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Neonatal Skin Maturation—Vernix Caseosa and Free Amino Acids

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Abstract: **Neonatal skin hydration decreases rapidly postnatally and then increases, indicating adaptive changes in stratum corneum water handling properties. Transition from high to low humidity at birth may initiate filaggrin proteolysis to free amino acids. Neonatal skin with vernix caseosa retained is more hydrated than skin with vernix removed. This study examines the potential roles of free amino acids and vernix in postnatal adaptation of infant stratum corneum in vivo. Specifically, the ontogeny of free amino acid generation in neonatal stratum corneum and the role of vernix caseosa in postnatal adaptation were examined using high performance liquid chromatography. Free amino acids were quantified for infant skin samples collected at (i) birth and 1 month and (ii) birth and 24 hours after vernix caseosa retention or removal and compared to neonatal foreskin, vernix caseosa, and adult stratum corneum using *t*-tests, analysis of variance, or univariate procedures. Free amino acids were extremely low at birth, significantly higher 1 month later but lower than in adults. Vernix caseosa retention led to significantly higher free amino acids 24 hours after birth compared to infants with vernix caseosa removed, and it paralleled the higher stratum corneum hydration of vernix caseosa-retained skin. Vernix caseosa contained free amino acids, with glutamic acid and histidine levels higher than in infants. Free amino acids in vernix caseosa-retained skin appear to originate from vernix caseosa. Free amino acids were lower in neonatal foreskin than adult forearm stratum corneum. Arginine was higher than citrulline at birth, but levels were comparable in older infants. The free amino acid increase at 1 month may be initiated by the humidity transition at birth and supports results in animals. The findings have implications for infant skin care practices.**

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Birth marks a major, rapid transition from the high humidity aqueous intrauterine environment to the characteristically low humidity (typically approximately 20–40%) in nurseries and homes. Following birth, full-term neonates have a competent stratum corneum (SC) barrier as indicated by low transepidermal water loss (TEWL) (1). However, skin hydration decreases markedly during the first postnatal day and increases progressively over the next 2 weeks (2). By 1 month, rates of moisture accumulation (i.e., transepidermal water that accumulates within the upper SC under occlusion) are significantly higher than maternal volar forearm sites. Water holding capacity also increases. Barrier integrity (i.e., TEWL) remains low throughout the period. Newborn skin is significantly drier than the skin of older infants (1, 2, and 6 months) and their mothers (3). Functional tests of water binding show significant differences between age groups. Neonatal SC hydration decreases significantly after transitioning from a 10-minute water soak (bath, high humidity) followed by towel drying to a much drier environment following a 15-minute equilibration at approximately 30–45% relative humidity (4). Adult volar forearm skin soaked in water for 10 minutes has significantly reduced levels of water-soluble amino acids and as well as decreased skin hydration and increased surface pH (5,6). Proper hydration of the SC is essential for effective skin function, for example, to allow sufficient plasticization and flexibility during movement, to prevent fissuring, and for proper desquamation of the outermost SC layer (7,8). Emergence of a fully functional SC barrier following transition from aqueous uterine surroundings to a dry environment depends upon molecular mechanisms of endogenous water binding within the SC. The SC water-handling properties must be sufficiently robust to respond to local forces (e.g., friction, heat, humidity, bathing, clothing, secretions, etc.) throughout life (4).

Filaggrin is derived from profilaggrin in the epidermal keratohyalin granules and aggregates the SC keratin filaments. It later undergoes proteolysis to form the free amino acid component of natural moisturizing factor (NMF) (9), which is responsible for hydration, water-handling properties, and plasticity of the SC (10–12). Hairless mice kept at 80% RH, then transferred to 10% RH, exhibit decreases in skin hydration, water-holding capacity, and free amino acid concentration and reduced filaggrin levels (13). High humidity (100%) blocks activation of filaggrin proteolysis in newborn rats after birth (14). Furthermore, the products of filaggrin proteolysis may function to acidify the SC.

Water-soluble free amino acids (FAA) constitute 40% of NMF (14,15). Filaggrin deimination and subsequent proteolysis is the major source of free amino acids in the SC (16–18). Histidase converts histidine to urocanic acid (19). A significant fraction of the arginine is converted to citrulline (20,21). The presence of high amounts of urocanic acid and citrulline indicate NMF production from filaggrin (19). Glutamic acid is converted to pyrrolidone carboxylic acid (PCA) (22,23), accounting for 12% of the total NMF (15). Lactate, urea, sugars, and ions are also part of NMF.

Vernix caseosa (VC) is a multicomponent mixture of protein (10%), lipids (10%), and 80% water, which is associated with fetal corneocytes embedded in a hydrophobic lipid matrix (24). Vernix covers the fetus during the last trimester (25) and presumptively protects the epidermis from water exposure while facilitating epidermal cornification and SC formation (26). Retention of VC immediately following birth leads to significantly higher skin hydration at 24 hours of life compared to infants with VC removed (25). This result is consistent with our previous report that normal adult skin treated with VC has an increased SC water-binding capacity relative to untreated control (27).

Multiple processes may impact neonatal skin hydration including: (i) extraction of water-binding FAA from the SC by amniotic fluid results in decreased hydration at birth and (ii) abrupt transition from high-to-low ambient humidity at birth initiates filaggrin proteolysis to the free amino acid components of NMF, which subsequently increase SC hydration. The aim of the present study was to examine the potential roles of free amino acids and vernix caseosa in postnatal adaptation of infant SC in vivo. We hypothesized that FAA would increase postnatally and would be higher in vernix-retained versus vernix-removed skin at birth. We examined the potential roles of FAA and VC in postnatal adaptation by quantifying the FAA component of NMF in (i) full-term infant SC at birth and at 1 month of age, (ii) neonatal SC with vernix retained or removed after birth and 24 hours later, and (iii) native VC at varying environmental humidities. The effect of humidity on native VC was examined to determine whether FAA could be generated (e.g., from filaggrin proteolysis triggered by decreasing humidity at birth). Neonatal foreskin was used as an in vitro control for newborn skin because FAA levels could be determined as a function of SC depth. Adult forearm SC served as another control since it has been a common reference for infant developmental studies and for evaluating skin care products (2,3,28,29). The findings are consistent with reports on the influence of humidity on filaggrin proteolysis and NMF formation in animals and have clinical implications for premature infant SC maturation.

METHODS

Subjects

In study 1, healthy full-term neonates ($n = 17$) were enrolled shortly after birth at a level II nursery (The Christ Hospital, Cincinnati, OH). Infants in distress, with major congenital abnormalities, or < 38 weeks gestational age (GA) were excluded. Skin surface samples were collected immediately after birth and 24 hours later using D-squame® tapes (CuDerm Corporation, Dallas, TX). In study 2, healthy full-term neonates ($n = 13$) were enrolled shortly after birth. Infants were evaluated over 1 month to determine changes in skin barrier integrity and water-handling properties using methods described previously (2,30). Skin surface samples were collected from chest and back at birth and 1 month. Samples from both studies were retained and stored at −80°C until analysis. The Institutional Review Boards (Cincinnati Children's Hospital Medical Center, The Christ Hospital) approved the research protocols, and parents provided written informed consent.

Vernix Caseosa and Neonatal Foreskins

Vernix caseosa was harvested immediately after delivery by gentle removal from the skin surface of 10 healthy newborns at local hospitals (The Christ Hospital, University Hospital Cincinnati, OH) and stored in sealed, sterile tubes at 4°C until analysis. Neonatal foreskins were collected from healthy infants (The Christ Hospital) within 24–36 hours of birth and stored in sterile media prior to sampling and analysis. The Institutional Review Board (IRB) approved the protocol.

Adult Subjects

A cohort of healthy adult subjects ($n = 11$) served as study controls, and FAA from volar forearm SC were determined as previously reported (6).

Extraction of Free Amino Acids from Vernix Caseosa

Vernix samples from 10 infants were pooled to create a representative sample (20 g) for analysis. Aliquots of 250 mg were processed in one of three different solvent systems, water, urea/sodium dodecyl sulfate (SDS), and chloroform/methanol (Folch extraction) as follows: (i) Vernix was homogenized in 20 volumes of deionized water for 1 minute using a Tissue Tearor (Model 985-370; Biospec Products, Inc., Bartlesville, OK) and filtered through a 70 micron nylon screen, (ii) Vernix was homogenized in 20 volumes of 300 mM urea (Ameresco, Solon, OH) with 2% SDS (Sigma Chemical Company, St. Louis, MO) for 1 minute and filtered through a nylon mesh filter, and (iii) Vernix was homogenized in 20 volumes of chloroform:methanol (2:1) for 5 minutes (31). Twelve volumes of deionized water were added, and the mixture was rehomogenized for 1 minute, quickly filtered, and allowed to stand until the two phases had separated. The water phase was collected and used for amino acid analysis. Acid extraction was not considered as an option due to potential confounding effects from acid hydrolysis of peptides in vernix. Replicates were analyzed for water-soluble amino acids. The amino acid profiles from the deionized water and the urea/SDS methods were similar to the major amino acids in filaggrin (Table 1), and the yields were higher than for the Folch method. The urea/SDS method was chosen because it yielded better High Performance Liquid Chromatography (HPLC) peak resolution.

TABLE 1. *Mean Amino Acid Concentrations (pmoles/μg protein) ± Standard Error of the Mean for the Water, Urea/SDS, and Folch Extraction Systems*

Amino acid	Water ($n = 5$)	Urea/SDS ($n = 5$)	Folch ($n = 5$)
Glycine (gly)	10.1 ± 0.4	51.1 ± 7.8	9.2 ± 0.5
Serine (ser)	53.1 ± 3.4	98.3 ± 5.4	30.5 ± 1.9
Glutamic acid (glu)	119.5 ± 7.6	239.5 ± 19.6	99.9 ± 4.8
Histidine (his)	383.4 ± 23.1	438.5 ± 96.7	86.5 ± 11.6
Arginine (arg)	69.4 ± 4.0	141.6 ± 11.0	60.4 ± 8.4
Citrulline (cit)	62.6 ± 4.1	121.5 ± 9.0	56.5 ± 3.6

The effect of humidity on the FAA profile was determined by exposing vernix to controlled conditions of 24%, 39%, 50%, 77%, 98%, and 100% RH for 48 hours (32). Vernix (250 mg, three replicates) was spread onto double layers of N-terface® (Winfield Laboratories, Inc., Richardson, TX), a permeable film substrate to simulate application to the skin surface. A second set (250 mg, three replicates) was maintained at each condition in bulk (no spreading). Samples were extracted in urea/SDS and analyzed as described above.

Filaggrin Determination

A pooled vernix sample was analyzed for filaggrin. Separate aliquots were extracted in urea/SDS, phosphate buffer solution (PBS), and 8 M urea in Tris HCL. Western blot analysis confirmed the presence of filaggrin (data not shown).

D-Squame SC Collection

Surface samples from infant skin and neonatal foreskins were collected using 22 mm diameter D-Squame®

(CuDerm Corporation) tapes. Infant samples were taken from the chest and back, close to the midline in studies 1 (three serial) and 2 (two serial). Tapes were applied with consistent pressure, removed after 30 seconds, and stored at −80°C until analysis. The first tape was discarded from the foreskins to remove residual blood then 10 (10) serial tapes were obtained for analysis. Ten serial tapes were also collected from a volar forearm site of healthy adults ($n = 11$) as previously reported (6).

Rationale and Analysis of Water-Soluble Amino Acids

Free amino acids constitute about 40% of the natural moisturizing factor (NMF) (14,15). Deimination of filaggrin and subsequent proteolysis is the major source of free amino acids in the SC (16–18). Histidine undergoes conversion to urocanic acid via histidase (19). A significant fraction of the arginine is converted to citrulline (20,21). Therefore, the presence of high amounts of urocanic acid and citrulline indicate NMF production from filaggrin (19). Glutamic acid is converted to pyrrolidone carboxylic acid (PCA) (22,23), about 12% of the total NMF (15). Lactate, urea, sugars, and ions are part of NMF. Based on the available analytical and detection methods, we evaluated the FAA component.

The tapes were extracted with 300 μL 6 mM hydrochloric acid and 10 μL of 2 μmol/mL α-amino-n-butyric acid (Sigma Chemical Co.) at room temperature for 3 hours (6). Free amino acids were quantified using reverse-phase HPLC and fluorescence detection (Waters Corporation, Milford, MA). The tapes were then extracted with 300 mM urea (Ameresco) with 2% SDS (Sigma Chemical Company) and analyzed for total soluble protein with the Pierce BCA protein assay kit (Pierce Biotechnology Inc, Rockford, IL) assay (33). Free amino acids were normalized to the total protein and reported as picomoles per microgram.

Skin Hydration

Skin hydration was measured as previously described (25,34) using a NOVA meter (NOVA Technology Corporation, Gloucester, MA). Stratum corneum hydration was taken as the first reading, and the moisture accumulation was determined over 20 seconds.

Statistical Analysis

The results are expressed as mean ± standard error of the mean (SE), and $p \leq 0.05$ was considered statistically significant. The ontogeny of infant FAA was determined using paired *t*-tests. Group comparisons (e.g., infant birth, infant 1 month, adult) and effect of SC depth (adult forearm, foreskins) were evaluated with univariate general linear models (GLM) and analysis of variance (ANOVA) (SigmaStat, SPSS, Inc., Chicago, IL). Log_{10} transformation was used to improve the normality of the foreskin and adult data.

RESULTS

Subjects

In study 1, SC samples were obtained from 12 sites (nine infants) from the vernix-retained group and nine sites (eight infants) from the vernix-removed group. Chest and back SC were collected at birth and 1 month for four infants in study 2. All data were used (2,25). The mean GA was 38.9 ± 0.9 for VC retained and 39.6 ± 1.3 for VC removed ($p < 0.05$). The mean vernix coverage (25) was 76.5% for VC retained and 9% for VC removed. The moisture accumulation rate was significantly higher for VC retained after birth (1.1 ± 0.6, $p < 0.05$) and directionally higher (0.10 ± 0.05, $p = 0.08$) 24 hours later compared to VC removed (0.24 ± 0.12 and 0.0 ± 0.06, respectively). These results were consistent for a larger group reported previously (25).

FAA in Vernix

Relative to the amino acid profile for filaggrin (35), native vernix had higher levels of glutamic acid and histidine and lower levels of glycine and serine ($p < 0.05$, Table 2). No significant differences occurred in FAA levels as a function of relative humidity (Table 3).

Effect of Vernix Retention on FAA

Vernix retention led to higher levels of FAA, 24 hours after birth ($p < 0.05$) (Fig. 1A). Gestational age was included as a covariate in the analysis to correct for group differences in age. The three most abundant FAA, glycine, serine, glutaminc acid ($p < 0.005$), as well as arginine ($p = 0.05$) and citrulline ($p = 0.03$), were significantly higher for VC-retained samples, and histidine was directionally higher ($p = 0.06$). Free amino acids at 24 hours paralleled the higher SC hydration. Native vernix had considerably higher glutamic acid, histidine, and citrulline levels but lower amounts of serine than VC-treated skin ($p < 0.05$) (Fig. 1B). Glycine and arginine levels were comparable. The total soluble protein was higher in SC when vernix was retained (55.8 ± 5.8 μg/mL) than when it was removed (16.8 ± 5.6 μg/mL, $p < 0.001$).

TABLE 2. *Amino Acid Compositions of Native Vernix and Adult Forearm Skin Relative to Fully Hydrolyzed Filaggrin (31)*

	Bulk vernix (pmoles/μg soluble protein)	Indexed to glycine	Adult SC (pmoles/μg soluble protein)	Indexed to glycine	Filaggrin (31) (pmoles/μg filaggrin)	Indexed to glycine
Glycine	78.5 ± 19	1.0	483.3 ± 134	1.0	2981	1.0
Serine	114.6 ± 13	1.4	870.6 ± 248	1.8	2640	0.9
Glutamic acid	284.6 ± 27	3.6	312.6 ± 101	0.6	1100	0.4
Histidine	459.4 ± 92	5.8	242.4 ± 37	0.5	900	0.3
Arginine	151.4 ± 12	1.9	493.0 ± 105	1.0	500	0.2
Citrulline	134.6 ± 13	1.7	289.7 ± 49	0.6	70	0.02

The values are from the HPLC analysis as pmoles amino acid per microgram soluble protein (extracted from tapes) for native vernix and adult skin. For comparison, the relative amino acids in filaggrin are given as pmoles per microgram of protein based on the composition from full acid hydrolysis (31). The FAA for vernix, adult skin and filaggrin are also shown as indices, i.e., normalized to glycine.

TABLE 3. *Effect of Relative Humidity on Total FAA, Arginine, and Citrulline in Vernix Caseosa Reported as Mean ± SE*

Relative Humidity (%)	Total FAA (pmoles/μg protein)	Arginine (pmoles/μg protein)	Citrulline (pmoles/μg protein)
Spread vernix			
24	1330.5 ± 104.5	198.7 ± 62.6	49.6 ± 18.0
39	1360.2 ± 106.9	128.6 ± 49.0	31.5 ± 6.9
50	1048.5 ± 69.5	57.0 ± 7.0	25.6 ± 3.8
77	1456.9 ± 43.2	72.1 ± 29.7	33.6 ± 13.1
98	865.3 ± 22.7	35.2 ± 5.2	14.2 ± 3.8
100	888.7 ± 116.9	31.1 ± 8.5	14.2 ± 3.7
Unspread vernix			
24	1324.5 ± 114.8	94.0 ± 13.6	16.4 ± 3.8
39	1489.9 ± 46.5	27.2 ± 1.7	9.1 ± 0.5
50	1243.2 ± 78.2	23.8 ± 3.7	9.2 ± 1.1
77	1361.1 ± 105.4	28.8 ± 5.1	9.8 ± 1.4
98	883.2 ± 121.0	26.6 ± 2.0	12.5 ± 2.2
100	1029.1 ± 41.3	28.9 ± 6.0	14.3 ± 3.3

Ontogeny of FAA in Infants

Free amino acid levels were very low at birth and increased significantly over the first month for the total and individual FAA with the exception of glutamic acid ($p < 0.05$, Fig. 2A, B). The increases paralleled the rise in SC hydration (2). Free amino acid levels were substantially lower in infants at 1 month of age than in adults for the total and individual amino acids ($p < 0.05$) (Fig. 2A, B). Glycine and serine values were higher, and the glutamic acid level was lower in 1-month SC than in vernix ($p < 0.05$, Figs. 1B and 2B). At 1 month, citrulline was higher versus VC-retained skin at birth and lower than native vernix.

FAA in Neonatal Foreskin

The use of neonatal foreskins ($n = 3$) permitted FAA determination at greater SC sampling depths than was possible from newborn infant skin in vivo. Total FAA, glutamic acid, histidine, arginine, and citrulline levels were significantly lower in the first collected foreskin tape versus adult forearm ($p < 0.05$). Free amino acids were summed for tapes 1, 3, 5, and 10 and normalized to the total cumulative protein. Free amino acid, histidine, and

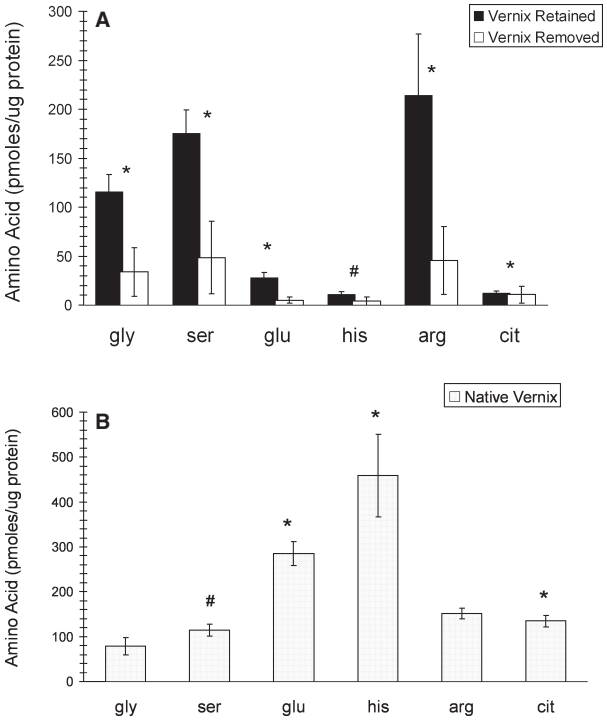

Figure 1. Effect of vernix retention on FAA levels in infant stratum corneum. (**A**) Vernix retention led to significantly higher quantities of FAA 24 hours after birth ($p < 0.05$). The most abundant amino acids glycine ($p < 0.005$), serine ($p < 0.010$), glutaminc acid ($p < 0.005$), as well as arginine ($p < 0.05$) and citrulline ($p < 0.05$), were higher in the vernix retained group ($p < 0.05$). Histidine was directionally higher in the vernix-treated group ($p = 0.06$). *Indicates significant difference between VC-retained and VC-removed ($p < 0.05$). #Indicates directional difference ($p = 0.06$). (**B**) The relative amino acid concentrations in vernix treated skin differed from vernix caseosa (normalized to protein). Vernix had considerably higher levels of glutamic acid, histidine, and citrulline, and lower serine than the sample from vernix-retained skin ($p < 0.05$). Glycine and arginine amounts were comparable. *Indicates significantly higher than VC-retained skin ($p < 0.05$). #Indicates significantly lower than VC-retained skin.

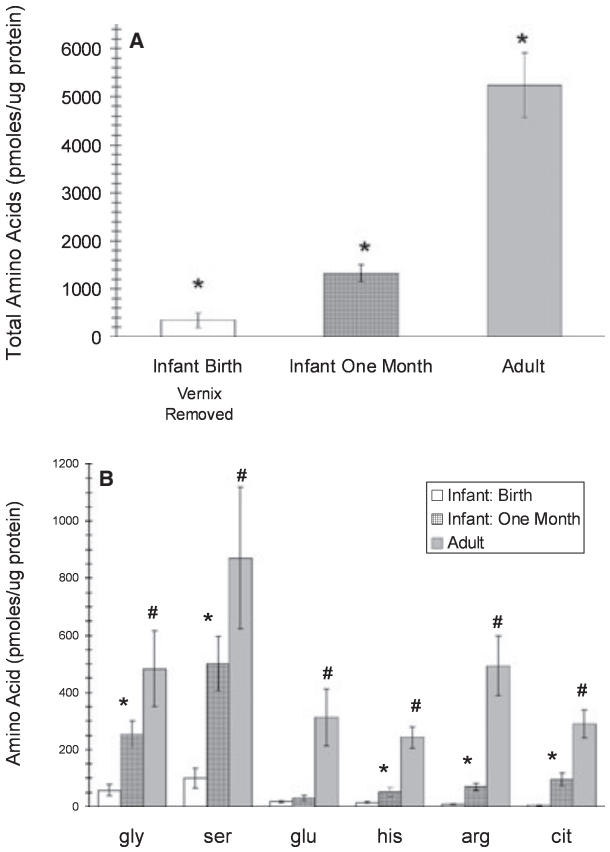

Figure 2. Ontogeny of free amino acids in infant stratum corneum. (**A**) Free amino acid levels were very low at birth and increased significantly over the first month for the total and (**B**) individual FAA except glutamic acid ($p < 0.05$, paired *t*-test). (**A**,**B**) Free amino acids were substantially lower in infants at 1 month of age than in adults for total and individual amino acids ($p < 0.05$). * Indicates significant difference for birth versus 1 month ($p < 0.05$). #Indicates significant difference versus infants at birth (vernix removed) and 1 month of age ($p < 0.05$).

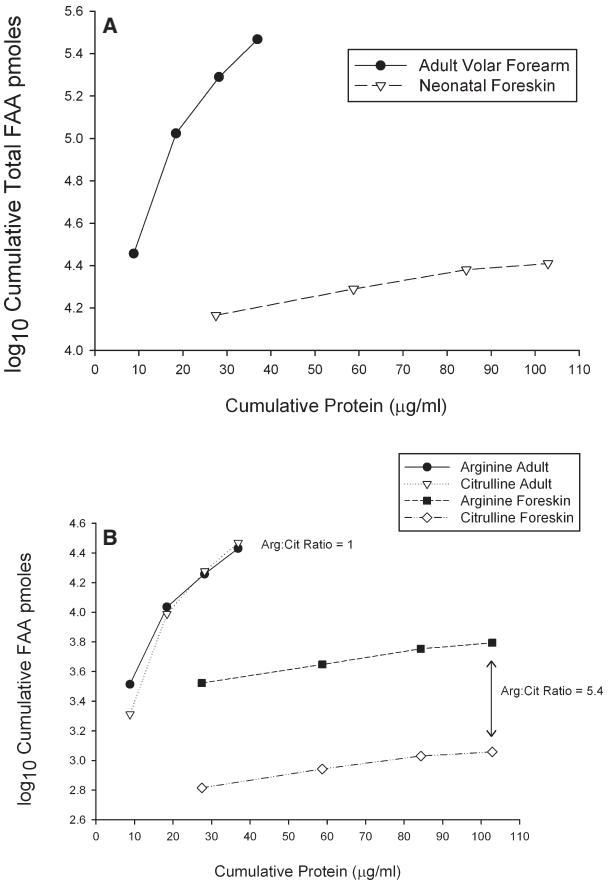

Figure 3. Free amino acid in neonatal foreskin compared to adult volar forearm. (**A**) Comparison of protein levels demonstrated that different depths within the SC were being assessed in neonatal foreskins versus adult volar forearm samples. Values are shown as $\log_{10}$ of the cumulative FAA versus the cumulative protein (tapes 1, 3, 5, and 10). (**B**) Arginine and citrulline were compared for cumulative amounts versus the cumulative protein. Arginine was significantly higher than citrulline for foreskin throughout the upper SC (paired *t*-test, $p < 0.05$).

citrulline levels were lower in foreskin ($p < 0.05$), and the glutamic acid level was directionally lower ($p = 0.06$). A confounding factor was that the amount of protein from foreskin (102.9 ± 12.5 μg/mL) was higher than that from forearm (36.9 ± 3.4 μg/mL) ($p < 0.05$) indicating that different SC depths were being compared. To account for this discrepancy, comparisons were made at "comparable" cumulative protein levels (i.e., foreskin tape 1 compared with cumulative forearm tapes 1, 3, and 5). Arginine was *higher* and citrulline was *lower* in foreskin at each SC level ($p < 0.05$, Fig. 3A, B). These two amino acids were not different throughout adult SC, except in tape 1 where arginine was directionally higher ($p = 0.09$). Arginine was higher than citrulline in VC ($p < 0.005$), VC-retained, and VC-removed skin ($p < 0.05$) (Fig. 1B). At 1 month, infant arginine and citrulline levels were comparable, as they were in adult skin (Fig. 2B).

DISCUSSION

The primary aim of this study was to examine the potential roles of free amino acids and vernix caseosa in postnatal adaptation of infant SC in vivo. The results suggest that vernix provides water-binding moieties (FAA) that can facilitate the sudden adaptation from amniotic fluid immersion in utero to the dry ambient conditions following birth. In general, in the absence of vernix, the FAA levels were extremely low at birth, increased over the first month but remained markedly lower than typical adult levels (Fig. 2A,B). The relative amounts of histidine and glutamic acid in vernix were higher than expected from filaggrin proteolysis alone, indicating that vernix may contain other sources of soluble amino acids. Histidine and glutamic acid were

significantly higher in vernix than in vernix-retained infant SC. Both amino acid products of filaggrin proteolysis undergo further change. Histidine is converted to urocanic acid by the enzyme histidase (36), although its presence in vernix has not been reported. The enzyme is up-regulated during keratinocyte differentiation (37) and has optimum activity at neutral pH (38). Histidase activity (i.e., conversion of histidine to urocanic acid) is not responsible for the postnatal pH decrease in animals (39). Consequently, it is unlikely that the pH decrease we observed for vernix-retained neonatal SC is due to conversion of histidine from vernix (25). Glutamic acid is converted to pyrrolidone carboxylic acid (PCA) via enzymatic (epidermal) and nonenzymatic processes, and levels decrease as PCA increases in normal skin (22,23). Therefore, the FAA in VC-retained skin are attributed to the vernix rather than to proteolysis of filaggrin to form FAA in the upper stratum corneum secondary to a reduction in ambient humidity (Fig. 1A,B) (14). However, it is possible that the higher FAA levels of glutamic acid and histidine in vernix are due to incomplete conversion to PCA and UCA.

Despite prolonged fluid immersion prenatally, low skin hydration is a consistent finding in full-term neonates within the first day after birth (2,25,40). Low hydration may result from several interacting factors including a lack of water binding FAA in the upper SC, via extraction into the amniotic fluid in utero (soaking effect) (4,6), or to delayed or impaired filaggrin proteolysis at high humidity, or both (14). In hairless mice, abrupt decreases in environmental humidity evoke increased DNA synthesis, reduced total free amino acid generation, and decreased filaggrin immunoreactivity due to decreased epidermal keratohyalin granules induced by high humidity and dry skin (13,14,40,41). Low hydration may indicate an underdeveloped SC due to lack of transglutaminase activity (42). However, this explanation is less likely given the generally excellent barrier integrity, indicated by the low TEWL values observed in full-term newborn skin (43).

Low FAA in infant SC at birth is consistent with reports in neonatal and adult rat skin (14). Profilaggrin appears at gestational day 19 in rat skin, and filaggrin is found at day 20, concurrent with SC development (14). At birth (day 21), filaggrin is present throughout the SC, markedly decreased on postnatal day 1, and even lower on day 2. High (100%) or low humidity (30–70%) prevents filaggrin proteolysis, which occurs optimally at 80–95% RH. The finding of reduced FAA in neonates at birth and 1 month extends the finding of lower NMF in 3- to 12-month infants versus adults with Raman confocal microspectroscopy (3). The finding of higher FAA in VC-retained skin may explain the increased SC acidity (lower pH) when vernix is retained at birth versus higher acidity when it is removed (25). We reported continuing acidification of the SC over weeks following birth in hospitalized premature and full-term infants (44). The higher FAA at 1 month in the present study is consistent with increasing acidification. An alternative explanation for the low FAA levels is that they are extracted from the SC into the amniotic fluid. Water exposure leads to significantly lower FAA in adults (6,45). The relatively higher levels of arginine and citrulline in vernix and adult skin may reflect (i) deimination of arginine to citrulline followed by proteolysis to FAA and (ii) conversion of histidine to urocanic acid (19,46,47). Additional perspective regarding the low FAA could be obtained by evaluating a highly occlusive treatment (e.g., Aquaphor®, Beiersdorf AG. Hamburg, Germany, or similar anhydrous petrolatum-based cream) on newborn skin immediately after birth. An occlusive treatment would serve as a control for the vernix-removed skin. Exposure to 100% humidity blocked filaggrin proteolysis in neonatal animals (14). Treatment of both normal and compromised (tape-stripped) skin with complete occlusion for 5 days led to significantly lower FAA levels in adults (48,49). Therefore, the levels of FAA in occluded newborn skin would likely be lower than in vernix-retained skin. Use of the newer noninvasive methods (i.e., Raman spectroscopy) would permit analysis of NMF at deeper levels in occluded infant skin.

To further explore the cause of low FAA in newborn infants, we measured the FAA as a function of SC depth in foreskins, compared to 10 serial samples from adult forearm skin. Similar to neonatal chest and back skin, FAA were significantly lower in foreskin than in adult SC. Arginine residues in filaggrin must be deiminated to citrulline to allow separation from the intracorneocyte matrix and facilitate proteolysis to FAA (46,50). The arginine/citrulline ratio is relatively high in filaggrin (i.e., 8:1) (35). Interestingly, despite the SC depth, foreskin citrulline levels were not only diminished relative to adults, but the ratio of arginine to citrulline was consistently higher in foreskin (Fig. 3B). A similar high arginine to citrulline ratio was observed in newborn skin following retention of vernix following birth (Fig. 1A). Filaggrin was found in vernix by Western blot analysis, consistent with the proteonomic analysis by Tollin et al (51). Vernix contains caspase 14 (51), an enzyme that converts deiminated filaggrin to peptide intermediates (52), which are hydrolyzed to generate FAA including citrulline (50).

For adult SC, infants at 1 month and vernix, the citrulline levels were higher, relative to arginine (molar basis), than in infant SC at birth, neonatal foreskin, and in filaggrin itself. This finding suggests that the arginine is converted to citrulline after the proteolysis of filaggrin

has occurred. Another potential source of citrulline from arginine is through the activity of nitric oxide synthase, an enzyme that generates citrulline and NO from arginine residues (53). Both arginine and NO facilitate various aspects of wound healing (54). While it has not yet been established, the possibility exists that nitric oxide synthase could be present in vernix and that it could generate citrulline from arginine along with volatile NO. In such a physiological scenario, vernix retained on the skin surface would function as a source of available arginine.

One limitation of this study is that we examined only FAA, which constitute 40% of the NMF composition (15). The other components such as PCA, urocanic acid, lactate, urea, sugars, and ions were not measured. Lactate is positively correlated to SC hydration (12). We have assumed filaggrin to be the source of citrulline; however, keratin 1 is another potential source. Keratin 1 is expected to be at higher levels in dry skin (42), and it may have been a source of citrulline in dry infant SC. Another potential source of reduced FAA is the degradation of histidine by skin microflora (bacteria) (55). We did not evaluate the skin sites for microflora and cannot address the impact of skin bacterial colonization on FAA levels. All tape specimens were immediately stored at −80°C to prevent microbial degradation between sample collection and analysis. Glycerol is another low molecular weight hygroscopic moiety that is expected to be present in vernix and stratum corneum. Glycerol is produced from lipase degradation of triglycerides in sebum and from circulation by way of aquaporin 3 channels (56). Vernix contains lipids, including fatty acids, and triglycerides (57). Although specific lipases have not been reported, generation of glycerol from vernix triglycerides is likely to occur. Glycerol in vernix and the epidermis would contribute to SC hydration.

Another limitation was the variation in skin sites where SC was collected from the chest and back in infants and from the volar forearm in adults. Regional differences in FAA are expected, given the differences in hydration and SC integrity. Variations in specific FAA were found for the cheek versus the back, cheek versus forearm, and calf versus forearm (10,58,59). We examined the regional differences in FAA among adults using the present analytical method and found levels to be higher for the calf versus forearm and lower for the jaw versus the chest and back (60). The forearm, chest, and back sites were not markedly different. The use of neonatal foreskin as a means of investigating the effects of SC depth on FAA levels is suboptimal. However, we could not obtain biopsies or surgical specimens from normal newborn skin. Noninvasive methods such as in vivo Raman spectroscopy for assessing NMF components as a function of depth were not available. The sites for sampling and the use of foreskin were chosen proactively while recognizing the limitations.

During the last trimester, vernix begins to coat the skin from head to toe and back to front, presumably under hormonal control with lipids generated by the sebaceous gland and cells from the hair follicle (61). It is "extruded" onto the interfollicular epidermis to cover the whole surface (62). In this working hypothesis, corticotropic-releasing factors (CRF) from either the placenta or hypothalamus initiate adrenocorticotropic hormone (ACTH) release from the pituitary gland. Adrenocorticotropic hormone adrenal gland stimulation promotes synthesis and release of androgenic steroids (e.g., dihydroepiandrosterone), which are converted to active androgens within the sebaceous gland. Production of superficial lipid film (sebum) in the immediate vicinity of the hair follicle modulates the transepidermal water gradient and protects the epidermis from exposure to water, thereby facilitating cornification of the underlying epidermis (63).

Multiple biological functions have been demonstrated or proposed for vernix (61). It contains lysozyme, lactoferrin and other microbials with demonstrated antiinfective properties (64–67). Films of vernix in vitro impede penetration of the exogenous enzyme chymotrypsin (found in meconium, similar to proteolytic fecal enzymes) and do not impede native enzyme activity necessary for epidermal development (68). In animals with barrier compromise (via tape stripping), vernix enhances SC formation without increasing epidermal thickness (69). Vernix functions as a skin cleanser, although it is often viewed as a soil itself (70). In vitro measurements indicate a low surface energy for vernix suggesting that it creates a protective hydrophobic layer around the fetus (26). Vernix has the cytokines IL1α, IL1β, TNFα, IL-6, IL-8, and MCP1 (29). Vernix contains cholesterol, ceramides, and a number of fatty acids (including oleic, linoleic, and long chain species) (57,67). Fatty acids, particularly linoleic, activate peroxisome proliferator-activated receptor-α (PPARα), which increases the rate of barrier formation (71). Linoleic acid has antiinflammatory properties (72). These functions, coupled with the antiinfective properties, are essential for a premature infant who may not have exposure to vernix in utero.

In conclusion, the present study is the first to address the ontogeny of free amino acids, putatively natural moisturizing factor, in the human neonate beginning at birth. The results are consistent with previous reports in fetal and neonatal animals compared to adults (13,14). The role of VC in neonatal adaptation is important since vernix is a uniquely human material with no counterpart in neonatal mice or rats. The findings suggest

another adaptive function of vernix, namely to provide water-binding FAA to plasticize the skin before the longer term adaptive epidermal changes and subsequent increased SC hydration have occurred (2). They also raise questions for future research (e.g., the origin of vernix free amino acids). The effect of GA on the ontogeny of FAA generation is of interest, given the dryness, scaling, and low hydration commonly observed in premature infants as the SC barrier develops (30,73,74). The findings have implications for infant skin care practices and provide additional support for the retention of vernix on the skin after birth. Studies on the application of water-binding vernix-like creams to neonatal skin after birth are warranted.

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