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0022-202X/83/8101S-0081s$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:81s–85s, 1983

Vol. 81, No. 1 Supplement
Printed in U.S.A.

Membrane Glycoconjugate Visualization and Biosynthesis in Normal and Retinoid-Treated Epidermis

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Cell-membrane glycoconjugates can be visualized for ultrastructural and fluorescence studies with certain surface markers, such as lectins and antisaccharide antibodies. When frozen sections of mammalian epidermis are treated with a battery of rhodamine-conjugated lectins, the cell membranes display a pattern of increased sugar complexity during keratinocyte maturation. Although this vectorial sequence is disrupted following retinoid treatment, these changes occur only at high doses and late in the course of treatment, suggesting that retinoid-induced alterations may be secondary. Seemingly as a result of specific glycosidase activity within the cytosol of both granular and cornified cells, lectin staining suddenly disappears from stratum corneum cell membranes. Although lectins stain membrane glycoconjugates of cultured human keratinocytes, quantitative techniques are required to recognize differences in proliferating versus postmitotic cells and in cultures supplemented with various growth factors. Whereas retinoids consistently depress glycoprotein synthesis in cultured keratinocytes, in organ culture they stimulate epidermal glycoprotein, and particularly glycolipid, biosynthesis. These studies suggest (1) that visualization of membrane glycoconjugates with lectins can reveal important variations in normal and pathologic epidermal differentiation, (2) that lectins may reveal subtle quantitative alterations in differentiation in vitro, (3) that retinoid stimulation of glycoconjugate biosynthesis either displays important species differences or requires a higher level of organization than occurs in cell culture, and (4) that the retinoid effect on glycoconjugate biosynthesis in organ culture may provide another useful bioassay for retinoid potency.

MEMBRANE SUGARS, LECTINS, AND EPIDERMAL DIFFERENTIATION: IN VIVO STUDIES

Mammalian cell membranes contain numerous glycoproteins and glycolipids that subserve a variety of cellular functions [1]. As a result of controlled modulations in glycosylation and deglycosylation, these cells generate a rich variety of different oligosaccharide chains from a relatively small number of sugar "building blocks" [2]. Since both the composition and distribution of these sugar components are known to change during differentiation, lectins, proteins of plant or animal origin that bind to specific carbohydrate binding sites [3], have been among the most useful methods for visualizing these variations (Tables I, II). Holt et al. [4] initially noted inhomogeneous labeling of malphigian versus outer mammalian epidermis in mammalian skin with a limited number of lectins. Later, after a greater number of lectins were applied to frozen sections of epidermis, it became apparent that keratinocyte membrane sugars display a vectorial pattern of progressive, sequential sugar additions to the cell membrane during progression from the basal to the

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Abbreviations:
PNA: peanut agglutinin
TCA: trichloroacetic acid
TEWL: transepidermal water loss
Ulex: *Ulex europaeus*

granular layer [5,6] (Fig. 1). However, virtually all lectin labeling abruptly disappears from stratum corneum cell membranes (Figs. 2–5). Although some additional information can be gleaned about the location of otherwise occult sugars in keratinocyte membranes with controlled proteolytic or neuraminidase digestion [7], some sugar moities may still not be accessible to all the lectins. Thus the actual sequence of sugar moieties cannot be construed from lectin-labeling studies alone.

TABLE I. *Labeling systems for visualization of cell-surface sugars*

I. Direct method:
Lectin-*Fl* or *Rhd*
II. Indirect methods:
Fluorescence:
B-lectin + Avidin-*Fl* or *Rhd*
Anti-Sacc. *Ab* + *Fl* or *Rhd*-anti-species *Ab*
B-anti-Sacc. *Ab* + Avidin-*Fl* or *Rhd*
Hp-anti-Sacc. *Ab* + anti-*Hp Ab*-*Fl* or *Rhd*
Electron microscopy:
B-lectin + Avidin + *B*-marker
Hp-anti-Sacc. *Ab* + anti-*Hp Ab* + *Hp*-marker

Abbreviations: Fl: fluorescein; *Rhd:* rhodamine; *B:* biotinylated; *Ab:* antibody; *Hp:* hapten; markers: TMW, hemocyanin, ferritin, etc.

TABLE II. *Lectins and their sugar specificity*

Lectin	Sugar
Concanavalin A (Con A)	α-D-Mannose > α-D-glucose
Pisum sativum (PSA)	α-D-Mannose > α-D-glucose
Ricinus communis I (RCA I)	β-D-Galactose, lactose
Bandeirea Simp. I (BSL I)	α-D-Galactose
Ricinus communis II (RCA II)	α-Acetyl-galactosamine-β-D-galactose
Soybean agg. (SBA)	(α and β) *N*-Acetyl-galactosamine
Peanut agg. (PNA)	β-D-Galactose-β-(1-3)-*N*-acetyl-galactosamine
Phaseolus vulgaris-E (PHA-E)	β-D-Galactose-β-D-*n*-acetyl-glucosamine
Phaseolus vulgaris-L (PHA-L)	β-D-Galactose + ?
Wheat germ agg. (WGA)	(β--*N*-Acetyl-glucosamine)$_3$, sialic acid
Succinyl-WGA (Succ-WGA)	(β-*N*-Acetyl-glucosamine)$_3$
Bandeirea simp. II (BSLIII)	(α > β) *N*-Acetyl-glucosamine
Ulex europaeus agg. I (Ulex)	α-L-Fucose
Limulus polyphemus agg. (LPA)	Sialic acid

GLYCOSIDASES AND EPIDERMAL DIFFERENTIATION

The sudden loss of membrane sugars at the level of the stratum corneum could be rationalized by either endogenous enzymatic cleavage or by occlusion of the oligosaccharide chain by either bulk protein or lipid. The second possibility, namely, that bulk lipid or protein could obscure membrane glycoconjugates in the stratum corneum, has been largely excluded by showing that pretreatment with either proteolytic enzymes or with lipid solvents exposed no lectin-binding sites in the stratum corneum [7]. Therefore, we sought evidence of glycosidase activity in the outer epidermis and obtained such evidence in high-speed supernatant fractions of both granular and cornified cells [7] (Table III). These glycosidases displayed activity that was specific for those sugars which could be demonstrated with lectin staining (Figs. 2–5), and pertinently, no activity could be found for a variety of sugars that were not demonstrable either biochemically or with lectin staining [7] (Table III). Rather than representing one enzyme with activity against multiple substrates, the glycosidases comprise a family with separate

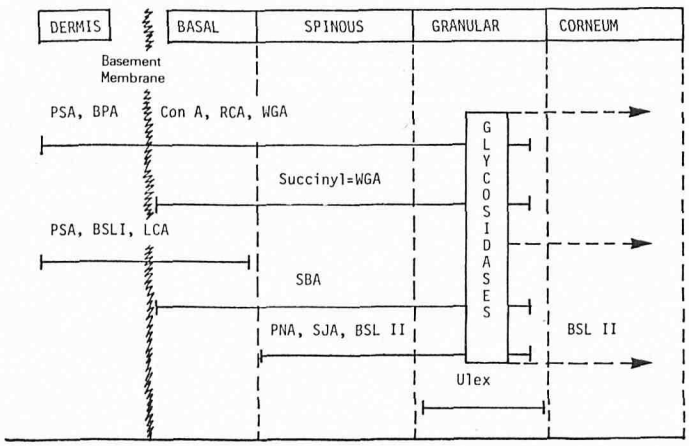
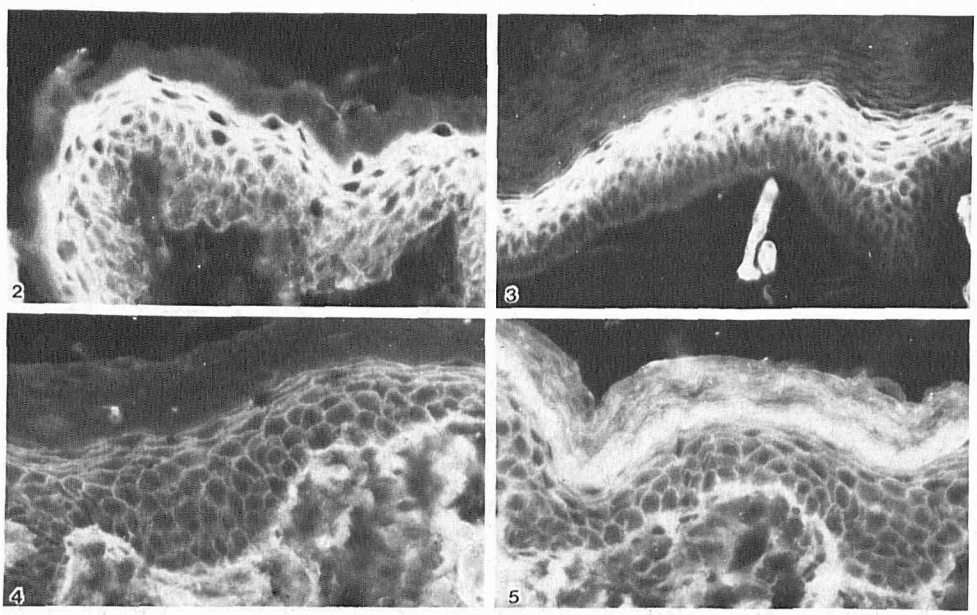


FIG 1. This diagram depicts the sequence of sugar modifications, as revealed by appropriate lectins, to keratinocyte cell membranes in rat, mouse, and human epidermis [4–7]. Virtually all staining disappears above the stratum granulosum, presumably due to the activity of a family of sugar-specific glycosidases [7]. (From [7].)



FIGS 2–5. This plate illustrates typical staining of frozen sections of neonatal mouse skin stained with peanut agglutin (Fig. 2), *Ulex* agglutinin (Fig. 3), *Ricinus communis* I agglutinin (Fig. 4), and wheat germ agglutinin (Fig. 5). Note intense staining over keratinocyte membranes of viable epidermis and absence of cytoplasmic staining with each lectin. Whereas *Ricinus* and wheat germ agglutinins stain all epidermal layers, peanut and *Ulex* agglutinin stain the stratum spinosum plus stratum granulosum (Fig. 2) and stratum granulosum alone (Fig. 3), respectively. Both dermal elements and the basement membrane are unstained by peanut and *Ulex* agglutinins, but these structures are stained by *Ricinus* and wheat germ agglutinins. The stratum corneum is unstained by all the lectins (staining by wheat germ agglutinin is nonspecific).

sugar-specific activities, as well as certain other definable characteristics [7] (Table IV).

Although biochemical data are not available on the sugar content of membrane glycoconjugates at various levels of epidermis, glucose levels are known to decrease dramatically in the outer epidermis [8]. Moreover, radiolabeled outer epidermal membrane glycoconjugates incorporate less precursor than lower-epidermal preparations [9] (see below). Furthermore, in separate studies on stratum corneum membrane glycosphingolipids, we and others have shown that sphingolipids are deglycosylated and essentially disappear from the outer stratum corneum [10,11]. These studies lend further limited support for the role of glycosidases in producing the observed pattern of abrupt loss of lectin labeling in the stratum corneum.

Membrane deglycosylation may have significance for both the water-barrier properties and the cohesiveness of the stratum corneum. Removal of charged sugar groups from stratum corneum membrane glycoconjugates should make the intercellular lipid-filled domains between corneocytes more hydrophobic. Whereas heavily glycosylated sphingolipids resist incorporation into broad lipid lamellar structures [12], cerebrosides and ceramides, such as those in the stratum corneum [13], form lamellar membranes even at high water-to-lipid ratios [12]. Ultrastructural studies support this model [13]. Whereas intercellular lipids in the granular layer are arrayed in small Con A-positive disks indicative of the presence of glycoconjugates, as these intercellular disks move into the stratum corneum, they transform into multilayered, Con A-negative neutral lipid-enriched sheets.

TABLE III. *Distribution of glycosidases within stratum granulosum/stratum corneum*[a]

Enzyme (lectin)	Concentration[b] (SG/SC)
β-D-*N*-Acetyl-glucosaminidase (WGA, BSL II)	++++/+++
α-D-Mannosidase (Con A, PSA, LCA)	++/++
β-D-Galactosidase (RCA, PNA)	++/++
β-D-*N*-Acetyl-galactosaminidase (SBA)	++/++
α-L-Fucosidase (Ulex)	++/++
α-D-Galactosidase (BSL I)	+/ND
β-D-Fucosidase (none known)	+/ND
α-D-Glucosidase (none known)	+/ND
β-D-Glucosidase (none known)	+/ND

[a] Results read semiquantitatively based on color intensity on a scale of 0 to 4+ over sugar controls.

[b] The 500-, 10,000-, and 50,000-g pellets obtained during the supernatant preparation contained β-D-*N*-acetyl-glucosaminidase activity and low levels of β-D-galactosidase, but otherwise all activity was found in supernatant (cytosol) fractions.

TABLE IV. *Characteristics of upper epidermal glycosidases*

1. Present in equal concentrations in stratum granulosum and stratum corneum
2. Activity is predominantly in cytosol
3. Demonstrate sugar specificity
4. Are inactivated by heat, but not freeze-thawing
5. Can be activated by certain detergents

LECTINS, STAINING OF MEMBRANE SUGARS IN VITRO

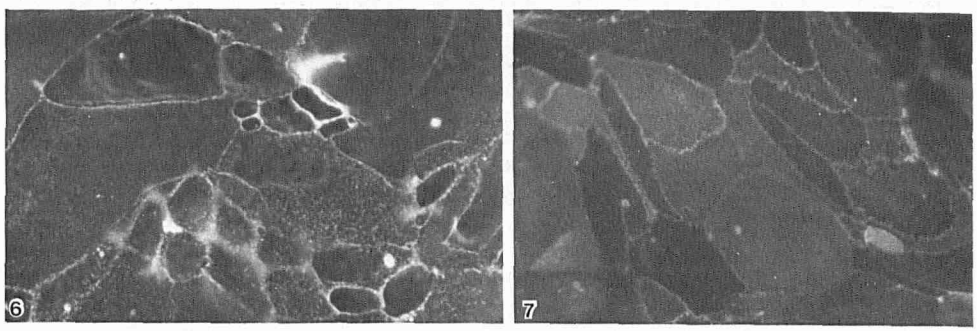
Despite the recent burgeoning of interest in human keratinocyte cultures, to date the only molecule that has been shown to vary during normal in vitro differentiation is involucrin, a protein precursor of the stratum corneum envelope. To determine whether lectins might provide a useful probe for the study of keratinocyte membrane sugars in vitro, we applied both rhodamine-conjugated and biotinylated lectins to human foreskin keratinocytes undergoing various degrees of differentiation. These studies demonstrated (1) that all the cells in each culture, regardless of their state of differentiation, display the same array of sugars found only on the most differentiated cells in vivo [15]. For example, even the most basaloid, proliferating cells bound peanut agglutinin (PNA) and *Ulex europaeus* (*Ulex*) (Figs. 6 and 7), lectins that in vivo bind only to suprabasilar cells [4–6] (Figs. 1–3). However, it should be noted that guinea pig keratinocytes, stained with lectins under similar conditions as here, reportedly do not bind *Ulex* [16], which may indicate that these cells are still more undifferentiated than their human counterparts. The intensity of fluorescence could be enhanced by using biotinylated lectins, followed by exposure to avidin-rhodamine (Table I; also compare Figs. 6 and 7).

Since human keratinocytes in all stages of differentiation bind the full spectrum of lectins, we applied quantitative techniques to detect differences in lectin binding under different growth conditions. In cultures exposed to a variety of different growth factors, both methods demonstrated diminution in *Ulex* staining following application of any (or all) of the growth factors [15] (Table V). In contrast, several other lectins (*Ricinus communis* agglutinin I, wheat germ agglutinin, and PNA) showed no consistent differences. These studies raise the possibility that lectins may be able to detect differences in human keratinocyte differentiation.

RETINOIDS AND MEMBRANE GLYCOSYLATION: IN VIVO STUDIES

Retinoids exert well-known, but poorly understood effects on epithelial differentiation (Fig. 8) [17]. One possible mechanism for their action is an effect on posttranslational glycosylation [18]. Phosphorylated retinoids act as carriers for certain monosaccharides, e.g., galactose and mannose, leading to the transmembrane insertion of these sugars into membrane glycoconjugates. It has been hypothesized that this mechanism may underlie the retinoids' important impact on substrate attachment and intercellular adhesion [18].

Yet it is unlikely that the retinoids' ability to cause mucous metaplasia in embryonic and neoplastic skin can be ascribed to the same glycosylation mechanism, since neither mannose nor galactose appear to be important constituents of epithelial mucins, as determined by lectin staining [19]. Moreover, since mucous metaplasia occurs primarily in immature or neoplastic



FIGS 6 and 7. Keratinocyte cultures stained with biotinylated *Ricinus communis* I agglutinin (RCA) plus avidin-rhodamine (Fig. 6) versus RCA-rhodamine alone. Both techniques specifically bind to cell membranes, demonstrating the microvillous-coated surfaces of more mature keratinocytes, but the intensity appears to be greater with the biotinylated lectin.

epidermis, it cannot explain the powerful impact of retinoids on normal postembryonic epidermis [17]. Using a variety of ultrastructural, histochemical, and functional assays, we demonstrated that retinoids stimulate epidermal DNA synthesis [21], while decreasing epidermal intercellular cohesion and integrity [20,21]. As a result, a deficit occurs in the permeability barrier that is manifested by dose- and time-dependent increases in transepidermal water loss (TEWL) [20]. This alteration in TEWL can be quantified providing a potentially unique bioassay for retinoid antikeratinizing potency [20].

Despite the fact that mucin deposition does not underlie the pathophysiologic alterations in retinoid-treated epidermis [20], certain alterations in membrane glycosylation staining do occur in retinoid-treated epidermis [23] (Figs. 9–13). Since these changes appear late in the course of retinoid treatment, i.e., after such pathophysiologic derangements as increased transepidermal water loss have already occurred [23], they are probably secondary to earlier biochemical events. One possibil-

TABLE V. *Influence of different growth factors on Ulex labeling of human keratinocyte cultures*

Condition[a]	Method	
	Microspectrofluorometry[b] (U)	Exposure time[c] (s)
20% Fetal calf serum (FCS)	83.7 (% change)	152 (% change)
FCS ± epidermal growth factor (EGF)	29.9 (−64%)	158 (+0.96%)
FCS ± hydrocortisone (HC)	41.6 (−52.3%)	202 (+24.8%)
FCS ± cholera toxin (ChT)	73.4 (−12.3%)	175 (+13.2%)
FCS ± in low Ca^{2+} (0.3 m*M*)	46.6 (−44.3%)	195 (+22.1%)
FCS ± EGF ± HC ± ChT	28.0 (−66.6%)	190 (+20.0%)
FCS ± low Ca^{2+} ± EGF ± HC ± ChT	22.4 (−73.2%)	214 (+29.0%)

[a] Second- or third-passage human foreskin keratinocytes, supported by Mitocycin C-treated 3T3 feeder cells, were grown to preconfluence (8–10 cell islands) in DME media supplemented with 20% FCS. Cells were washed with serum-free media several times and preincubated with serum-free, factorless media for 24 hours prior to addition of sera plus the following factors: EFG (20 ng/ml), hydrocortisone (0.4 μg/ml), and cholera toxin (10^{-10} m*M*).

[b] Data shown represent mean for measurements over the cell membrane of 20 different cells using a 1 μm slit aperture.

[c] Exposure time correlates inversely with intensity in an approximately straight-line fashion as determined with fluorescent covaspheres [7].

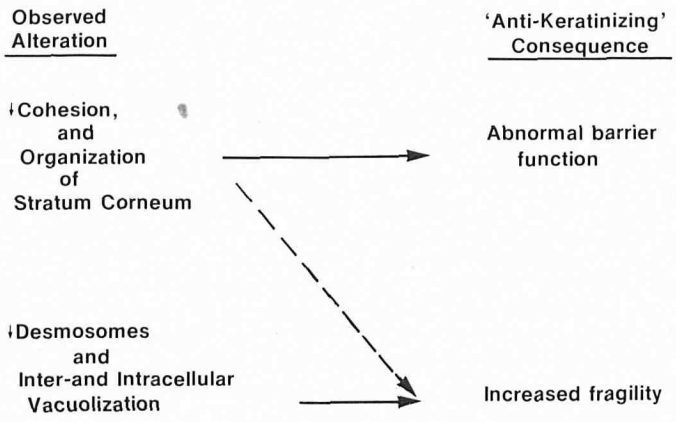
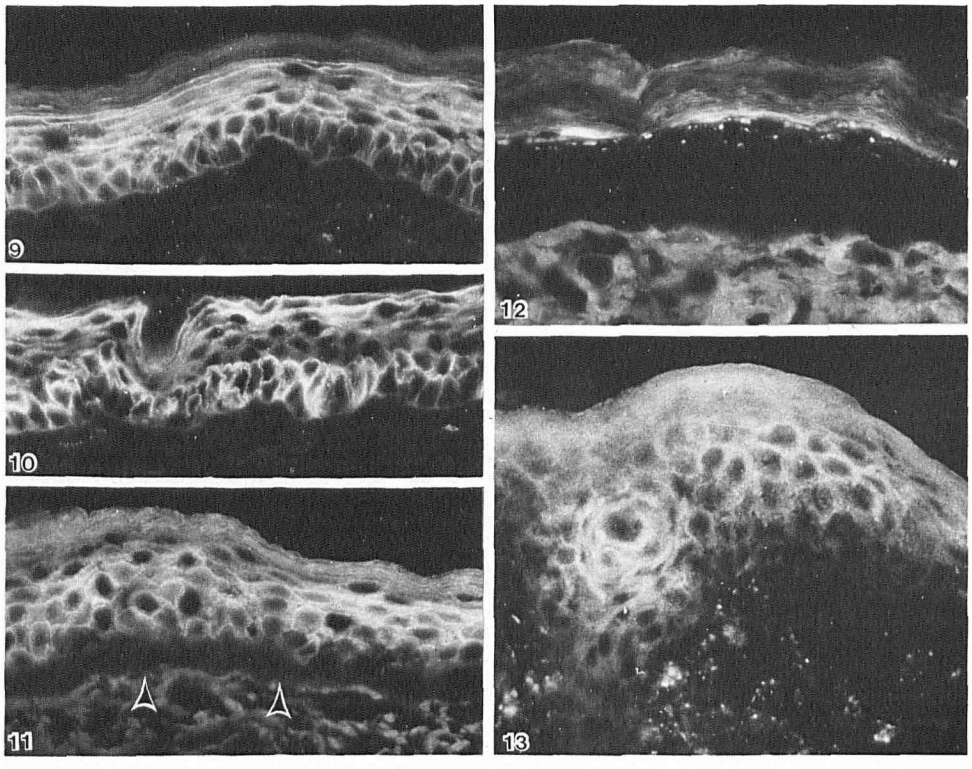


FIG 8. Diagram of apparent "antikeratinizing" action of retinoids based on studies in [30,31]. (Reprinted with permission from Lab Invest 44:531–540, 1981.)

TABLE VI. *Influence of retinoids on de novo glycoprotein biosynthesis in organ-cultured neonatal mouse epidermis*[a]

Etretinate concentration	Retinoid (% change from control)		
	Tretinoin	Isotretinoin	Etretinate
10^{-4} *M*	133 ± 23 (↑40%)	55 ± 3 (↓8%)	63 ± 5 (↓12%)
10^{-5} *M*	149 ± 13 (↑57%)	96 ± 2 (↑64%)	123 ± 30 (↑70%)
10^{-6} *M*	113 ± 23 (↑19%)	98 ± 8 (↑67%)	81 ± 10 (↑14%)
Control	95 ± 20	59 ± 7	71 ± 19

[a] Epidermis separated from dermis/subcutaneous fat after overnight incubations with retinoid in delipidized, sugar-free media plus [^{3}H]-*n*-acetyl-D-glucosamine (40 μCi/cc). At the end of incubations the samples were homogenized, solubilized with 2% SDS, and precipitated with 7% TCA. Both etretinate and control cultures contained 2% DMSO. The protein concentration of the solubilized sample was determined by the Lowry method, and data are specific activity ± SEM.



FIGS 9–13. Frozen sections of hairless mouse epidermis during treatment with etretinate (10–25 mg/kg/day). After 15 days of treatment, peanut agglutinin (PNA) stains basal layer (Fig. 9), which normally is unstained (see Fig. 2). After 17 days, PNA staining of the spinous layer is actually *less* than staining of the granular and basal layers (Fig. 10), but after 40 days, staining is only patchy throughout epidermis (Fig. 13). *Ulex* stains both the granular and spinous layers after 21 days (Fig. 11), in contrast to normal epidermis, where only the granular layer is stained (see Fig. 12). Wheat germ agglutinin (not illustrated) depicts droplets in the granular layer, but only after 30 or more days. (Reprinted with permission from J Am Acad Dermatol 4:801–808, 1982.)

TABLE VII. *De novo synthesis of epidermal glycoconjugates in etretinate-treated neonatal mouse skin (cpm/dish)*[a]

Fraction[b]	Etretinate[c]			Control[c]		
	1	2	3	1	2	3
Epidermis, upper	273	287	207	328	344	118
Epidermis, full-thickness	2092	3638	1189	681	501	—
Dermis	2752	2981	4573	4939	2524	—

[a] Specimens handled exactly as in Table VI, except that at the end of incubations with retinoid plus ^{3}H-*n*-acetyl-D-glucosamine (20 μCi/ml), samples were fixed in glutaraldehyde to permeabilize cells to unincorporated sugars, washed extensively, and then hydrolyzed in 6 *N* HCl at 90°C for 5 min.

[b] Upper epidermis obtained by in vivo administration of highly purified noncytotoxic preparations of staphylococcal epidermolytic toxin. Epidermal-dermal separation performed by incubation in 10 *M* dithiothreitol for 1 hour at 37°C.

[c] Concentration of etretinate was always 10^{-5} *M*. Both etretinate and control cultures contained 2% DMSO.

ity is that these shifting patterns may reflect modulations in glycosidase activity due to labilization of lysosomal hydrolytic enzymes [17]. Finally, whereas application of lectins to retinoid-treated epidermis does not reveal mucin deposition [23], both wheat germ agglutinin (WGA) and succinyl-WGA reveal a distinctive pattern of sugar deposition, in the form of droplets within the outer epidermis, late in the course of retinoid treatment (Fig. 12).

RETINOIDS AND MEMBRANE GLYCOCONJUGATE BIOSYNTHESIS: IN VITRO STUDIES

Although retinoids generally stimulate epithelial posttranslational glycosylation in vivo [17,18], studies in cultured cells have yielded conflicting results. To see whether retinoids stimulate epidermal glycosylation, we measured de novo glycoprotein synthesis in cultured human foreskin keratinocytes dosed with tretinoin (all-*trans*-retinoic acid), isotretinoin (13-*cis*-retinoic acid), and etretinate (RO 10-9359) (range 10^{-4}–10^{-8} *M*) for 12 to 96 hours in delipidized fetal calf serum containing [^{3}H]-D-glucosamine or [^{3}H]-D-galactose (10–15 mCi per dish). Briefly summarized, regardless of the retinoid type, dosage, labeled precursor, or length of incubation, synthesis was invariably inhibited by the retinoids (data not presented) [24]. This result conflicts with earlier studies in mouse keratinocyte cultures, where retinoids clearly stimulated glycosylation [25,26]. In contrast, in organ cultures of neonatal mouse epidermis dosed with the same concentrations of these retinoids under the same conditions, the retinoids stimulated incorporation into glycoproteins by 20 to 70 percent over controls [9,24] (Table VI). In keeping with their superior potency in several other systems [17], the synthetic retinoids isotretinoin and etretinate were more potent than tretinoin, but at higher concentrations (10^{-4} *M*) they appeared to be more toxic (Table VII).

We next showed that the retinoids' stimulatory effect is directed at the epidermis, since pieces of epidermis free of any dermis were stimulated by retinoids, but dermis alone was not stimulated [9] (Table VII), a finding in agreement with that of King and Tabiowo [27], who obtained similar results in pig epidermal slices. Moreover, when incorporation into outer epidermal sheets, obtained with highly purified fractions of the staphylococcal epidermolytic toxins, was compared with incorporation into whole epidermis, all the enhanced incorporation occurred in the malpighian of the epidermis [9]. These results indicate that despite persistence of increasingly complex membrane sugars into the stratum granulosum (as shown by lectin staining), de novo synthesis is limited to the lower epidermis.

Comparison of the data in Tables VI and VII also suggests that the retinoids exert a disproportionately greater effect on glycolipids than on glycoproteins [19]. Whereas in normal epidermis free sugars are distributed in about a 1:2 ratio in glycolipids versus glycoproteins [28], retinoids appear to stimulate glycolipid biosynthesis much more than glycoprotein synthesis, since the increase in total incorporation is much greater than the amount of incorporations into TCA-precipitable material [9]. However, regardless of their molecular target, it is possible that the present organ culture system could provide a powerful in vitro bioassay for the comparison of retinoid potency.

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