Epidermal lipid metabolism of cultured skin substitutes during healing of full-thickness wounds in athymic mice

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Cultured epidermal keratinocytes provide an abundant supply of biologic material for wound treatment. Because restoration of barrier function is a definitive criterion for efficacy of wound closure and depends on the lipids present in the epidermis, we analyzed lipid composition of the epidermis in cultured skin substitutes in vitro and after grafting to athymic mice. The cultured skin substitutes were prepared from human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan substrates. After 14 days of incubation, cultured skin substitutes were grafted orthotopically onto full-thickness wounds in athymic mice. Samples for lipid analysis were collected after 14 and 34 days of in vitro incubation, and 3 weeks and 4 months after grafting. Both in vitro samples show disproportions in epidermal lipid profile as compared with the native human epidermis, i.e., a low amount of phospholipids (indicating imbalance in proliferation and differentiation); a large excess of triglycerides (storage lipids); and low levels of free fatty acids, glucosphingolipids, cholesterol sulfate, and ceramides—suggesting abnormal composition of stratum corneum barrier lipids. Fatty acid analysis of cultured skin substitutes in vitro revealed insufficient uptake of linoleic acid, which resulted in increased synthesis of and substitution with monounsaturated fatty acids, mainly oleic acid. These abnormalities were partially corrected by 3 weeks after grafting; and 4 months after grafting, all epidermal lipids, with some minor exceptions, were synthesized in proportions very similar to human epidermis. Results of this study show that grafting of cultured skin substitutes to a physiologic host permits the recovery of lipid in proportion to that required for barrier formation in normal human epidermis. **(WOUND REP REG 1997;5:329-38)**

Cultured epidermal keratinocytes provide an abundant supply of biologic material for treatment of skin wounds. In addition, they serve as alternatives to animals for cutaneous toxicity testing and as models for studying skin biology and pathology. Although in recent years various human skin substitutes have been developed showing histologic resemblance to native epidermis, these cultured substitutes exhibit a number of abnormalities compared to native skin. In none of the cultured substitutes has epidermal homeostasis been achieved. Maturation of the epidermal analog proceeds too rap-

CER	Ceramides
CSS	Cultured skin substitute
EFA	Essential fatty acids
FAME	Fatty acid methyl esters
FFA	Free fatty acids
GAG	Glycosaminoglycans
GLC	Gas liquid chromatography
HPTLC	High-performance thin layer chromatography
MSL	Mouse-specific lipids
PL	Phospholipids
SC	Stratum corneum
TG	Triglycerides

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idly, resulting in a decease in the number of living cell layers, while the thickness of the stratum corneum (SC) increases as a function of incubation time.[1] This imbalance between proliferation and differentiation is further exemplified by the premature expression of a number of differentiation markers (involucrin, transglutaminase, keratins 6 and 16) similar to hyperproliferative epidermis.[2] Barrier function of all cultured human skin analogues described until now is significantly deficient,[3-6] organization and desquamation of cornified layers is impaired,[7] and epidermal lipids are synthesized and metabolized at rates different from those in vivo.[8] The organization of intercorneocyte lipids in cultured epidermis also deviates in a number of features from the native tissue as observed in ultrastructural[9,10] and x-ray diffraction[11] studies.

Previous studies have described generation of cultured skin substitutes (CSS) from collagen-glycosaminoglycan (GAG) sponges populated sequentially with human dermal fibroblasts and human epidermal keratinocytes.[12,13] Supplementation of serum-free culture media with free fatty acids (FFA) and vitamin E resulted in modulation of the synthesis of barrier lipids.[14] Clinical studies have shown that CSS serve successfully for treatment of burn wounds[15,16] and chronic wounds.[17] Grafting of human epidermis[18] or in vitro reconstructed epidermis[19] onto athymic mice has been used as a valid in vivo model for studying processes related to epidermal proliferation and differentiation. It has been shown that cultured keratinocytes retain their capability to generate a well-differentiated epidermis, exhibiting improved barrier properties and lipid composition.[19] Recently, measurements of surface electric capacitance showed development of epidermal barrier as a function of hydration during healing wounds treated with cultured skin substitutes.[20]

In this study, changes in structure and overall epidermal lipid metabolism in cultured skin substitutes after grafting to athymic mouse and in vitro were compared. Data collected after short-term (3 weeks) and long-term (4 months) postgrafting intervals show improvement of epidermal homeostasis and normalization of epidermal lipid and fatty acid profile. Understanding those metabolic processes in the epidermis during healing wounds is necessary for optimal modulation of culture media and the physiologic environment, which can allow preparation in vitro of a more physiologic, stable, and efficacious skin substitute.

MATERIALS AND METHODS

CSS were prepared as described in previous studies.[13,14,16] Secondary cultures of human keratinocytes (isolated from surgical discard of split-thickness skin from a pediatric donor) were used to prepare CSS by inoculation onto collagen-GAG substrates populated with human dermal fibroblasts from the same skin donor. Beginning with inoculation of cultured keratinocytes on day 0 of incubation, cultured cell-biopolymer composites were incubated 2 days, submerged in modified MCDB 153 medium (Dr. Boyce's laboratory; reference 14) containing 0.2 mmol/L calcium, 1 ng/ml epidermal growth factor (Gibco, Grand Island, N.Y.), and 0.5% bovine pituitary extract (Upstate Biologicals, Lake Placid, N.Y.) (vol/vol). On culture day 2, medium was changed to replace bovine pituitary extract with lipid supplement plus carnitine, increased serine,[14] and calcium 0.5 mmol/L. On culture day 3, CSS were lifted to the air-liquid interface, epidermal growth factor was decreased to 1 ng/ml, and calcium concentration increased to 1.0 mmol/L. On culture day 4, epidermal growth factor was removed, calcium concentration was increased to 1.5 mmol/L, and CSS were incubated another 11 or 31 days in these conditions with daily medium changes.

Grafting to athymic mice

CSS were grafted to athymic mice at day 14 of incubation, as described previously.[13,20] After grafting, the entire wound area was covered with a semipermeable adhesive film (OpSite; Smith & Nephew United, Largo, Fla.) to produce a liquid-tight compartment over the wound, into which 1.5 ml of modified keratinocyte growth medium containing antimicrobials was injected immediately after surgery. Constant conditions for irrigation media included 1.5 mmol/L calcium and 0.5 μg/ml of hydrocortisone. Dressed grafts were then covered with a self-adherent bandage to protect treated sites from mechanical disturbance. Dressings covering treated sites were injected with 1.0 ml/day of irrigants described previously for day 13 after grafting. On day 14 after grafting, dressings and stent sutures were removed from all animals. Mice were rebandaged on days 14 and 21 after grafting. On day 28, the dressings were removed from all animals, and tissue samples were collected at 3 weeks and 4 months after grafting. Presence of human keratinocytes was confirmed in all grafts by staining for HLA-ABC antigens. Animal studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Histology

Full-thickness skin samples were fixed in 2% glutaraldehyde/2% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) and embedded in glycol-methacrylate resin. Four μm- thick sections were mounted on glass slides and stained with 0.1% toluidine blue. Biopsies were examined and photographed on a Nikon FXA photo microscope.

Lipid extraction and separation

Epidermis was separated from the collagen-GAG substrate or the dermis by heating for 1 to 2 minutes at 60° C, washed in Hepes-buffered saline solution, and collected in chloroform:methanol (1:2, vol/vol). For control samples, human epidermis obtained from healthy young

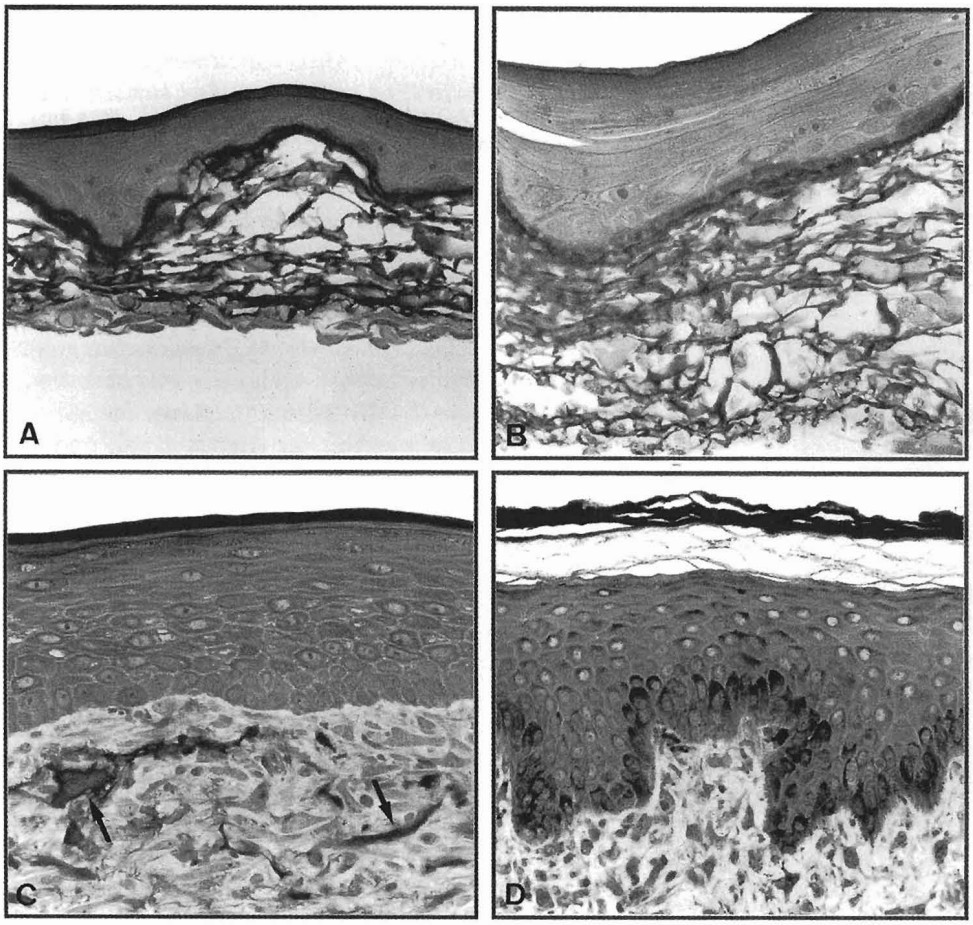

Figure 1 Histology of CSS before and after grafting to athymic mice. **A**, Day 13 of incubation in vitro. Epidermal analog consists of stratified keratinocytes with a cornified surface attached to a reticulated collagen-GAG substrate. **B**, Day 34 of incubation in vitro. Epidermal analog remains stratified and cornified, but nucleated cells accumulate lipid as cytoplasmic vacuoles and begin to degenerate. **C**, Three weeks after grafting (simultaneous with day 34 in vitro) healing epidermis has typical basal keratinocyte layer, thickened spinous layer, and SC forms under hyperkeratotic plaque. Connective tissue repair proceeds as collagen-GAG implant degrades in wound (*arrows*). **D**, Four months after grafting, human epidermis interdigitates with connective tissue, epidermal strata are normal, SC is fully formed and desquamating, and pigmentation of skin is complete. Original magnification ×208.

donors undergoing elective surgery. The extraction and separation was performed as previously described by Ponec and Weerheim.[21] Total lipids were extracted using a series of chloroform/methanol mixtures according to the method of Bligh and Dyer,[22] with the addition of 0.25 mol/L KCl to extract polar lipids. Lipids were then stored in chloroform:methanol (2:1, vol/vol) at –20° C under nitrogen until used. The extracted lipids were separated by one-dimensional, high-performance, thin-layer chromatography (HPTLC) on 20 × 10-cm glass plates coated with silica gel (Kieselgel 60, Merck, Darmstadt, Germany) using the "total and ceramide development systems," as described in detail previously. Serial dilutions of appropriate standard lipids were used for quantification. The quantification was performed after staining (copper acetate and copper sulfate in phosphoric acid) and charring, using a photodensitometer (Shimadzu CS-9000) with automatic peak integration (Shimadzu FDU-3). Three parallel samples were analyzed; data are presented as means ± SD.

Preparation and analysis of fatty acid methyl esters

One hundred μg of the total lipid extract was dissolved in 100 μl of toluene and transmethylated in 1 ml boron trichloride/methanol (10%) using microwave irradiation, which was carried out at the lowest power setting (85 W) for 4 hours. Fatty acid methyl esters (FAME) were dissolved in hexane and purified on a silica-gel column. FAME fractions were separated and analyzed on a Vega GC 6000 gas chromatograph (Carlo Erba Instruments, Italy) using a capillary column-CP Wax 52 (Chrompack, The Netherlands). An initial temperature of 80° C was increased

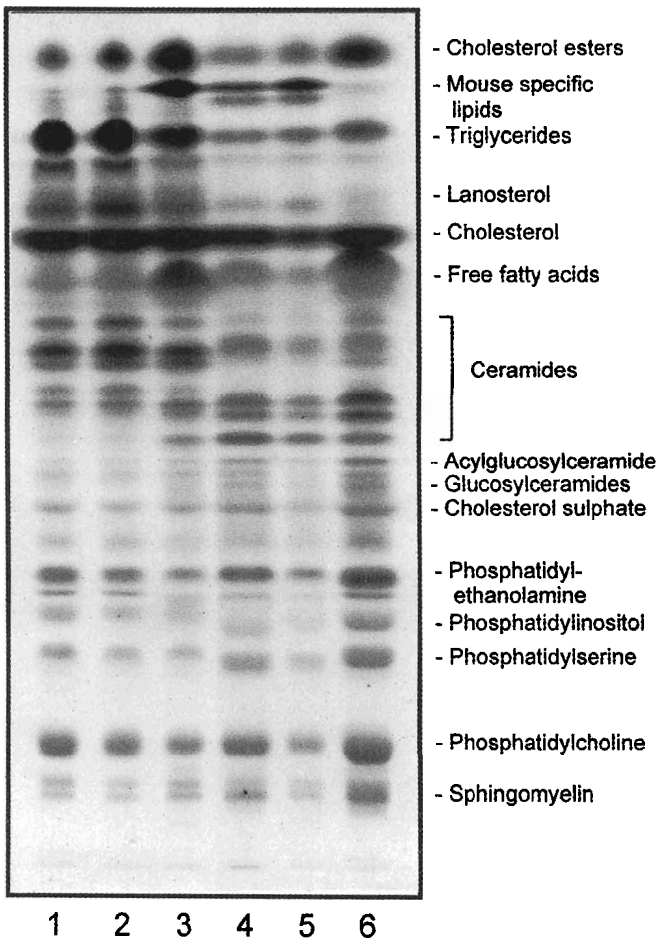

Figure 2 HPTLC separation of epidermal lipids extracted from CSS. cultured for 14 and 34 days in vitro (**1** and **2**), 3 weeks (**3**), 4 and 6 months after grafting to athymic mouse (**4** and **5**), and from native human epidermis (**6**).

to 160° C with a rate of 40° C/min, followed by a 2° C/min increase to 250° C, which was maintained until all peaks had eluted. The peaks were identified by comparison with FAME standards (Sigma Chemical Co., St. Louis, Mo.). Integration of peak areas and calculation of relative percentage were performed by a Baseline 810 (Carlo Erba Instruments, Italy) integrator. Heptadecanoic acid was used as the internal standard. Two parallel samples were used, data are presented as means ± range.

To determine fatty acid composition of individual lipid fractions (phospholipid [PL], triglycerides [TG], free fatty acids [FFA], ceramides [CER]), fractions of these lipids were scraped off the HPTLC plate after separation, extracted, transmethylated, and analyzed as described previously.

RESULTS

Photomicrographs of CSS from samples prepared for lipid analysis are shown in Figure 1. Morphologic organization of skin substitutes after 14 days of culture was analogous to human skin. Nucleated keratinocytes were attached to collagen-GAG substrates, and the epidermal analog was stratified with a cornified surface (Figure 1,*A*). CSS were grafted at this time, and parallel CSS were maintained until 34 days of culture, as shown in Figure 1,*B*. The epidermal analog remained stratified and cornified, but fewer nucleated cells were observed, and lipid vacuoles were present in keratinocytes attached to the substrate. These vacuoles most probably collect TG that accumulate during several weeks of culture as described below. Simultaneously, at 3 weeks after grafting of the CSS, an hypertrophic epidermis was observed, as shown in Figure 1,*C*. At this time point, the spinous layer of epidermis was thicker than normal, and the SC was formed but not yet mature. An hyperkeratotic plaque covered the healing skin and detached to expose the epidermal surface. Also at 3 weeks after grafting, the collagen-GAG matrix degraded and permitted ingrowth of fibrovascular tissue from the wound bed. By 4 months after grafting (Figure 1,*D*), the human epidermis stabilized and exhibited anatomic features of normal skin including: an SC with a uniform thickness and typical pattern of desquamation; interdigitations of epithelial and connective tissues that resemble rete ridges; and active synthesis and distribution of pigment by melanocytes. Pigmentation results from "passenger" melanocytes present in the keratinocyte cultures used to prepare CSS, and was expressed by focal colonies of melanocytes.[13] This skin anatomy can remain stable for the life of the murine host.

Composition of epidermal lipids

Total epidermal lipids extracted from CSS in vitro, healed human skin after grafting, and normal human epidermis were separated by HPTLC (Figure 2). Composition of all epidermal lipids is summarized in Table 1. In addition to the common epidermal lipids, skin samples from epidermal grafts contain a lipid fraction with a mobility between TG and cholesterol esters, which has been previously identified as mouse-specific lipid (MSL).[18] The MSL fraction represents 31.1 ± 4.7 wt.% of all epidermal lipids in samples collected 3 weeks after grafting, and 12.3 ± 4.7 wt.% at 4 months after grafting (Table 1).

Phospholipids

Low amounts of phospholipids (PLs) present in CSS in vitro remained unchanged 3 weeks after grafting (12.3%), but increased by 4 months after grafting up to 40% (Table 1). Because the relative PL content reflects the ratio between the number of viable and cornified cell layers, a low content in vitro and at early time points after grafting is suggestive of a relative increase in the number of cornified cells. This is also in agreement with observed hyperkeratotic plaques at 3 weeks after grafting (Figure 1,*C*). The PL fractions, consisting of sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine, are present in similar proportions in all

Table 1. Composition of epidermal lipids extracted from CSS before and after grafting to athymic mice and from native human epidermis

Epidermal lipids	In vitro		After grafting		Human epidermis
	14 days	34 days	3 wk	4 mo	
PL	18 ± 5.0	12.5 ± 2.4	12.3 ± 1.5	39.3 ± 7.3	50.1 ± 4.5
MSL	None	None	31.2 ± 7.1	12.3 ± 4.7	None
Other lipids	81.7 ± 6.0	87.5 ± 2.4	56.5 ± 2.1	48.4 ± 2.3	49.9 ± 4.6
CSO_4	2.8 ± 0.4	2.6 ± 0.6	4.9 ± 1.6	10.2 ± 1.6	9.1 ± 0.8
GSL	0.6 ± 0.1	0.5 ± 0.1	1.4 ± 0.4	3.7 ± 0.6	4.2 ± 2.3
AGC	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.1	1.7 ± 0.0	1.7 ± 0.5
CER	16.3 ± 2.0	15.2 ± 3.4	18.0 ± 1.6	22.5 ± 1.7	19.9 ± 0.5
FFA	3.7 ± 0.5	4.6 ± 0.6	14.1 ± 1.5	15.6 ± 0.9	20.1 ± 2.0
CH	25.7 ± 2.4	27.0 ± 2.8	25.5 ± 3.2	26.0 ± 3.1	24.8 ± 1.7
TG	46.4 ± 6.6	44.3 ± 6.3	16.2 ± 1.4	8.6 ± 2.3	7.0 ± 0.9
CE	3.3 ± 0.9	3.9 ± 0.9	19.3 ± 1.9	10.3 ± 1.0	13.8 ± 1.4

Data are presented as percentage of total epidermal lipids, means ± SD, $n = 3$. *AGC*, Acylglucosylceramides; *CH*, cholesterol; *CE*, cholesterol esters; *CSO_4*, cholesterol sulfate; *GSL*, glucosphingolipids.

Table 2. PL profiles in CSS before and after grafting to athymic mice and in native human epidermis

PL	In vitro		After grafting		Human epidermis
	14 days	34 days	3 wk	4 mo	
Sphingomyelin	15.0 ± 0.6	15.3 ± 1.5	26.1 ± 1.5	21.7 ± 2.0	23.0 ± 1.6
Phosphatidylcholine	35.8 ± 0.7	37.6 ± 1.9	38.3 ± 0.8	32.1 ± 0.5	31.2 ± 0.5
Phosphatidylserine	15.0 ± 1.0	13.6 ± 0.7	10.3 ± 0.4	14.9 ± 0.5	15.3 ± 0.5
Phosphatidylinositol	11.0 ± 0.6	9.8 ± 1.0	7.8 ± 0.9	8.5 ± 0.7	9.3 ± 0.4
Phosphatidyl-ethanolamine	23.1 ± 0.4	23 ± 8 1.1	17.6 ± 0.6	22.8 ± 0.6	21.2 ± 1.0

Data are presented as wt.% of PL content, means ± SD, $n = 3$.

analyzed samples (Table 2). CSS incubated in vitro for both 14 and 34 days contains a lower amount of sphingomyelin, which is compensated for by a relative increase in phosphatidylcholine content.

"Other" lipids

Comparison of epidermal lipid profiles of CSS incubated in culture for both 14 and 34 days to native human epidermis shows deficiencies in the content of free fatty acids, acylglucosylceramide, cholesterol sulfate, and cholesterol ester synthesis. TG are the major lipid group present. Also, a small amount of lanosterol was detected in the in vitro samples, suggesting abnormalities in cholesterol synthesis. The lipid profile normalizes partially at 3 weeks after grafting, although the amount of TG is still about two times higher than found in the native epidermis, whereas the FFA are lower than normal. Samples collected 4 months after grafting reveal similar values as native epidermis, although lower fatty acid content was still detected (Table 1).

The total CER content in CSS in vitro (16.3 wt.% and 15.2 wt.% of neutral epidermal lipids) is only slightly lower than the value 19.9 ± 0.4 wt.% in the native epidermis. However, the chromatographic separation clearly shows marked differences in the CER (Table 3). With our development system, the CER were separated into seven separate fractions according to their mobility on HPTLC silica plates, as similarly described by Wertz and Downing[23] and Robson et al.[24] In the native epidermis, CER 2, 3, and 5 are each present in about 20 wt.%, CER 7 about 15.5 ± 2.3 wt.%, and CER 1, 4, and 6 represent less than 10 wt.%. A different CER profile was obtained for CSS in vitro. Analysis of CSS incubated in culture for 14 or 34 days shows absence of CER 6 and 7, whereas the level of CER 2 exceeded 50 wt.%. Three weeks after grafting, CER 6 and 7 are present below the levels of normal skin, but they normalize by 4 months after grafting. CER 2 remains still slightly elevated even at 4 months after grafting.

Fatty acid composition of epidermal lipids

In Table 4, the fatty acid compositions of CSS after 14 and 34 days in culture, CSS 3 weeks and 4 months after grafting, and native human epidermis are compared. The data are expressed as wt.% of the total fatty acid in the sample. Medium long chain fatty acids, mainly unsatur-

Table 3. CER profiles in CSS before and after grafting to athymic mice and in native human epidermis

	In vitro		After grafting		Human
CER	14 days	34 days	3 wk	4 mo	epidermis
1	13.4 ± 0.8	12.7 ± 0.6	11.1 ± 1.0	9.0 ± 0.6	9.7 ± 1.5
2	52.5 ± 1.2	53.5 ± 0.9	40.6 ± 3.2	29.1 ± 2.8	20.8 ± 2.2
3	16.4 ± 0.3	16.5 ± 0.8	11.2 ± 0.5	14.0 ± 2.4	20.2 ± 3.2
4	9.2 ± 1.0	8.1 ± 0.9	10.3 ± 0.4	6.5 ± 0.3	7.1 ± 1.1
5	7.1 ± 0.4	6.1 ± 0.0	12.5 ± 1.6	20.9 ± 2.0	19.8 ± 1.2
6	0	0	4.3 ± 0.1	7.0 ± 0.3	6.7 ± 2.4
7	0	0	9.9 ± 1.7	17.1 ± 0.7	15.5 ± 2.3

Data are presented as wt.% of CER content, mean ± SD, $n = 3$.

Table 4. Composition of total epidermal fatty acids in CSS before and after grafting to athymic mice and in native human epidermis

Epidermal	In vitro		After grafting		Human
fatty acids	14 days	34 days	3 wk	4 mo	epidermis
16:0	9.2 ± 0.6	9.3 ± 0.2	9.4 ± 0.1	11.2 ± 0.5	10.4 ± 0.7
16:1	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	1.9 ± 0.2	1.8 ± 0.8
18:0	9.7 ± 0.9	9.6 ± 0.2	10.0 ± 1.0	10.9 ± 0.8	10.8 ± 0.1
18:1ω9	28.4 ± 1.9	29.0 ± 0.8	12.9 ± 1.0	14.8 ± 1.5	15.5 ± 0.7
18:1ω7	10.3 ± 0.3	9.2 ± 0.1	2.5 ± 0.4	1.9 ± 0.0	1.6 ± 0.5
18:2	1.9 ± 0.3	8.7 ± 0.1	6.4 ± 0.4	12.3 ± 0.6	24.9 ± 0.3
18:3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:0	1.6 ± 0.3	1.9 ± 0.1	6.2 ± 1.8	4.3 ± 0.1	1.4 ± 0.1
20:1	3.0 ± 0.9	3.0 ± 0.2	5.0 ± 0.1	3.2 ± 1.1	0.4 ± 0.1
20:2	0.4 ± 0.3	0.1 ± 0.1	1.1 ± 0.4	0.1 ± 0.1	0.0 ± 0.0
20:3	2.1 ± 0.4	1.5 ± 0.6	1.1 ± 0.2	0.5 ± 0.3	1.2 ± 0.1
20:4	4.8 ± 2.6	4.1 ± 0.4	3.9 ± 0.2	4.1 ± 0.2	4.8 ± 0.3
22:0	2.4 ± 0.3	2.8 ± 0.3	7.6 ± 2.2	4.5 ± 0.6	2.3 ± 0.0
22:1	1.2 ± 0.2	1.3 ± 0.1	8.2 ± 0.5	3.3 ± 0.2	0.2 ± 0.1
22:2	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
22:4	1.3 ± 0.4	1.0 ± 0.0	1.7 ± 0.9	0.3 ± 0.2	0.0 ± 0.0
24:0	10.2 ± 1.3	11.8 ± 0.7	11.2 ± 2.5	8.2 ± 2.2	9.6 ± 0.8
24:1	4.7 ± 0.3	4.7 ± 0.3	2.9 ± 1.8	1.1 ± 0.1	0.7 ± 0.1
25:0	0.7 ± 0.1	0.5 ± 0.3	1.5 ± 0.3	4.6 ± 0.5	2.6 ± 0.1
26:0	8.7 ± 1.4	8.5 ± 1.8	4.5 ± 0.5	5.8 ± 1.3	8.4 ± 1.1
27:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.4	0.9 ± 0.1
28:0	1.7 ± 0.1	1.7 ± 0.4	1.2 ± 0.1	3.1 ± 0.9	3.5 ± 0.8
29:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.1
30:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.5 ± 0.1

Data are presented as wt.% of total fatty acids content, mean ± range, $n = 2$.

Table 5. Ratios of 18-carbon fatty acids (C18) in CSS before and after grafting to athymic mice and in native human epidermis

C18 Ratio	In vitro		After grafting		Human
	14 days	34 days	3 wk	4 mo	epidermis
18:1/18:0	3.7 ± 0.1	3.7 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	1.4 ± 0.2
18:2/18:0	0.2 ± 0.0	0.2 ± 0.0	0.7 ± 0.1	1.1 ± 0.2	2.4 ± 0.2
18:1ω9/18:1ω7	2.7 ± 0.3	3.1 ± 0.1	4.0 ± 0.1	8.6 ± 2.1	10.2 ± 2.4

Ratios presented as means ± range, $n = 2$.

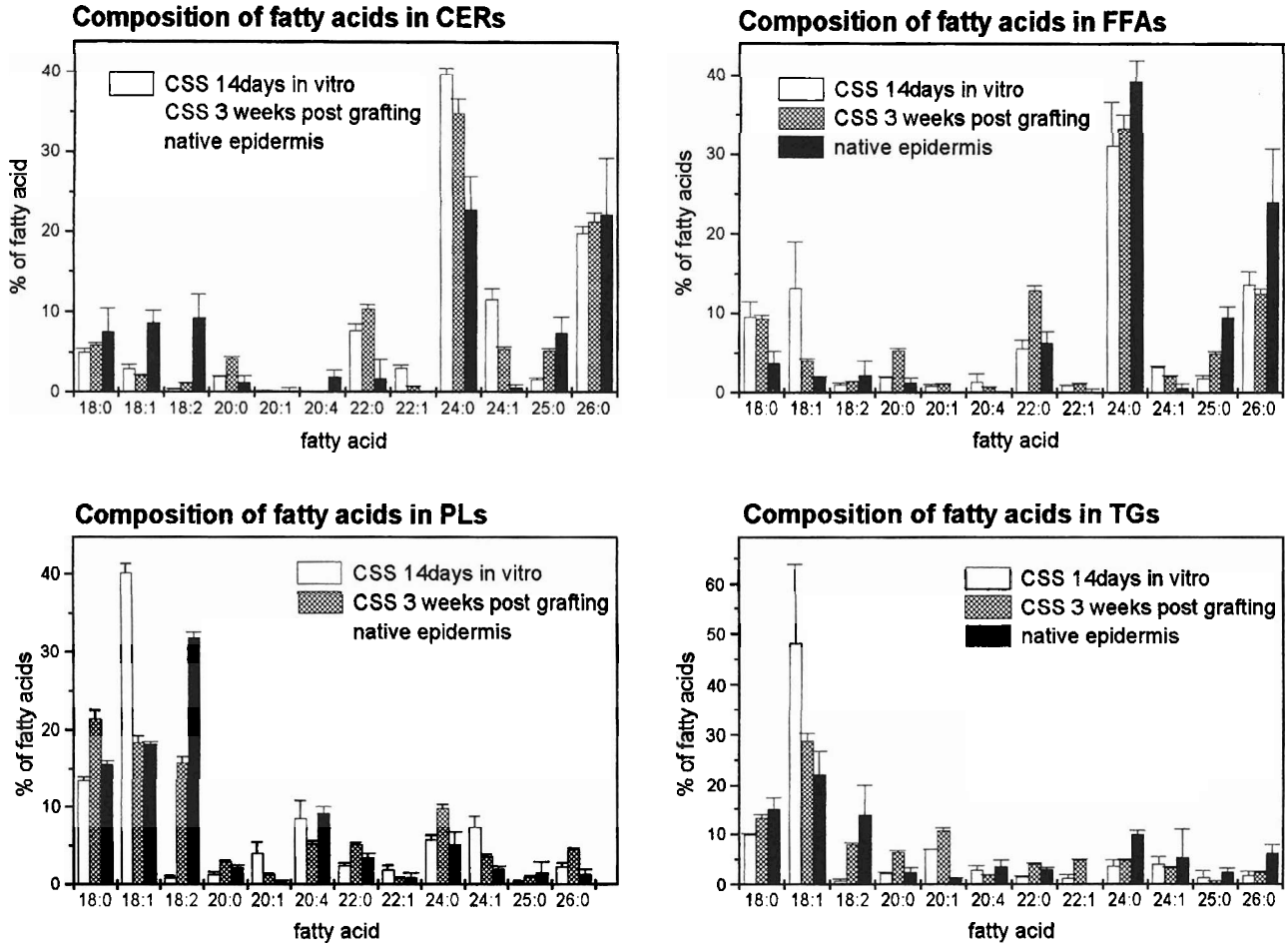

Figure 3 Composition of fatty acids in CER, FFA, PL, and TG fractions isolated from CSS cultured for 2 weeks in vitro, 3 weeks after grafting onto athymic mice, and from native human epidermis. Data are expressed as % of total fatty acids and presented as means ± range, $n = 2$.

ated, such as 20:1, 20:2, 22:1, 24:1, 22:4, are slightly elevated in CSS in vitro in comparison with native levels; saturated fatty acids (20:0, 22:0) approximate the same values as in vivo (Table 4). Fatty acid composition of epidermal grafts shows higher amounts of 20:0, 20:1, 22:0, and 22:1, originating probably from the MSL.[34] Major long chain fatty acids present in human epidermis are 24:0 (9.6% ± 0.8%) and 26:0 (8.4% ± 1.1%). These fatty acids show no substantial difference in their values in the cultured CSS or after grafting.

Detailed examination of the content of 18-carbon fatty acids (C18) shows imbalance in oleic and linoleic acid content in CSS in vitro. The oleic acid level reached about twice the value found in the native skin, whereas only very low amounts of linoleic acid were detected. The ratio of 18:0/18:1/18:2 was 1/3.7/0.2 in CSS incubated for both 14 and 34 days in vitro, which changes 3 weeks after grafting to the ratio of 1/1.6/0.7. Total recovery was not reached by 4 months, and the ratio 1.5/1.1/8.6 still differed from that obtained for the native epidermis (1.4/2.4/10.2) (Table 5). Two isomers of oleic acid were detected in all samples: 18:1ω9 and 18:1ω7. A decrease in 18:1ω9/18:1ω7 ratio, when the CSS in vitro (2.7 ± 0.3 and 3.1 ± 0.1) was compared to the native tissue (10.2 ± 2.4), indicated disproportions in synthesis of oleic acid isomers in vitro (Table 5).

Individual epidermal lipid fractions from CSS incubated in culture for 14 days, 3 weeks after grafting, and native human epidermis separated by HPTLC, were transmethylated and analyzed for fatty acid composition. Fatty acids that revealed most profound differences are shown in Figure 3. In general, both CER and FFA contained predominantly long chain fatty acids; whereas phospholipids and triglycerides contained short chain fatty acids in all samples, although the content of individual fatty acids shows differences between in vitro and native epidermis, with partial normalization by 3 weeks after grafting. FFA and CER contain higher amount of 20:0 and 22:0 in samples collected 3 weeks after grafting, suggesting that the mouse host might influence synthesis of medium long chain fatty acids.

DISCUSSION

Results of this study show that in vitro CSS exhibit deficiencies in epidermal lipid composition compared with normal skin, and that grafting to a physiologic host (athymic mouse) permits lipid metabolism to normalize. After grafting, cultured keratinocytes synthesize all epidermal lipids in very similar proportions to those present in healthy human epidermis. Therefore, abnormalities in epidermal lipids in vitro that limit barrier formation result from deficiencies in culture conditions.

Similar to previously reported keratinocyte culture systems,[25] CSS reveal disproportions in lipid composition, which are associated with physiologic instability in vitro and impaired barrier function. The fact that PLs as a component of cell membranes disappear in the SC, whereas neutral lipids and sphingolipids increase in relative abundance, is generally accepted. The content of PLs in native epidermis varies from 40% to 50%, and indirectly represents the proportion of viable cells present in the epidermis. Low PL content in CSS indicates a relatively low number of viable cell layers. Moreover, decrease of PLs in samples incubated for 34 days versus 14 days reflects the limited life span of these cultures, because the ratio of PLs to other lipids effectively represents thickness of viable cell layers versus SC. A low relative content of total PLs at 3 weeks after grafting corresponds to the presence of hyperkeratotic epidermis at this time point. After removal of dressings from the grafts, epidermal hypertrophy resolves slowly, and the PL content increases during wound healing, showing recovery of the balance between keratinocyte proliferation and differentiation and improved stability of CSS. This biochemical result is consistent with the normal proportion of epidermal layers observed in histology at 4 months after grafting.

Apart from the obvious "thickening" of cultured SC with time, prolongation of culture time does not affect the composition of other epidermal lipids. A large excess of TG in combination with low levels of FFA, glucosphingolipids, and cholesterol sulfate in CSS in vitro, and in other keratinocyte culture systems,[25] gradually normalizes after grafting, suggesting that these deficiencies result from culture conditions. Factors present in the culture media include, but are not limited to: absolute concentrations of substrates for intermediary metabolism (glucose, acetate, glutamine, pyruvate), and transport factors (insulin, insulin-like growth factors). Other stimuli of proliferation (i.e., epidermal growth factor) in CSS further exaggerate lipid abnormalities.[14,26] Results of this study indicate that reduction in substrates, and cofactors for carbohydrate metabolism, will reduce abnormal proportions of epidermal lipids in vitro. Because the final goal in the development of skin substitutes is formation of functioning SC that would exhibit the same barrier function as native skin, special attention should be paid to the composition of SC barrier lipids. Although the amounts of cholesterol and total CER in vitro reveal values similar to those obtained for the native epidermis, HPTLC analysis clearly shows a different CER pattern. A low amount of CER 5, and the absence of CER 6 and 7 containing α-hydroxy fatty acids,[23,24] identifies deficiencies in hydroxylation of long chain fatty acids and sphingoid bases in vitro.[25] Based on this finding, it is postulated that stimulation of hydroxylation may allow synthesis of CER 6 and 7, and contribute to improved barrier function. Hydroxylation of collagen by fibroblasts is stimulated by ascorbic acid, which is a cofactor for the hydroxylase enzyme, proline 4-monooxygenase. Media used in this study contained no ascorbic acid. It is possible that addition of a cofactor, such as ascorbic acid, for an hydroxylase enzyme may facilitate synthesis of CER 6 and 7 and improve epidermal barrier in vitro.[25]

Although HPTLC provides us with a useful tool for separation and identification of different epidermal lipid classes based on their polarities, with the HPTLC approach it is impossible to establish the molecular structure of individual lipids that is critical for the proper organization of lipids in membranes and lipid bilayers of the SC. Fatty acid profiles of either total lipid extract or individual lipid fractions show different fatty acid compositions in CSS, which identifies deviations in fatty acid metabolism in vitro. CSS contains a low amount of linoleic acid (18:2), whereas the amounts of monounsaturated fatty acids, mainly oleic acid (18:1), are elevated as compared with the native epidermis. Similar observations have been made by Marcelo et al.[27,28] in keratinocyte cultures grown in essential fatty acid – deficient medium. Results reported here suggest that although culture media for CSS are supplemented with linoleic acid, its cellular uptake is not sufficient. This might be due to the low concentration of linoleic acid in the medium, inefficient release of the linoleic acid from the carrier molecule, or low permeability of the dermal substrate. However, a low uptake of linoleic acid may also result from poor incorporation into barrier lipids that depend on hydroxylation, as described by Wertz and Downing.[29] The lack of linoleic acid is compensated for by abundant synthesis of oleic acid and its subsequent elongation, resulting in enrichment in medium long chain fatty acids (20:1, 22:1, 24:1) in CSS. Because the linoleate-containing lipids play a critical role in formation and maintenance of the epidermal barrier,[28,30,31] and oleate-linoleate substitution in sphingolipids was found to occur in association with defective barrier function in essential fatty acid deficiency,[32] disproportions in oleate-linoleate content are most probably partly responsible for the impaired barrier of CSS in vitro. Therefore, special attention should be paid to an efficient supplementation of the culture media with linoleic acid, and stimulation of its uptake by keratinocytes. Arachidonic acid (20:4) is present in CSS in vitro in its normal levels, suggesting sufficient supplementation and concentration

in the culture media. It remains to be established whether supplementation with arachidonic acid is necessary at all. Namely, during generation of CSS, the culture is kept for 3 days under submerged conditions, under which transformation of linoleic acid into arachidonic acid occurs.[1,33]

Finally, the physiologic host (athymic mouse) does not seem to affect the recovery of lipid metabolism of CSS after grafting, because the total epidermal lipid profile is similar to that of healthy human epidermis (with the exception of CER 2, which still remains elevated at 4 months after grafting and some disproportions in fatty acid profile). Some minor differences in the lipid and fatty acid profiles might also be attributed to the original source of cultured and control human keratinocytes. Although all epidermal grafts, as well as the mouse host, contained an additional apolar lipid fraction with a mobility (polarity) between TG and cholesterol esters, described by Higounenc et al.[18] as "mouse-specific lipids," they were shown not to be a constituent of the human graft. Rather, they are most probably a contamination by mouse lipids, such as sebaceous lipids, that enter the human graft by simple diffusion. However, fatty acids derived from mouse-specific lipids can also contribute to differences in total epidermal fatty acid profile.

On the basis of our observations, we conclude that CSS epidermal lipid profile normalizes after transplantation onto athymic mice, which permits the restoration of an epidermal barrier and successful wound closure. This conclusion is in an agreement with the results on measurements of surface electric capacitance (hydration of the SC) of grafted CSS demonstrated by Boyce et al.[20] Data included in this report support the use of CSS grafted to a physiologic host as a valuable model for understanding carbohydrate-lipid metabolism in epidermis and to allow preparation in vitro of a more physiologic and stable skin substitute. CSS with more fidelity to native skin may be expected to contribute to improved healing of acute and chronic wounds.

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REFERENCES

1. Ponec M, Weerheim AM, Kempenaar JA, Mommaas AM, Nugteren DH. Lipid composition of cultured keratinocytes in relation to their differentiation. J Lipid Res 1988;29:949-62.
2. Ponec M. Human epidermis reconstructed on de-epidermized dermis: expression of differentiation-specific protein markers and lipid composition. Toxicol In Vitro 1991;5:597-606.
3. Ponec M, Wauben-Penris PJJ, Burger A, Kempenaar J, Boddé HE. Nitroglycerin and sucrose permeability as markers for reconstructed human epidermis. Skin Pharmacol 1990;3:126-35.
4. Régnier M, Asselineau D, Lenoir MC. Human epidermis reconstructed on dermal substrates in vitro: an alternative to animals in skin pharmacology. Skin Pharmacol 1990;3:70-85.
5. Mak VH, Cumpstone MB, Kennedy AH, Harmon CS, Guy RH, Potts RO. Barrier function of human keratinocyte cultures grown at the air-liquid interface. J Invest Dermatol 1991;96:323-7.
6. Simonetti O, Hoogstraate AJ, Bilaik W, Kempenaar JA, Schrijvers AHG, Boddé HE, Ponec M. Visualization of diffusion pathways across the stratum corneum of native and in vitro reconstructed epidermis by confocal laser scanning microscopy. Arch Dermatol Res 1995;287:465-73.
7. Vičanová J, Mommaas A, Mulder AA, Koerten HK, Ponec M. Impaired desquamation in the in vitro reconstructed human epidermis. Cell Tissue Res 1996;286:155-22.
8. Ponec M. Lipid biosynthesis. In: Leigh IM, Lane EB, Watt FM, editors. The keratinocyte handbook. Cambridge: Cambridge University Press;1994.p.351-63.
9. Boddé HE, Holman D, Spies F, Weerheim AM, Kempenaar JA, Mommaas AM, Ponec M. Freeze fracture electron microscopy of in vitro reconstructed epidermis. J Invest Dermatol 1990;95:108-16.
10. Fartasch M, Ponec M. Improved barrier structure formation in air-exposed human keratinocyte culture systems. J Invest Dermatol 1994;102:366-74.
11. Bouwstra JA, Gooris GS, Weerheim A, Kempenaar J, Ponec M. Characterization of stratum corneum structure in reconstructed epidermis by x-ray diffraction. J Lipid Res 1995;36:496-504.
12. Boyce ST, Hansbrough JF. Biologic attachment, growth, and differentiation of cultured human epidermal keratinocytes on a graftable collagen and chondroitin-6-sulfate has substrate. Surgery 1988;103:421-31.
13. Boyce ST, Medrano EE, Abdel-Malek ZA, Supp AP, Dodick JM, Nordlund JJ, Warden GD. Pigmentation and inhibition of wound contraction by cultured skin substitutes with adult melanocytes after transplantation to athymic mice. J Invest Dermatol 1993;100:360-5.
14. Boyce ST, Williams ML. Lipid supplemented medium induces lamellar bodies and precursors of barrier lipids in cultured analogues of human skin. J Invest Dermatol 1993;101:180-4.
15. Boyce ST, Greenhalgh DG, Housinger TA, Kagan RJ, Rieman RT, Childres CP, Warden GD. Skin anatomy and antigen expression after burn wound closure with composite grafts of cultured skin cells and biopolymers. Plast Reconstr Surg 1993;91:632-41.
16. Boyce ST, Goretsky MJ, Greenhalgh DG, Kagan RJ, Rieman RT, Warden GD. Comparative assessment of cultured skin substitutes and native skin autograft for treatment of full thickness burns. Ann Surg 1995;22:743-52.
17. Boyce ST, Glatter R, Kitzmiller WJ. Treatment of chronic wounds with cultured cells and biopolymers. Plast Reconstr Surg 1995;91:623-41.
18. Higounenc I, Spies F, Boddé H, Schaefer H, Démarchez M, Shroot B, Ponec M. Lipid composition and barrier function of human skin after grafting onto athymic nude mice. Skin Pharmacol 1994;7:164-75.
19. Higounenc I, Démarchez M, Régnier M, Schmidt R, Shroot B, Ponec M. Improvement of epidermal differentiation and barrier function in reconstructed human skin after grafting onto athymic nude mice. Arch Dermatol Res 1994;286:107-14.
20. Boyce ST, Supp AP, Harriger MD, Pickens WL, Wickett RR, Hoath SB. Surface electrical capacitance as a noninvasive index of epidermal barrier in cultured skin substitutes in athymic mice. J Invest Dermatol 1996;107:82-7.

21. Ponec M, Weerheim A. Retinoids and lipid changes in keratinocytes. In: Packer L, editor. Methods in enzymology, vol 190, retinoids, part B. San Diego: Academic Press; 1990.p.30-41.
22. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-7.
23. Wertz PW, Downing DT. Ceramides of pig epidermis: structure determination. J Lipid Res 1983;24:759-65.
24. Robson KJ, Stewart ME, Michelsen S, Lazo ND, Downing DT. 6-hydroxy-4-sphingenine in human epidermal ceramides. J Lipid Res 1994;35:2060-8.
25. Ponec M, Weerheim A, Kempenaar J, Mulder A, Gooris GS, Bouwstra J, Mommaas AM. The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. J Invest Dermatol. 1997;109:348-55.
26. Ponec M, Gibbs S, Weerheim A, Kempenaar J, Mulder A, Mommaas AM. Epidermal growth factor and temperature regulate keratinocyte differentiation. Arch Dermatol Res 1997;289:317-26.
27. Marcelo CL, Duell EA, Rhodes LM, Dunham WR. In vitro model of essential fatty acid deficiency. J Invest Dermatol 1992;99:703-8.
28. Marcelo CL, Dunham WR. Fatty acid metabolism studies of human epidermal cell cultures. J Lipid Res 1993;34:2077-90.
29. Wertz PW, Downing DT. Epidermal lipids. In: Goldsmith L, editor. Physiology, biochemistry and molecular biology of the skin. New York: Oxford University Press; 1991.p.205-36.
30. Prottey C. Investigations of functions of essential fatty acids in the skin. Br J Dermatol 1977;64:29-38.
31. Elias PM, Brown BE. The mammalian cutaneous permeability barrier: defective barrier function in essential fatty acid deficiency correlates with abnormal intercellular lipid deposition. Lab Invest 1978;39:574-83.
32. Wertz PW, Cho ES, Downing DT. Effect of essential fatty acid deficiency on the epidermal sphingolipids of the rat. Biochim Biophys Acta 1983;753:350-5.
33. Iseroff RR, Ziboh VA, Chapkin RS, Martinez DT. Conversion of linoleic acid into arachidonic acid by cultured murine and human keratinocytes. J Lipid Res 1987;28:1342-9.
34. Vičanová J, Boyce ST, Bouwstra JA, Weerheim AM, Harriger MD, Ponec M. Stratum corneum lipid composition and structure in cultured skin substitutes is restored to normal after grafting onto athymic mice. J Invest Dermat. In press.