Recessive x-Linked Ichthyosis: Role of Cholesterol-Sulfate Accumulation in the Barrier Abnormality

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Cholesterol sulfate is a multifunctional sterol metabolite, produced in large amounts in squamous keratinizing epithelia. Because patients with recessive x-linked ichthyosis display not only a 10-fold increase in cholesterol sulfate, but also a 50% reduction in cholesterol, we assessed here whether cholesterol sulfate accumulation and/or cholesterol deficiency produce abnormal barrier function in recessive x-linked ichthyosis. Patients with recessive x-linked ichthyosis display both an abnormal barrier under basal conditions, and a delay in barrier recovery after acute perturbation, which correlate with minor abnormalities in membrane structure and extensive lamellar-phase separation. Moreover, both the functional and the structural abnormalities were corrected by topical cholesterol. Yet, topical cholesterol sulfate produced both a barrier abnormality in intact skin and extracellular abnormalities in isolated stratum corneum, effects largely reversed by coapplications of cholesterol. Together, these results suggest that cholesterol sulfate accumulation rather than cholesterol deficiency is responsible for the barrier abnormality. Despite the apparent importance of cholesterol sulfate-to-cholesterol processing for normal barrier homeostasis, neither steroid sulfatase activity nor mRNA levels are upregulated following acute perturbations. These results demonstrate both a potential role for cholesterol sulfate-to-cholesterol processing in normal permeability barrier homeostasis, and that basal levels of steroid sulfatase are sufficient to accommodate acute insults to the permeability barrier.
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Cholesterol sulfate appears to possess multiple, possible functions in keratinizing epithelia, where it is: (i) both a marker of squamous metaplasia (Jetten *et al*, 1989) and a possible inducer of differentiation (Denning *et al*, 1995); (ii) a negative feedback regulator of cholesterol synthesis *in vitro* (Williams *et al*, 1987); (iii) an indicator of pathologic desquamation in recessive x-linked ichthyosis (RXLI) (Williams and Elias, 1981); and (iv) an inducer of excess scale in normal skin (Maloney *et al*, 1984). Although cholesterol sulfate is also present in minute quantities in extracutaneous tissues, it occurs in much larger quantities in keratinizing epithelia. In epidermis, its levels peak at around 5% of total lipid in the stratum granulosum, declining to about 1% in the outer stratum corneum (Long *et al*, 1985; Ranasinghe *et al*, 1986; Elias *et al*, 1988). Cholesterol sulfate, like cholesterol, is localized to membrane domains in the stratum corneum of both normal and RXLI subjects (Elias *et al*, 1984; Koppe *et al*, 1978).

Whether cholesterol sulfate is an important bulk precursor of the cholesterol requirement for permeability barrier homeostasis is not known. In RXLI, due to absence of the enzyme steroid sulfatase, which catalyzes the desulfation of cholesterol sulfate to cholesterol (Shapiro *et al*, 1978; Kubilus *et al*, 1979), cholesterol sulfate levels in the stratum corneum are ≈10-fold elevated, whereas free cholesterol levels are ≈50% reduced (Williams and Elias, 1981). Substantial cholesterol, however, is delivered directly to the stratum corneum interstices by the exocytosis of epidermal lamellar body contents, which are enriched in cholesterol (Grayson *et al*, 1985; Feingold *et al*, 1990), a mechanism that could suffice for barrier requirements.

If cholesterol sulfate processing provides an important quota of cholesterol for the barrier, then one would expect to encounter permeability barrier abnormalities in RXLI. Prior clinical studies on barrier function in RXLI have been indecisive: some studies report minor abnormalities in basal barrier function (e.g., Frost *et al*, 1968); whereas others claim that barrier function is normal (Lavrijsen *et al*, 1993) or near-normal (Johansen *et al*, 1995). In this study, we first evaluated barrier function in patients with RXLI; and second, we employed several approaches to assess the role of cholesterol sulfate accumulation *versus* cholesterol deficiency in development of the barrier abnormality. Our studies indicate that cholesterol sulfate processing is important for normal barrier homeostasis, because cholesterol sulfate accumulation appears to be responsible for the barrier abnormality in RXLI. Yet, neither steroid sulfatase activity nor mRNA levels are regulated by experimental perturbations to barrier function.

MATERIALS AND METHODS

Test materials Cholesterol (Sigma, St Louis, MO) and cholesterol sulfate (Research Plus, Bayonne, NJ) were dissolved alone or together at a 2% concentration in propylene glycol:isopropanol (7:3, vol/vol). Solutions were applied to test area A twice daily for 1 wk, and one additional time immediately after acute barrier abrogation (see below). Vehicle alone was applied to test area B twice daily for 1 wk and once more after acute abrogations. Cream or lotion based vehicles were avoided in order to isolate the effects of sterols *versus*

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Abbreviation: RXLI, recessive x-linked ichthyosis.

vehicle. Test area C served as an untreated control area. Cholesterol sulfate solutions were used for animal studies only (see below).

Studies in human volunteers Thirty male Caucasian volunteers, aged 15–75, gave informed consent to participate in the study (protocol approved by UCSF Committee on Human Research.) Fifteen subjects, who carried the diagnosis of RXLI, previously confirmed by enzyme assay, agreed to studies of basal function, whereas five of these subjects agreed to further functional and morphologic studies. Control subjects comprised five age- and sex-matched volunteers, without skin disease. All participants were in good general health; none were taking oral medications; and all topical treatments were terminated at least 2 wk prior to these studies. All measurements were obtained from the ventral forearm, 3 cm below the antecubital fossa, where the scaling symptoms were relatively mild in the RXLI volunteers.

Animal studies Cholesterol sulfate (2%) or vehicle alone was applied twice daily for 5 d to the contralateral flanks of groups of 6–12 wk old, male hairless mice (n = 6). Transepidermal water loss (TEWL) was measured daily, with an electrolytic water analyzer (Meeco, Warrington, PA) immediately prior to the next morning's application. Biopsies were obtained for electron microscopy after the last TEWL measurements, and processed as described below.

In another set of studies, tape strippings of normal murine skin were floated immediately on drops of 0.5% trypsin in phosphate-buffered saline. Either 2% cholesterol, 0.5% cholesterol sulfate, cholesterol sulfate 0.5% plus cholesterol (2%), or vehicle was added to the outer surface of the samples for 2 h. At the end of incubations, the samples were fixed for electron microscopy (see below).

Assessment of basal barrier function TEWL was measured over the three test areas (4 × 1.3 cm^2 each) on the volar forearms of human subjects using an Evaporimeter (ServoMed), and recorded in mg per cm^2 per h over background. Three sites were measured within each test area, and averaged to generate one data point for subsequent analysis. TEWL values were registered 45 s after application of the probe to the skin. The mean temperature was 21°C (range, 18–24°C), the average relative humidity was 39% (range, 28%–52%), and the average atmospheric pressure was 8.9 mmHg (range, 5.3–13.8 mmHg). A closed box with a Plexiglas top was used to protect the measurement zone from excess air convection, and a gold-plated cover with a screen and grid was used for probe protection.

Assessment of barrier integrity and recovery Barrier integrity, i.e., stratum corneum cohesion, was tested in the same areas used for the basal TEWL measurements, using sequential cellophane (Tesa Tuck, New Rochelle, NY) tape strippings. TEWL was measured on three sites within each area after each group of 10 sequential tape strippings until a comparable degree of barrier disruption was obtained in all volunteers, defined as a TEWL of ≈2.0 mg per cm^2 per h. Ten microliters of 2% cholesterol in propylene glycol:propanol or the vehicle alone were applied to each test area immediately after barrier abrogation. TEWL was determined again in all test areas at 6 h, 24 h, and 1 wk after barrier abrogation.

Tissue preparation Male hairless mice (ages 6–12 wk) (Charles River Laboratories) were used for studies on enzyme regulation. Tape-stripped or acetone-treated *versus* untreated (TEWL ⩾ 4 mg per cm^2 per h) skin was compared at various time points from 4 to 12 h after barrier disruption. Epidermis was separated from dermis by incubation in 10 mM ethylene diamine tetraacetic acid in Ca^{++}- and Mg^{++}-free phosphate-buffered saline, pH 7.4, at 37°C for 30–40 min, followed by gentle vortexing. Epidermal preparations then were homogenized (3 × 15 s) with a Polytron homogenizer, followed by sonication at 35% power (2 × 10 s) on ice. The homogenization buffer consisted of 10 mM Tris, pH 7.5, containing 0.15 M sucrose and 2 mM ethylene diamine tetraacetic acid. Crude homogenates were first centrifuged at 10,000 × *g* for 10 min, then at 100,000 × *g* for 60 min, both at 4°C. Steroid sulfatase activity was measured in the microsomal pellet, as described below. Protein content was measured by a variation of the method of Bradford (Johansen *et al*, 1995).

Steroid sulfatase assay Steroid sulfatase activity was assayed in acetone-treated or tape-stripped *versus* untreated hairless mouse epidermis, as described by Milewish *et al* (1990), with modifications. Assays were performed in 0.1 M Tris buffer (pH 7.4) containing 5.6 mM glucose. Microsomes, isolated from epidermis (0.1 mg) were incubated with 15 μM [^{3}H]dehydroepiandrosterone sulfate (5 μCi) for 2 h at 37°C, with a final assay volume of 1.1 ml. The product, [^{3}H]dehydroepiandrosterone, was extracted from the reaction mixture with benzene (Kubilus *et al*, 1979; Epstein *et al*, 1984), and an aliquot was counted by liquid scintillation spectrophotometry.

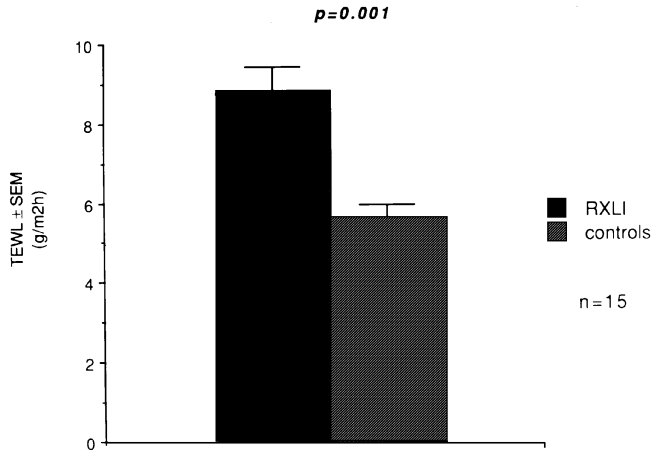

Figure 1. Barrier function in RXLI *versus* age/sex-matched control subjects. n = 15 in each group; differences are significant by Student's t test.

mRNA isolation, northern blotting, and densitometry Epidermis (0.2–0.4 g) was obtained from the treated *versus* untreated torsos of male hairless mice, and processed for mRNA extraction (Chomczynski and Sacchi, 1987). Poly(A)$^+$ mRNA was isolated by olio(dt) chromatography (type 77F, Pharmacia LKB), as described previously (Wood *et al*, 1992). Eight to 10 micrograms of Poly(A)$^+$ mRNA (extracted from the epidermis of two mice) were loaded per lane onto a formaldehyde/1% agarose gel. After electrophoresis, the gel was stained with acridine orange for visualization of the integrity of residual ribosomal RNA bands. RNA was transferred to Nytran membranes and subsequently fixed by baking at 80°C for 2 h. cDNA probes were [^{32}P]-labeled by the random priming method according to the manufacturer's instructions (Amersham, Arlington Heights, IL). Labeled probes were purified by exclusion chromatography through G-50 mini spin columns. Hybridization to RNA was performed as described previously (Wood *et al*, 1992). Briefly, northern blots were prehybridized for 1 h at 65°C in hybridization buffer and exposed to the radiolabeled probe overnight in the same buffer at 65°C. The first wash was carried out for 30 min at room temperature, and the second wash was performed at 65°C for 1 h. The blots were exposed to film at –70°C, and then scanned with a model GS-670 imaging densitometer (Bio-Rad, Hercules, CA). All values are presented as ratios of relative intensity of enzyme mRNA:relative intensity of cyclophilin mRNA, which was chosen to normalize for RNA loading, because it did not change after barrier disruption.

Electron microscopy Human volunteers with RXLI were treated for 1 wk with 2% cholesterol *versus* vehicle. Cyanoacrylate strippings were obtained from cholesterol- and vehicle-treated sites as well as from untreated sites. Additional biopsies from each site were obtained 6 h after barrier abrogation in three subjects. Biopsies of either 1% cholesterol sulfate- or vehicle-treated hairless mouse skin were also obtained after twice daily applications for 5 d. All samples, including samples incubated with lipids *in vitro*, were fixed in half-strength Karnovsky's fixative overnight, washed in 0.1 M sodium cacodylate buffer, and post-fixed in 0.5% ruthenium tetroxide in 1.5% potassium ferrocyanide (Hou *et al*, 1991), followed by ethanol dehydration and embedding in an Epon-epoxy mixture. Ultrathin sections were contrasted further with lead citrate and viewed in a Zeiss 10 A electron microscope (Carl Zeiss, Thornwood, NY) operated at 60 kV. Micrographs were photographed randomly by an uninvolved observer and interpreted blindly by the authors.

Statistical analysis Statistical analysis utilized the Student's t test with a two-way analysis of programs (Stat-Works). Data were expressed as mean ± SEM with ⩽ 0.05 considered significant. When the results from the Student's test were checked further by Wilcoxon and/or ANOVA analysis, the difference between RXLI and normals remained significant.

RESULTS

RXLI patients display abnormal barrier function To assess the potential importance of cholesterol sulfate processing for normal barrier homeostasis, we first measured barrier function in patients with RXLI *versus* an age-matched group of male controls. As seen in **Fig 1**, barrier function is abnormal in RXLI *versus* normal, age-matched, and sex-matched subjects under basal conditions, with an almost 40% mean increase in TEWL levels *versus* controls (n = 15; $p < 0.001$). We next compared the kinetics of barrier recovery after similar initial insults

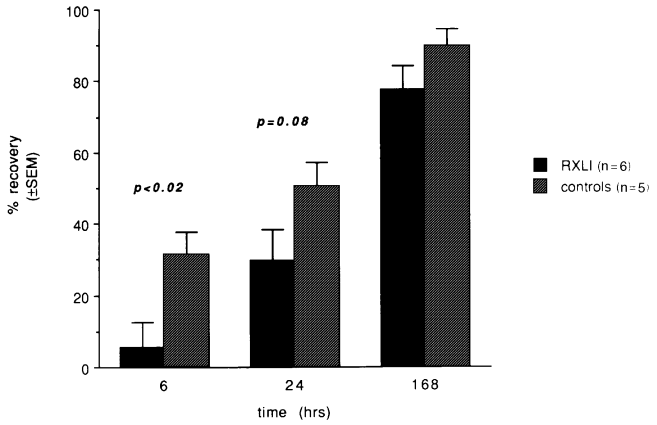

Figure 2. Barrier recovery after acute perturbations. Data are for six RXLI and five age/sex-matched controls after tape stripping of volar forearms. The differences at 6 h are significant by Student's t test.

(i.e., initial TEWL levels $\geq$ 2.0 mg per cm^2 per h). Although both the RXLI and the control group (n = 5 each) display normalization of barrier function (100% recovery) by 1 wk, divergence in recovery rates is apparent at earlier time points; i.e., 6 and 24 h (**Fig 2**; $p < 0.02$ and $p = 0.08$ for 6 and 24 h, respectively). These results show that patients with RXLI demonstrate a significant barrier abnormality under both basal and stressed conditions.

Topical cholesterol normalizes barrier recovery rates in RXLI To assess the possibility that the delay in barrier recovery in RXLI could be attributed to impaired cholesterol sulfate processing, we next measured barrier recovery in RXLI *versus* control human skin after topical cholesterol applications. For these studies, we employed a 2% concentration of cholesterol, which displays few effects in normal skin, but improves the barrier abnormality in aging skin (Mao-Qiang *et al*, 1993; Ghadially *et al*, 1996; Zettersten *et al*, 1997). As reported previously (Mao-Qiang *et al*, 1993), topical cholesterol either has no effect or slightly delays barrier recovery in normal skin (6 h, NS), and the vehicle has no effect (**Fig 3*A***). In contrast, topical cholesterol significantly accelerates barrier recovery in RXLI subjects (**Fig 3*B***; $p < 0.001$ and 0.037 at 6 and 24 h, respectively). Moreover, control sites in RXLI subjects, treated with vehicle alone, exhibit delays in recovery. Furthermore, a comparison of data from cholesterol-treated RXLI to cholesterol-treated control sites reveals no differences in the kinetics of recovery (**Fig 3*C***; NS); i.e., cholesterol completely normalizes the kinetics of barrier recovery. Together, these results show that cholesterol repletion normalizes barrier recovery in RXLI.

Barrier dysfunction in RXLI is associated with abnormalities in extracellular lamellar organization (Fig 4*A*, *B*) Although prior electron spin resonance studies revealed abnormal lipid interactions in the stratum corneum of RXLI patients, these changes were linked solely to the desquamation abnormality (Rehfeld *et al*, 1988). Hence, we next assessed extracellular lamellar membrane ultrastructure in skin biopsies from three untreated RXLI patients *versus* several historical, age-matched, and sex-matched controls, utilizing ruthenium tetroxide post-fixation. In all three subjects, we observed extensive separation into lamellar and nonlamellar domains, as well as abnormalities in the organization of the extracellular lamellae in the stratum corneum [**Fig 4*B*** (vehicle-treated); untreated RXLI (not shown) appears similar to vehicle-treated sites].

To ascertain whether these membrane abnormalities could be attributable to a deficiency of cholesterol, we next compared stratum corneum ultrastructure from cholesterol- *versus* vehicle-treated sites from cyanoacrylate tape strippings from three subjects with RXLI. Although prior studies have shown that topical cholesterol improves the desquamation abnormality (Lykkesfeldt and Hoyer, 1983), its effects on barrier function in RXLI have not been assessed. As seen in **Fig 4(*A*)**, 1 wk of twice daily cholesterol applications largely normalized extracellular membrane structure; i.e., treated sites were indistinguishable from unaffected controls, and nonlamellar domains also largely disappeared. In contrast, vehicle-treated sites in RXLI subjects remained highly abnormal (**Fig 4*B***). These results show first, that the barrier abnormality in RXLI is attributable to abnormalities in extracellular membrane structure and/or formation of separate lamellar and nonlamellar domains. Second, they show that cholesterol repletion largely normalizes both of these abnormalities.

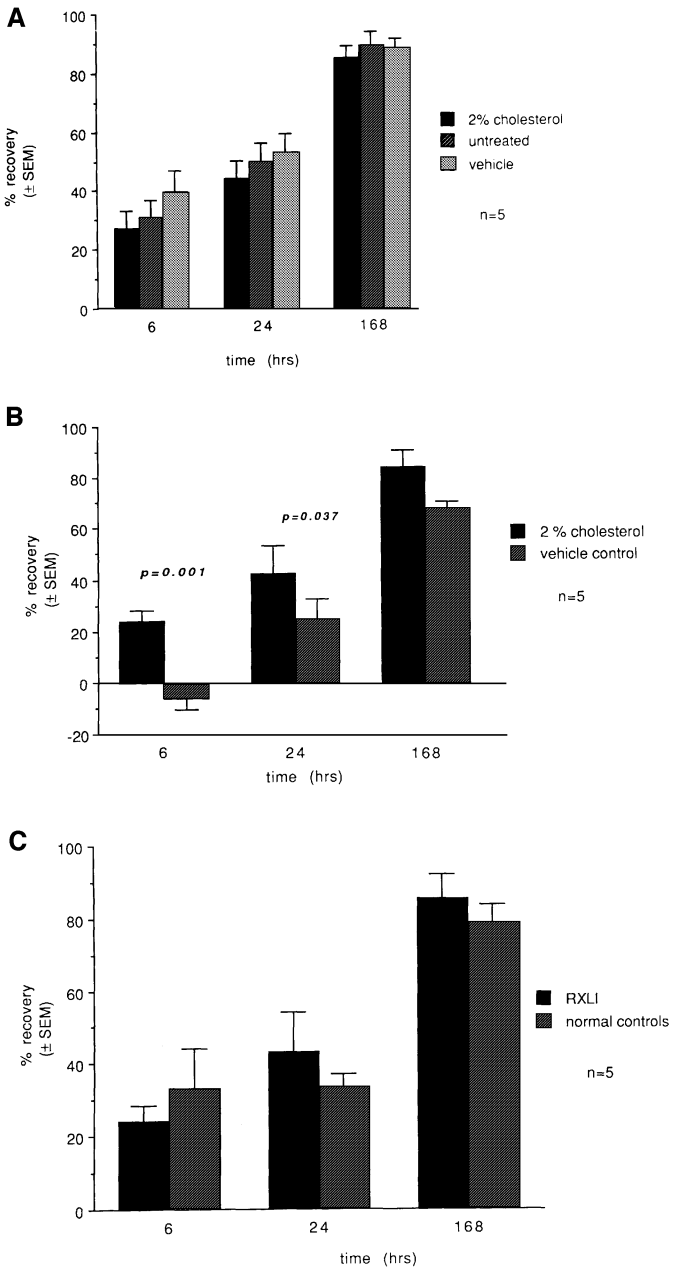

Figure 3. Barrier recovery in RXLI *versus* age/sex-matched controls after cholesterol treatment. Data shown are for (*A*) cholesterol *versus* vehicle in normal control subjects, (*B*) cholesterol *versus* vehicle treatment in RXLI subjects, and (*C*) cholesterol-treated RXLI *versus* untreated normal control subjects. n = 5 in each group. Statistical comparisons are by Student's t test.

Topical cholesterol sulfate also produces a barrier abnormality in normal skin The ability of topical cholesterol to normalize the function and ultrastructure in RXLI has several potential explanations (see below). Therefore, to ascertain further whether the barrier abnormality in RXLI is due to a failure of cholesterol sulfate-to-cholesterol processing resulting in cholesterol deficiency, or to an accumulation of excess cholesterol sulfate, we next assessed the effects of repeated topical applications of 2% cholesterol sulfate to normal

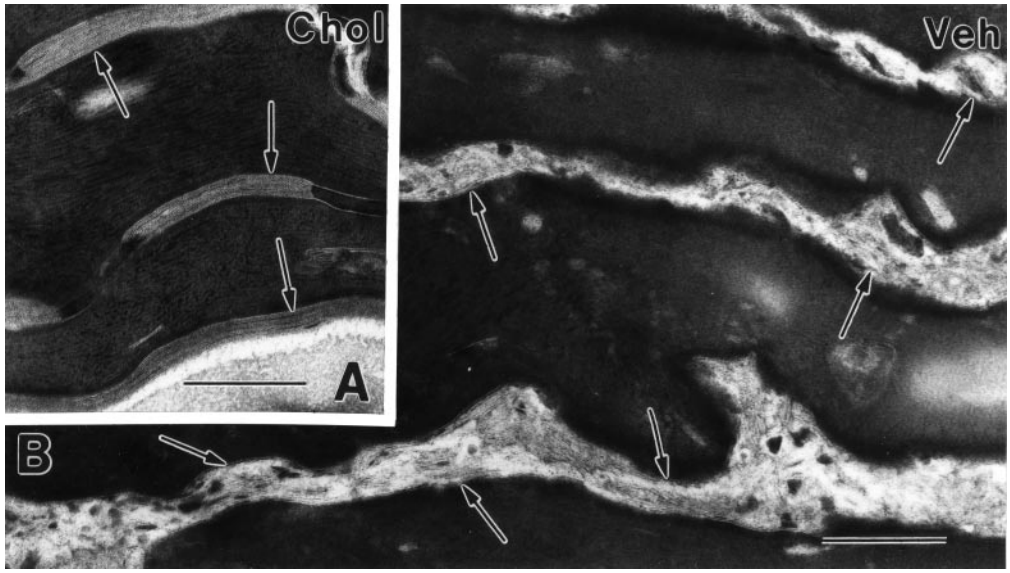

Figure 4. Ultrastructure of vehicle-*versus* cholesterol-treated RXLI. The same membrane abnormality is present in three different patients, and is not influenced by vehicle (*B*). Lamellae are fragmented and disrupted (*arrows*), with extensive, nonlamellar domains present within the extracellular spaces. In contrast to vehicle, 2% cholesterol treatment twice daily for 1 wk completely normalizes the structure of extracellular lamellae (*A*, *arrows*). (*A*, *B*) RuO_4 postfixation. *Scale bar*: 0.5 μm.

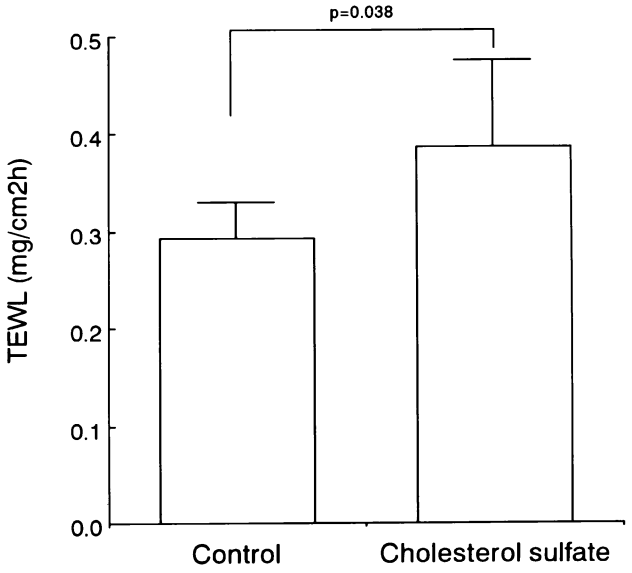

Figure 5. Repeated applications of topical cholesterol sulfate results in a barrier abnormality. Twice daily applications of 2% cholesterol sulfate *versus* vehicle. Data shown are after 5 d treatment, and significances were calculated by Student's t test. n = 6 sites each.

hairless mouse skin (for ethical reasons, we did not perform these studies in human subjects). Prior studies have shown that topical cholesterol sulfate, but not topical cholesterol, produces an ichthyosiform dermatosis in intact hairless mouse skin (Maloney *et al*, 1984). As in these prior studies, twice daily applications of 2% cholesterol sulfate *versus* vehicle alone for 5 d results in a mild ichthyosiform eruption, characterized by histologic hyperkeratosis, but no significant epidermal hyperplasia or inflammation (not shown). As seen in **Fig 5**, cholesterol sulfate-treated animals also display a moderate, but significant increase in TEWL levels ($p < 0.05$). Co-applications of cholesterol with cholesterol sulfate prevent development of the ichthyosiform abnormality (as in Maloney *et al*, 1984), as well as the permeability barrier alterations (not shown). Finally, RuO_4 post-fixed preparations from CSO_4-treated, but not vehicle-treated samples display both extensive phase separation and disruption of lamellar membrane structure (**Fig 6*A*** *versus* ***B***), as in RXLI (cf., **Fig 4*B***). These studies show that applications of excessive cholesterol sulfate to normal (steroid sulfatase replete) skin produce a barrier defect that is comparable functionally and structurally to RXLI.

Excess cholesterol sulfate produces membrane abnormalities in normal stratum corneum To distinguish further whether the barrier abnormality in RXLI is due to cholesterol sulfate accumulation, or metabolic consequences of excess cholesterol sulfate [e.g., downregulation of cholesterol synthesis (Williams *et al*, 1985, 1987)], we next applied cholesterol sulfate topically to isolated tape strippings of murine stratum corneum. As seen in **Fig 7(*A*)**, cholesterol sulfate produces direct effects on the SC extracellular membrane organization. Both extensive nonlamellar domain formation and disruption of lamellar membrane structure are evident. In contrast, coapplication of cholesterol with cholesterol sulfate largely prevents development of the abnormality, producing minimal domain separation alone (**Fig 7*B***), and neither cholesterol alone nor the vehicle alter membrane structure in most areas (**Fig 7*C***; cholesterol alone not shown). These results show that cholesterol sulfate produces both direct effects on stratum corneum membrane organization and lamellar/nonlamellar domain separation.

Acute changes in barrier function do not regulate steroid sulfatase Whereas the above studies suggest that cholesterol sulfate processing is important for normal barrier homeostasis, we next asked whether barrier function regulates steroid sulfatase, the enzyme which catalyzes this reaction. As seen in **Fig 8**, mRNA levels for steroid sulfatase do not change 4 h after barrier disruption. Moreover, enzyme activity does not change significantly at various time points (2, 4, 12 h) after tape stripping in comparison with untreated and 0 time controls (= immediately after stripping; data not shown). Together, these results indicate that steroid sulfatase levels are not regulated by acute alterations in barrier status.

DISCUSSION

Prior studies have addressed multiple, potential functions of cholesterol sulfate in squamous epithelia (**Fig 9**), including roles in: (i) differentiation; (ii) desquamation; and (iii) cholesterol metabolism. A role for this molecule in differentiation is supported by (i) the high concentration of cholesterol sulfate in the outer nucleated layers of epidermis (Elias *et al*, 1984); (ii) the observation that cholesterol sulfate content in mucosal epithelia normally is low, but increases dramatically with hypovitaminosis A-induced squamous metaplasia (Rearick and Jetten, 1986; Rearick *et al*, 1987); (iii) stimulation of the η isoform of epidermal protein kinase (PKC) by cholesterol sulfate (Denning *et al*, 1995); (iv) evidence for transactivation of the involucrin gene in keratinocytes by PKCη following exposure to cholesterol sulfate (Ikuta *et al*, 1994); and (v) finally, the hyperorthokeratosis in RXLI could be interpreted as an imbalance in proliferation *versus* differentiation (the former is normal, but the latter is increased) (Williams, 1991). These results suggest that cholesterol sulfate plays an important role in

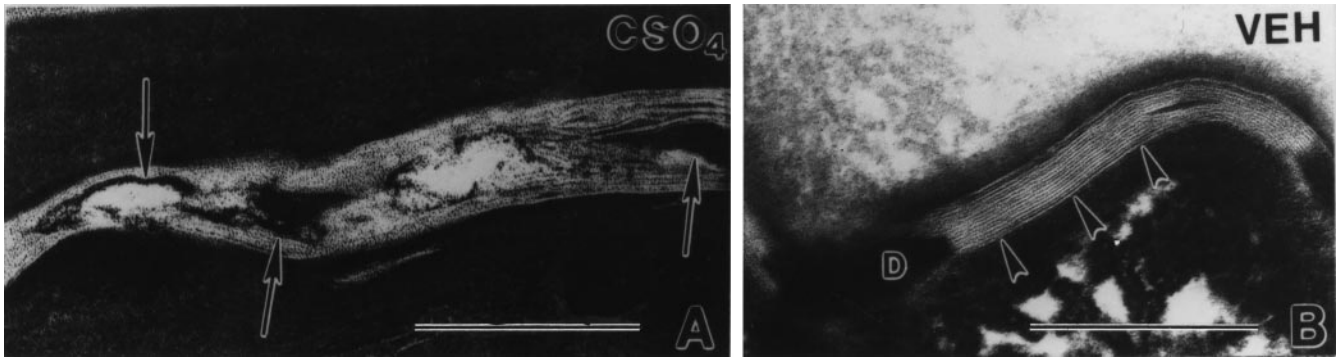

Figure 6. Ultrastructure of (*A*) cholesterol sulfate- *versus* (*B*) vehicle-treated normal mouse skin. Applications were made twice daily for 5 d. *Arrows* depict both membrane fragmentation and phase separation in cholesterol sulfate-treated sites, but only minimal nonlamellar domain formation with vehicle alone. RuO_4 postfixation. *Scale bar*: 0.5 μm.

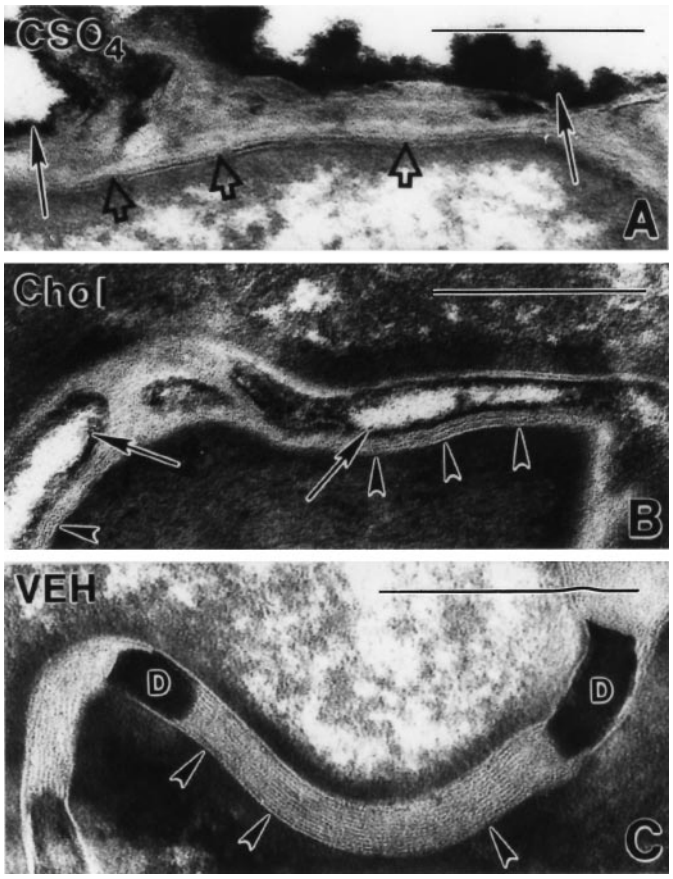

Figure 7. Cholesterol sulfate (CSO_4) produces membrane abnormalities and nonlamellar domain formation in isolated stratum corneum. Murine SC tape strippings were incubated with either 2% CSO_4 alone (*A*); 2% cholesterol (Chol) plus 2% CSO_4 (*B*); cholesterol alone (not shown); or vehicle (Veh) alone (*C*) for 2 h. (*A*, *B*) *Open arrows* depict membrane structural abnormalities; *single arrows* depict nonlamellar domains; *arrowheads* depict normal extracellular lamellar membranes. C, vehicle alone; D, desmosomes. (A–C) RuO_4 postfixation. *Scale bar*: 0.5 μm.

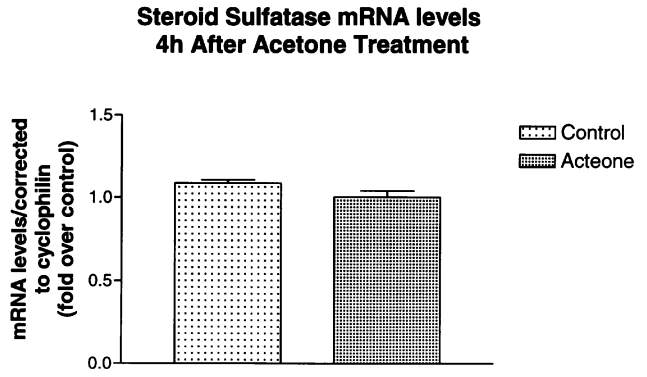

Figure 8. Steroid sulfatase mRNA levels do not change after barrier disruption. Data shown are for immediately before and 4 h after acetone treatment. No significant differences were observed at these or other time points (2, 12 h data not shown).

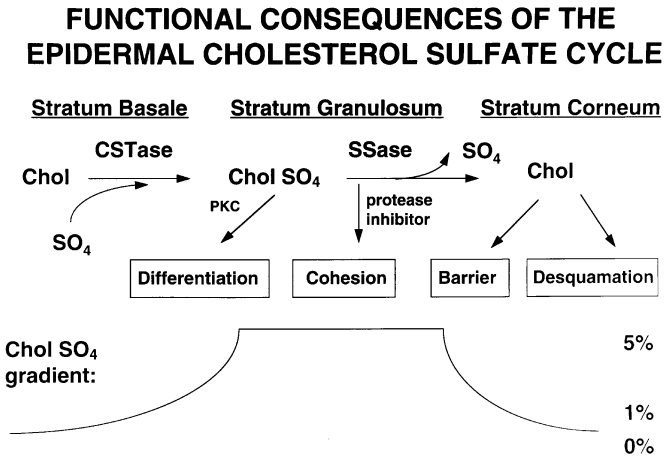

Figure 9. Cholesterol sulfate is a plurifunctional molecule. Diagram illustrates several potential effects of this molecule on epidermal function in relation to the concentration gradient of cholesterol sulfate within the epidermis.

epidermal differentiation, and that the signaling pathway involves PKCη.

The role of cholesterol sulfate in the modulation of desquamation is supported by (i) the development of ichthyosis in patients with RXLI, who generate a 10-fold excess of cholesterol sulfate in the face of steroid sulfatase deficiency (Williams and Elias, 1981), and (ii) the development of excess scale in animals treated with topical cholesterol sulfate (Maloney *et al*, 1984), and as shown again in this study. The following four molecular mechanisms have been proposed to explain these ichthyosiform changes: (i) The inability of cholesterol sulfate to form eutectic mixtures with other SC extracellular lipids, which would result in phase separation (Rehfeld *et al*, 1986). This mechanism is supported by prior electron spin resonance work (Rehfeld *et al*, 1988), and by our observations here on RuO_4 post-fixed material, which also demonstrate this phenomenon morphologically. (ii) Cationic cross-linking of the sulfate moiety with Ca^{++} ions is possible in the SC interstices, although evidence to support this hypothesis has not been forthcoming (Williams, 1991). (iii) Cholesterol sulfate inhibits extracellular proteases that participate in desmosomal and stratum corneum dissolution (Sato *et al*, 1998), and desmosomes are retained in excess at all levels of the SC in RXLI (Mesquita-Guimarães, 1981).

(iv) Finally, altered membrane dynamics could occur from the reduced cholesterol, rather than from the excess cholesterol sulfate levels in RXLI (Williams and Elias, 1981; Williams, 1991).

This last finding, i.e., that cholesterol levels are 50% reduced in the stratum corneum of RXLI, led to the hypothesis that cholesterol sulfate might be an endogenous, feedback regulator of cholesterol synthesis. Indeed, in both cultured human fibroblasts and keratinocytes, cholesterol sulfate inhibits cholesterol synthesis at pathophysiologically relevant concentrations, and with an efficacy approaching LDL-cholesterol and 25-OH-cholesterol (Williams *et al*, 1987), two classic feedback regulators of cholesterol synthesis. Furthermore, prior catabolism to cholesterol is not required, i.e., cholesterol sulfate itself is the regulator (Williams *et al*, 1985). Finally, cholesterol sulfate displays detergent effects that allow it to cross cell membranes (Ponec and Williams, 1986; Tempesta *et al*, 1995), followed by binding to cytosolic constituents (Ponec and Williams, 1986), again supporting its potential capacity as a feedback regulator. Yet, cholesterol sulfate does not appear to inhibit epidermal cholesterol synthesis *in vivo* (Menon *et al*, 1985).

The 50% reduction in cholesterol in RXLI has an alternate explanation, i.e., failure of cholesterol sulfate-to-cholesterol processing, an hypothesis explored here. Therefore, we examined the possibility that cholesterol sulfate processing by steroid sulfatase could provide a second source of bulk cholesterol, required for permeability barrier homeostasis. Prior studies in fetal skin, where steroid sulfatase activity peaks coincide with barrier formation (Hanley *et al*, 1997), support this view. Moreover, we showed here that RXLI patients display a permeability barrier abnormality even under basal conditions, supporting three of four prior studies (Grice and Bettley, 1967; Frost *et al*, 1968; Lavrijsen *et al*, 1993; *versus* Fartasch, in press). Furthermore, we found that the barrier abnormality becomes even more apparent when the barrier is first stressed, i.e., the kinetics of barrier recovery are abnormal over the initial 24+ hours, approaching normal rates only after 1 wk. That this defect is due to decreased steroid sulfatase within affected keratinocytes is shown in gene replacement studies, where grafted RXLI keratinocytes that are stably transfected with the steroid sulfatase gene reconstituted an epidermis with an intact barrier (Freiberg *et al*, 1997). In addition, the ultrastructure of both RXLI and cholesterol sulfate-treated normal stratum corneum, utilizing the RuO_4 post-fixation method, reveals not only the previously described desmosome abnormality (e.g., Williams *et al*, 1987; Fartasch, 1997), but also membrane structural abnormalities and extensive domain separation. These microscopic lesions would be expected to produce a barrier defect. Our results differ from those of Fartasch (1997), who described normal extracellular membrane structure in three patients with RXLI. Possible explanations for this discrepancy include either seasonal or phenotypic variations in disease severity in the two study groups. Finally, topical cholesterol corrects both the barrier and the membrane abnormalities in RXLI. Whereas these results support the hypothesis that defective cholesterol sulfate processing contributes to the barrier abnormality, they do not address directly whether the abnormality is due to excess cholesterol sulfate, inhibition of cholesterol synthesis, or a deficiency of bulk cholesterol.

Although downregulation of cholesterol synthesis seems unlikely (Menon *et al*, 1985), topical cholesterol corrects (i) the desquamation abnormality (Lykkesfeldt and Hoyer, 1983; Maloney *et al*, 1984); (ii) the membrane and extralamellar phase abnormalities in RXLI *in vivo* (these studies); and (iii) cholesterol sulfate-induced abnormalities *in vitro*. These data are consistent with a pathogenic role for excess cholesterol sulfate, because: (i) topical cholesterol sulfate alone produces a barrier abnormality in intact skin, where no processing defect exists; and (ii) *in vitro* experiments demonstrate the ability of cholesterol sulfate to produce abnormalities in extracellular organization, particularly phase separation, indicating a likely direct effect of excess cholesterol sulfate on barrier homeostasis. Thus, these studies together suggest that the principal mechanism contributing to the barrier abnormality in RXLI is accumulation of cholesterol sulfate.

These studies also demonstrate that cholesterol reverses the potentially pathogenic effects of excess cholesterol sulfate on stratum corneum membrane structure and barrier function. Prior studies demonstrated benefits of topical cholesterol both to treat excess scale in RXLI (cited in Lykkesfeldt and Hoyer, 1983), and to prevent the emergence of abnormal desquamation with topical cholesterol sulfate applications to normal murine skin (Maloney *et al*, 1984). The mechanism(s) of cholesterol's benefit, however, is not clear. When applied alone to a damaged barrier in young skin, cholesterol is either detrimental or exerts no effect (Mao-Qiang *et al*, 1993). In contrast, topical cholesterol (Ghadially *et al*, 1996) and cholesterol-enriched, physiologic lipid mixtures (Zettersten *et al*, 1997) accelerate barrier recovery in chronologically aged skin. In the case of aging, cholesterol appears to overcome a profound decrement in cholesterol metabolism (Ghadially *et al*, 1996). In these studies, one can hypothesize two potential mechanisms: First, topical cholesterol could overcome inhibition of endogenous cholesterol synthesis by excess cholesterol sulfate, a mechanism that is operative *in vitro* (Williams *et al*, 1985, 1987), but not demonstrated in preliminary *in vivo* studies (Menon *et al*, 1985). This mechanism, however, cannot explain the ability of cholesterol to neutralize the negative effects of cholesterol sulfate on isolated, normal stratum corneum, as shown here. A second mechanism might relate to an ability of cholesterol to either bind cholesterol sulfate, analogous to a chelating agent or exchange resin, or to normalize the eutectic properties of SC extracellular lipids (Rehfeld *et al*, 1986). Further studies will be needed to evaluate these and other potential mechanisms for the ameliorating effects of topical cholesterol.

Finally, despite the apparent importance for cholesterol sulfate processing by steroid sulfatase, we could not demonstrate that this enzyme is regulated in relation to barrier abrogations. Neither enzyme activity nor mRNA levels are upregulated after acute disruption. It is likely therefore that the levels of steroid sulfatase that are present under basal conditions suffice for ongoing, or even increased demands for cholesterol sulfate processing. This interpretation parallels our work with other stratum corneum extracellular lipid processing enzymes, where an excess of enzyme seems to be present, e.g., β-glucocerebrosidase (≈20-fold excess is present basally) (Holleran *et al*, 1993, 1994).

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