) at room temperature. HPTLC plates were prewashed overnight in *solvent system I*, consisting of methanol-chloroform-water (20:85:1). After evaporation of all solvents under a stream of hot air, ten samples and four standards with increasing concentrations were applied. After the samples were dried, the plates were developed in chambers saturated with the appropriate solvent mixture. Three consecutive separation steps were performed: *Solvent system I* was used for the separation of polar lipids. The solvent front was allowed to migrate to 6.0 cm above the origin. After drying the plate with cool air, this step was repeated. *Solvent system II*, n-hexane-diethyl ether-glacial acetic acid (80:20:10), was developed to a distance of 8.5 cm above the origin for the separation of neutral lipids. The plates were then dried by hot air, ensuring the complete removal of acetic acid. The plates were finally developed with *solvent system III*, petroleum benzine, to full length for the separation of squalene and hydrocarbons from the fraction of sterol and wax esters.

Detection of lipids by degradative charring. Following chromatography the plates were sprayed with 10% (w/v) cupric sulfate hydrate in 8% (w/v) phosphoric acid and charred on a heat block at 180°C for 40 min.

Scanning and quantitation. After charring, HPTLC plates were scanned with an LKB 2202 UltroScan laser densitometer in absorbance mode (632.8 nm). Densitometric signals were integrated with a Spectra Physics 4270 integrator. Comedo and stratum corneum lipids were identified by comigration with authentic lipid standards. Although not all bovine ceramide standards did exactly comigrate with all human ceramide fractions, they were used for mass calibration purposes. Mass determinations of comedo and stratum corneum lipids were performed by use of appropriate lipid standard calibration curves [23, 42].

Statistical analysis. The statistical significance of the differences between mean lipid values before and after the 6th week of

Table 2. Comparison between the average composition of sebum lipids and comedo lipids (n = number of patients)

Lipid fraction	Mean lipid values (weight percent)			
	Sebum[a]	Comedones		
		(n = 10)	(n = 3)[b]	(n = 5)[c]
Polar lipids	–	1.9	–	–
Cholesterol sulfate	–	0.9	–	–
Total ceramides	–	14.1	–	–
Free sterols	1.5	6.8	12.0	9.0–13.0
Glycerides and free fatty acids	57.5	52.5	62.0	48.0–55.0
Wax esters and sterol esters	29.0	16.1	18.0	7.0–13.0
Squalene	12.0	8.2	8.0	7.0–10.0
Hydrocarbons	–	–	–	<3.0

Sources: [1, 3][a]; [24][b]; [14][c]

Table 3. Composition of plantar stratum corneum lipids before and after the 6th week of oral isotretionin treatment (n = 10)

Lipid fraction	Lipid weight (% ± SD)		Change (%)
	Before Rx	During Rx	
Polar lipids	3.7±0.9	3.8±1.1	+ 2.7
Cholesterol sulfate	1.5±0.4	1.6±0.9	+ 6.7
Total ceramides	36.0±8.0	34.6±5.0	− 3.9
Free sterols	28.7±5.6	31.3±3.2	+ 9.1
Free fatty acids	19.8±5.4	20.4±6.6	+ 3.0
Triglycerides/ diglycerides	2.1±1.3	1.8±1.0	−14.3
Sterol esters	8.0±2.1	7.2±1.7	−10.0
Squalene	0.5±0.6	0.4±0.2	−20.0
Alkanes	Traces	Traces	

isotretinoin treatment was analyzed with Student's t-test for paired data.

Results

Table 1 shows the composition of comedo lipids before and after the 6th week of systemic isotretinoin therapy. The average total yield of lipids before therapy was 33.1% ± 7.5% of total comedo weight and 21.0% ± 8.3% after the 6th week of treatment. The free fatty acid fraction could be identified as the predominant lipid class of macroscopically noninflamed open and closed comedones comprising 45.4 weight % of total comedo lipids, followed by the fraction of wax and sterol esters and total ceramides. The ceramide fraction consisted of glucosylceramides and six distinct ceramide subfractions with different polarity. In relation to the average lipid composition of sebum (Table 2), comedo lipid analysis revealed a

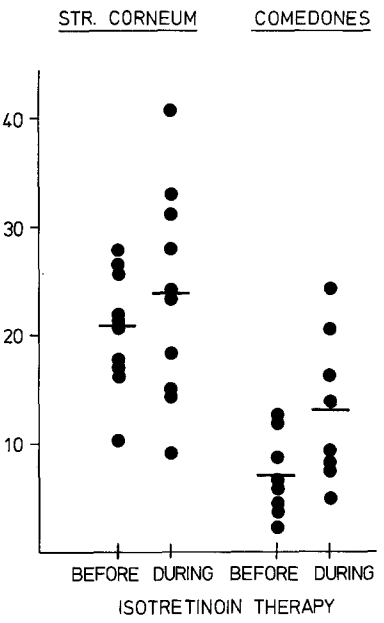

Fig. 1. Increase of the mass ratio of free sterols/cholesterol sulfate in plantar stratum corneum (n = 10) and comedones (n = 8; $p < 0.05$) after 6 weeks of oral isotretinoin treatment

moderate reduction in glycerides and free fatty acids, wax and sterol esters, as well as squalene. Compared with sebum lipids, the presence of substantial amounts of ceramides (14.1 weight %) and a fourfold amount of free sterols (6.8 weight %) were observed in comedones. Minor amounts of cholesterol sulfate in the range of 1 weight % could be consistently detected in comedonal lipid extracts. With regard to the mean percent changes of individual comedo lipids from pretreatment values, isotretinoin caused a decrease of the glyceride fraction by 36.1%, whereas free sterols and total ceramides increased by 33.8% and 19.1%, respectively.

Table 3 presents the distribution (weight percent) of plantar stratum corneum lipids – which are free from lipids of sebaceous origin – before and during isotretinoin treatment, indicating the predominance of free sterols and ceramides. No significant changes of plantar stratum corneum lipids have occurred after 6 weeks of isotretinoin therapy.

Changes of the mass ratio of free sterols and cholesterol sulfate, which have been recognized as an important factor for the regulation of normal desquamation, are presented in Fig. 1. Whereas only a minor elevation of the free sterols/cholesterol sulfate ratio of 15.2% (from a mean pretreatment value of 20.7 ± 5.4 to a mean treatment value of 23.9 ± 9.7) was observed in stratum corneum, a significant increase of 85.9% from a mean pretreatment value of 7.1 ± 3.7 to a mean value of 13.2 ± 6.8 ($p < 0.05$) was demonstrable in

comedones after 6 weeks of isotretinoin treatment. The increase of this ratio is due to an increase in free sterols, but not to changes of the relative amount of cholesterol sulfate.

Discussion

Isotretinoin influences follicular hyperkeratinization in acne [18, 29], but neither the nature of its antikeratinizing activity nor its mechanisms of action are completely known. In hairless mice oral isotretinoin administration produces epidermal and stratum corneum loosening. Dishesion correlates with loss of desmosomes and accumulation of intra- and intercellular amorphous material in the upper epidermis [5]. Disintegration of comedonal desmosomes is documented in patients with cystic acne following 20 weeks of oral isotretinoin therapy [43].

Although patients with acne have consistently been observed to have elevated levels of sebum secretion, yet no precise mechanisms relating increased sebum secretion rates to comedogenesis are known. However, recent lipid biochemical advances in our understanding of several disorders of keratinization emphasize the importance of intercorneocyte lipids for cohesive properties of horny cells [4, 6, 21, 38, 41]. In analogy to the ultrastructural organization of the lipid-enriched intercellular spaces of stratum corneum, electron microscopy of follicular casts and early comedones of prepuberal children reveals similar intercellular structures with alternating dense and less dense lamellar configurations arranged in series of tightly packed membrane-like sheets or bilayers [19]. Despite the new insights into lipid-corneocyte interactions, so far no attention has been paid to the role of intracomedonal lipids for comedogenesis and comedo shedding. We show that oral low-dose isotretinoin treatment over 6 weeks reduces the relative amount of comedonal triglycerides, diglycerides, free fatty acids and squalene, the major lipids of sebaceous origin [3]. In contrast, the relative levels of free sterols and ceramides, which are primarily of epidermal origin [6, 38, 41], increased within comedones causing a significant elevation of the ratio of free sterols and cholesterol sulfate by 86% in a favorable direction for enhanced corneocyte desquamation [5–7, 10, 21, 30–32, 40, 41]. Already after 6 weeks of isotretinoin therapy, this ratio approaches levels measured in regularly desquamating stratum corneum [41]. Although it is still uncertain, whether the isotretinoin-induced changes of the comedonal lipid composition are primary effects on the follicular epidermal lipids or secondary changes due to suppression of sebaceous lipogenesis, the latter is more likely because other systemic retinoids without sebum-suppressive activity are of no use in acne.

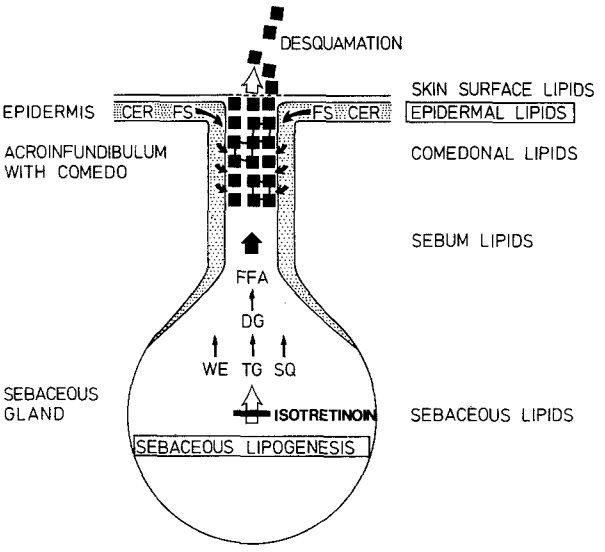

Fig. 2. Hypothetical scheme of the comedolytic effects of isotretinoin: besides disintegration of comedonal desmosomes, the restoration of epidermal lipid homeostasis of the follicular epithelium due to inhibition of increased sebaceous lipogenesis seems to be of critical importance for the antikeratinizing activity of isotretinoin. *CER*, ceramides; *FS*, free sterols; *FFA*, free fatty acids; *TG*, triglycerides; *WE*, wax esters; *SQ*, squalene

The isotretinoin-induced compositional alterations of comedonal lipids closely resemble changes of skin-surface lipids following isotretinoin treatment, with an elevation of the percentage of free cholesterol and a decrease in the percentage of wax esters and squalene [33, 35–37]. This correlation supports our hypothesis that a localized dilutional effect due to increased sebaceous gland secretion on the proportion of free sterols might be the important etiologic factor in comedogenesis. The isotretinoin-induced inhibition of sebaceous lipogenesis seems to be the prerequisite for the restoration of epidermal lipid homeostasis of the follicular epithelium, confirmed by the predominant increase of intracomedonal ceramides and free sterols, known to be the major lipid fractions derived from epidermis (Fig. 2).

The unaffected profile of plantar stratum corneum lipids after 6 weeks of isotretinoin treatment supports the conclusion that isotretinoin-induced changes of the composition of intracomedonal lipids are a secondary phenomenon due to the inhibition of sebum secretion, although 6 weeks of isotretinoin treatment might not be sufficient for the detection of significant lipid-biochemical changes of human plantar stratum corneum with its prolonged cell turnover in comparison with other skin areas. Simultaneously, other direct effects of isotretinoin on the follicular epithelium might be operative as well.

In contrast to comedonal lipid data reported by Nicolaides et al. [24] and Gershbein et al. [14], we consistently have been able to identify considerable amounts of ceramides (14.1%). The relative amount of cholesterol sulfate which is detected in the range of 1 weight % of total comedonal lipids is not affected by isotretinoin treatment.

Recently, it could be demonstrated that comedonal ceramides contain diminished proportions of linoleic acid which has led to the hypothesis that a localized insufficiency of linoleic acid due to dilutional effects of increased sebum production might be an etiologic factor in comedogenesis [34, 39]. Whether low concentrations of linoleate in sebum impose a state of essential fatty acid deficiency on the cells of the follicular epithelium, thereby inducing the characteristic responses of hyperkeratosis and decreased barrier function [2], *or* whether decreased concentrations of free sterols within the follicular epithelium associated with a low ratio of free sterols to cholesterol sulfate, thereby inducing local conditions resembling the retention hyperkeratosis of x-linked recessive ichthyosis [9], or even whether both factors are operative in comedogenesis, remains to be elucidated.

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