The Expression of Congenital Ichthyosiform Erythroderma in Second Trimester Fetuses of the Same Family: Morphologic and Biochemical Studies

Karen A. Holbrook, Ph.D., Beverly A. Dale, Ph.D., Mary L. Williams, M.D., Tracy B. Perry, M.Sc., Mary S. Hoff, B.S., Emily F. Hamilton, M.D., Chris Fisher, Ph.D., and Vyta Senikas, M.D.

Departments of Biological Structure (KAH, MSH, CF), Medicine (Dermatology) (KAH, BAD), Periodontics, and Oral Biology (BAD), University of Washington, Seattle, Washington; Departments of Dermatology and Pediatrics (MLW), University of California San Francisco Medical Center and the VA Medical Center, San Francisco, California, U.S.A.; and Department of Obstetrics and Gynecology (TBP, EFH), Royal Victoria Hospital, McGill University, Montreal, Canada

The first-born offspring of first-cousin parents was affected with a keratinization disorder thought to be nonbullous congenital ichthyosiform erythroderma (CIE). In each of three subsequent pregnancies, the parents elected to have prenatal diagnosis based on evaluation of fetal skin biopsies. The epidermis of fetus 1 was identical to normal 21-wk estimated gestation age (EGA) fetal epidermis, but because keratinization begins normally around 24 wk EGA, the procedure was repeated 4 wk later. A thin epidermis with a few layers of stratum corneum indicated a normal fetus and a healthy infant was born at term. Skin biopsy samples from fetus 2 gave conflicting results; the epidermis of one sample appeared normal but the second had 5–15 layers of incompletely keratinized cells superficial to basal and intermediate layers. The hair canals of both samples were hyperkeratotic. Pelleted amniotic fluid cells contained aggregates of incompletely keratinized epidermal cells and concentric rings of keratinized cells. The fetus was thought to be affected and the pregnancy terminated. Regional variation in epidermal thickness and keratinization was noted upon gross examination of the fetus and by histology of the skin. Marked hyperkeratinization of follicles was evident in all regions. No abnormal keratins were expressed in the affected epidermis but epidermal lipids analyzed from two body regions had a lower triglyceride content and a higher content of free sterols compared with age-matched, normal fetal epidermis. Immunolabeling for markers of differentiation revealed variable stages of epidermal differentiation according to region. Four structurally identical biopsy samples were obtained from a third fetus. The epidermis appeared normal for age and hair canals were keratinized to various extents. The pregnancy was continued and at 33 wk a male infant was born with a severe ichthyosis of the face and scalp and fine, white scaling on the body. The epidermis of both the severely and mildly affected regions of the newborn had a thick, compact stratum corneum and other features of CIE. Scars from all four fetal biopsies were identified on the trunk, in areas which appeared less affected clinically. This study reports, for the first time, the criteria for prenatal diagnosis of CIE and the variable expression of this disorder in the midtrimester fetus. More importantly, it demonstrates the risks and pitfalls of this in utero diagnosis based on epidermal morphology. J Invest Dermatol 91:521–531, 1988

Two forms of autosomal recessive, congenital ichthyosis can be distinguished on the basis of clinical, kinetic, histologic, and biochemical criteria [1–3]. Patients with nonbullous, congenital ichthyosiform erythroderma (CIE) have fine, white scales on the trunk and pronounced erythroderma, often expressed with regional variation [1–4]. The characteristic scale of patients with lamellar ichthyosis (LI) is large, dark, and plate-like. Affected individuals may have severe ectropion and an unremitting course of the disease [1–4]. In both, the labeling index of epidermal cells is greater than in normal epidermis but in CIE it is increased nearly twofold in comparison with LI [3]. There is a corresponding decrease in transit time [4]. The histopathology of epidermis in CIE includes acanthosis, focal parakeratosis, and a thickened stratum corneum. In contrast, the viable epidermis in LI is not as thickened, but hyperkeratosis is more extensive, and there is prominent papillomatosis [1–8]. In both CIE and LI, follicles are hyperkeratotic and often plugged with keratinous debris. The keratin and filaggrin proteins of normal epidermis are unaltered in both diseases, although additional keratin peptides that are markers of hyperproliferation [9] are expressed. The total lipid content in CIE and LI scale is similar, but CIE is distinguished by elevated levels of n-alkanes, reduced quantities of triglycerides, and free fatty acids [1,2]. The scale of patients with LI shows a significant increase in both sphingolipids, free sterols [1,2], and a decrease in sterol (cholesterol) ester [10].

Prenatal diagnosis of several, severe ichthyoses and other genetic disorders of which ichthyosis is a component has been possible on the basis of biochemical studies of amniotic fluid from the 14–16 wk EGA (estimated gestational age) fetus and urine from the mother (recessive x-linked ichthyosis [11]), and by morphologic examination of skin biopsies obtained from 19–21 wk fetuses at risk. Fetuses affected with bullous congenital ichthyosiform erythroderma (BCIE or epidermolytic hyperkeratosis) can be identified by a characteristic clumping of keratin filaments in interme-
diate layer cells of the epidermis [12,13] and in desquamated epidermal cells in the amniotic fluid [13,14]. Harlequin ichthyosis is recognized in utero by early-onset hyperkeratinization, which results in an excessively thickened stratum corneum in the 19-22 wk-old fetus [15,16]; the diagnosis of Sjögren-Larsson syndrome has been made on the basis of hyperkeratotic hair follicles, precocious keratinization of the interfollicular epidermis, and hypergranulosis of both the follicular and interfollicular epidermis [17]. Thus it is possible to diagnose disorders of keratinization at 19-21 wk EGA even though keratinization of the epidermis does not occur normally until 22-24 wks EGA [18].

We have had the opportunity to study fetal skin biopsy samples and pelleted amniotic fluid cells from three fetuses of the same family at risk for CIE using a combination of light and electron microscopy, immunohistochemistry and protein and lipid biochemistry. The skin of the first fetus was normal, that of the second was affected, and that of the third was affected, although the fetal skin biopsy samples were interpreted as normal. We report the findings from studies of skin from these three fetuses obtained at the time of biopsy, at autopsy, and after the birth of the third and affected fetus. The characteristics of expression of the disease in utero that complicate the prenatal diagnosis are discussed.

MATERIALS AND METHODS

Patient History A 22-year-old, healthy white female of Italian descent and her 23 year-old husband, first cousins, were first seen in the Genetics Clinic at The Montreal Children’s Hospital in 1979 after the delivery of a colloidon baby described as having generalized large, thick scales. A biopsy of the skin taken from the thigh of the infant at 3 wk of age revealed hyperkeratosis, parakeratosis, and minimal acanthosis of the epidermis with a slight perivascular infiltrate in the dermis. The diagnosis was reported as consistent with lamellate type of congenital ichthyosiform erythroderma or “ichthyosis vulgaris x-linked.” The infant was subsequently adopted and lost to followup. The data we have obtained by evaluating fetal skin biopsy samples, autopsy specimens, and by examining an affected newborn from a subsequent pregnancy, suggest that the disorder in this family is consistent with CIE [1-8].

In 1981, the parents sought genetic counseling for a second pregnancy (fetus 1) and were informed of the possibility of prenatal diagnosis by fetoscopy with fetal skin biopsy. Skin samples were obtained from the fetus at 21 wks EGA and examined by light (LM) and electron microscopy (EM). There were no apparent abnormalities of the epidermis. However, because the epidermis is normally not keratinized at this age, and there had been no experience in prenatal diagnosis of this disorder, two more biopsies were taken at 25 wk EGA. The skin from the fetus at this age also appeared normal, and a healthy female infant was delivered at 40 wk gestation. The sites of the biopsy were recognized by small scars on the trunk and leg.

In January of 1985, the parents returned for prenatal diagnosis of the fetus at risk in the third pregnancy (fetus 2). Six skin samples were obtained at 21 wks EGA for morphology (two biopsies) and biochemistry (four biopsies). An amniotic fluid sample was withdrawn prior to biopsy. One of the samples revealed an abnormal epidermis [19] and the parents elected to terminate the pregnancy at 22 wk gestation. The fetus was examined at autopsy, clinical photographs were taken, and samples of skin were obtained from multiple body regions for further morphologic and biochemical studies. The small wounds created by the biopsy procedure were identified near the ventral midline superior to the umbilicus, in the pectoral region, and on the ventral-lateral trunk.

In the summer of 1985 the parents again returned to Royal Victoria Hospital to arrange for fetoscopy and prenatal diagnosis of the fetus of the fourth pregnancy (fetus 3). At 19 wk, 6 d gestation, four biopsy samples and amniotic fluid were obtained for morphologic examination. On the basis of skin structure, the fetus was thought to be normal and the pregnancy was continued. A premature, male infant was born at 33 wk gestation with a severe, regional ichthyosis affecting primarily the head. Fine, white scale was apparent on the body. The condition was dissimilar from a colloidon baby. Small scars on the left lateral abdomen, chest, and axilla corresponded to the biopsy sites. All areas biopsied appeared mildly affected.

Fetoscopy and Fetal Biopsy Biopsy samples were obtained from the fetuses at risk following the procedure published elsewhere [19]. Permission to perform the biopsies in all cases was obtained from the Royal Victoria Hospital Departmental Ethics Committee. Skin biopsy specimens for microscopy were placed immediately in 2% glutaraldehyde fixative prepared in 0.1 M cacodylate buffer; those for biochemistry were placed in a carrying medium and frozen. Approximately 15 cc of amniotic fluid were withdrawn prior to biopsy and mixed with an equal volume of buffered glutaraldehyde. All specimens were shipped to the University of Washington (for LM, EM and protein biochemistry) or to the University of California San Francisco (for lipid analysis).

Light and Electron Microscopy Samples of skin for LM and EM were removed from the primary fixative, washed in 0.1 M cacodylate buffer, and post-fixed in 2% OsO₄ in distilled water for 1 h at room temperature. The specimens were then dehydrated through a graded series of alcohols into propylene oxide and embedded in Epon 812 by conventional methods [19]. All of the samples were oriented so that sections could be cut through the full thickness of the skin.

Sections for LM (1 μm) and EM (800 Å) were cut serially through a portion or all of the sample. The histologic sections were stained with toluidine blue and the thin sections were stained with saturated uranyl acetate and lead citrate. Photomicrographs were taken at various intervals in the biopsy sample using a Zeiss Photomicroscope. Thin sections were examined in a Philips 420 STEM in the transmission mode at 60 kV.

The amniotic fluid was spun in Eppendorf microfuge tubes at 12,800 × G for 1.5 min. The cell pellets were processed into plastic within the tubes. The tips containing the embedded cells were cut out of the tube and affixed to metal chucks which could be placed in the ultramicrotome for sectioning. Multiple pellets of amniotic fluid cells from fetuses 2 and 3 were prepared in this manner and sectioned for LM and EM.

Protein Biochemistry The frozen samples of skin were transferred to 5 mM ethylenediamine-tetracetic acid (EDTA) in phosphate buffered saline (PBS) and incubated for 3 min at 50°C. The samples were plunged into cold PBS and the epidermis separated from the dermis. Epidermal proteins were extracted by homogenization at 4°C in 8 M urea/0.05 M Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.1 M 2-mercaptoethanol, and the protease inhibitors PMSE (100 μg/ml) and aprotinin (100 μg/ml). The proteins were separated using the discontinuous buffer system of Laemmli [20] in polyacrylamide gradient gels (7.5%-15%). The samples were boiled for 3-5 min with 2% SDS and 3% 2-mercaptoethanol and 10-14 μg protein was loaded per lane for electrophoresis. Coomassie Brilliant blue was used to stain the proteins in the gel.

Proteins from skin homogenate, amniotic fluid were transferred electrophoretically to nitrocellulose membrane using a BioRad Transblot apparatus (overnight at 4°C 50 V in Tris-glycine buffer, pH 7.5, with 20% methanol). The blots were incubated for 1 h in 3% BSA to block additional protein binding sites, then incubated sequentially in anti-keratin monoclonal antibodies at 1/500 dilution. The blots were incubated for 3-5 min with 2% SDS and 3% 2-mercaptoethanol and 10-14 μg protein was loaded per lane for electrophoresis. Coomassie Brilliant blue was used to stain the proteins in the gel.
Lipid Biochemistry  Skin samples for lipid analysis were handled only with forceps, wrapped in aluminum foil, frozen at -70°C, and shipped to San Francisco on dry ice. The tissue was thawed and any visible subcutaneous fat was carefully scraped away from the dermis. Full-thickness samples were floated dermis side down in calcium-free, magnesium-free phosphate buffered saline (Dulbecco’s) containing 1 mM EDTA (Sigma) at 4°C for 1–4 h. The epidermis was peeled from the dermis using solvent-rinsed forceps. The completeness of the separations was monitored on representative samples by histology.

Tissue lipids were extracted according to the method of Bligh and Dyer [22] as modified [23,24]. In samples to be analyzed for cholesterol sulfate, 0.1 M KCl [24] was included to prevent loss of this lipid into the water phase. All lipids were stored in benzene under nitrogen at -20°C until further analyzed. Lipid composition was analyzed using silica-quartz rod microchromatography/flame ionization (latroscan THIO-Mark III-TCL Analyzer; Ancal Inc., Los Osos, CA), as previously described [25]. This method offers the advantage of analyzing complex lipids from small amounts of starting material and compares favorably with other methods of analysis [26–28]. Briefly, 20 μg of lipid in chloroform: methanol (2:1, vols) was spotted on each chromarod and the rods sequentially developed in 1) hexane: diethyl ether: water (80:20:1 vols) to 2/3 the rod length then hexane alone to top for neutral lipids; and 2) chloroform: methanol: water (50:25:3, vols) for polar lipids. After each development the rods were heated briefly and run through a flame ionization detector. All of the chromarods (Type SII) were pretreated with known standards in a mixture whose composition was similar to our sample lipids. Each sample was analyzed on a minimum of three rods. Data were expressed as the means of these determinations.

Immunohistochemistry Monoclonal antibodies AE1, AE2, and AE3 against keratin polypeptides [29] were a generous gift from Dr. James Rheinwald, Dana Farber Cancer Research Institute, Boston. Anti-involucrin was purchased commercially (Biomedical Technologies, Inc. Stoughton, MA) and the monoclonal antibody that recognizes pro-filaggrin and filaggrin (AKH1) was prepared as reported previously [30].

Biopsy and autopsy specimens were fixed briefly in Carnoy’s fixative, rinsed in absolute alcohol, rehydrated by 70% alcohol, and embedded in paraffin. Sections of 5-μm thickness were stained by the avidin-biotin-peroxidase complex (ABC) method [31]. Antibody dilutions were 1/1000 to 1/5000 for the monoclonal antibodies and control ascites fluid. Sections were incubated first in normal horse serum, then in the primary antibody for 30 min, in biotin-conjugated-horse-anti-mouse IgG and IgM for 30 min, and in avidin-dextran with biotin-conjugated peroxidase for 45 min (Tago, Inc., Burlingame, CA). Sections were rinsed extensively in PBS between incubations. The color reaction was developed by incubating the sections in the substrate described above for 10 min.

RESULTS

Fetus 1: Light Microscopy and Electron Microscopy Three biopsy samples were obtained at 21 wks EGA from unknown sites. The nonkeratinized, interfollicular epidermis of each was 5 cell layers thick (basal plus intermediate layers) and covered with a thin layer of stratum corneum that could be mistaken for epidermal keratinization. A thin layer of stratum corneum covers the 25-wk fetal epidermis. a: Remnants of desquamated periderm at the epidermal surface demonstrate that the stratum corneum is not unusually thick, hence, it is likely to be normal. a: ×250; b: ×180; d: ×250.

cornified cells, 1–2 layers of granular cells, 3–4 spinous cell layers, and a single basal layer (Fig 1c). Granular cells contained normal-appearing keratohyaline granules, lamellar granules, and other cytoplasmic organelles. Cells of the stratum corneum were electron-dense and devoid of organelles and lipid droplets. Remnants of the periderm and a few desquamated cells remained associated with the stratum corneum (Fig 1d). These findings were consistent with a normal fetus and the pregnancy was continued. A healthy female infant was delivered at 40 wk gestation.

Fetus 2: Light and Electron Microscopy

Fetal Skin Biopsy Samples These data have been reported in a preliminary paper documenting successful prenatal diagnosis of the affected fetus [19]. Briefly, the interfollicular epidermis of one biopsy sample appeared normal (Fig 2a). The interfollicular epidermis of the other sample had a single layer of basal cells, 3–4 layers of spinous cells, 1–3 layers of granular cells, and 3–15 layers of flattened, parakeratotic cells above the granular layer (Fig 2b,c). The periderm covered the epidermis of both specimens (Fig 2a,c,e). In the abnormal biopsy, basal cells were poorly aligned, widely separated, and more electron-dense than spinous cells (Fig 2d, e). Spinous cells contained small, keratin filament bundles that appeared fragmented (Fig 2d, e). The granular cells were vacuolated and there were large, keratohyaline granules associated with small,
sparse bundles of keratin filaments (Fig 2c, e). Normal lamellar granules were not evident, although granular and cornified cells had pockets of infolded plasma membrane that contained cellular debris (Fig 2c, e). The incompletely keratinized cells contained vacuoles, mitochondria, and remnants of the nucleus and organelles (Fig 2c, e). They had cornified cell envelopes and were joined by typical (non-modified) desmosomes (Fig 2e).

Most hair follicles were extensively keratinized; hair canals were occluded and hairs were seldom evident (Fig 2a). Cornified cells lining the hair canals appeared more completely keratinized, but

Figure 2. Light (a and b) and electron (c-f) micrographs of skin biopsy samples from fetus 2 obtained at 21 wk EGA. a: The epidermis in one biopsy sample is 4–5 cell layers thick (see vertical line) and covered with periderm (P). Note the occlusion of a hair canal (HC) with keratinized material. b: The second biopsy sample has a normal number of layers of viable cells, a granular layer (G), and multiple layers of incompletely keratinized cells (see vertical line). Note periderm (P) at the epidermal surface. c: Six layers of incompletely keratinized cells are superficial to the granular layer (G) and covered with periderm (P). d: Basal cells (B) are more electron-dense than superficial cells and separated by spaces that contain extracellular debris (asterisk). Small keratin filament bundles in spinous cells (S) are evident only where associated with desmosomes (arrows). e: The small filament bundles (arrowheads) are evident in a spinous cell (S); granular cells (G) contain keratohyaline granules, remnants of organelles, and infoldings of the cell membrane (arrows), but no true lamellar granules. The granular cells are bounded by a cornified cell envelope. f: Hair canal showing normal-appearing granular cells (G). The keratinized cells (KC) are more completely keratinized than those of interfollicular epidermis but show spaces (arrows) where lipid was dissolved during tissue processing. a, b: X 350; c, d: X 4300; e, f: X 10,650.
The nails and hair appeared normal and there was no remarkable skin, recognized as white, translucent plaques in contrast to the dorsal and ventral surfaces of the trunk lateral to the midline. The pregnancy was terminated at 22 wk EGA and the fetus examined for evidence of regional variation in keratinization and to identify biopsy sites. Hyperkeratotic skin, recognized as white, translucent plaques in contrast to the more typically red skin, was found around the ears and mouth and along the dorsal and ventral surfaces of the trunk lateral to the midline. The nails and hair appeared normal and there was no remarkable thickening of the palms or soles. [The gross observations contrast with the histology (see below)]. The biopsy sites were located with difficulty, suggesting that wounds heal well in utero.

Samples of skin for LM and EM, immunohistochemistry, and biochemical analysis of epidermal proteins and lipids were obtained from the scalp, chest, lateral and medial surfaces of the leg, palm and sole, upper arm, upper back, lateral thigh, and face.

The histologic studies revealed regional variability in expression of the disorder in utero (Fig 4; Table I). Numerous layers of incompletely keratinized, superficial cells (similar to the affected fetal biopsy sample) were characteristic for the face, scalp, palm, and sole (Fig 4a). The last three regions also had several additional layers of densely staining, keratinized cells proximal to the viable epidermal layers (Figs 4c, d). The more normal appearing stratum corneum cells showed fewer profiles of organelles and lacked the nucleus, but contained vacuoles that were suggestive of retained lipid (Fig 4d). The epidermis of the chest, upper arm, upper back, thigh, and medial and lateral leg was somewhat less thickened (Fig 4b).

All samples of hairy skin showed excessive keratinization of hair canals (Figs 4a–c).

### Protein Biochemistry

**Fetal Skin Biopsy Samples** Proteins were extracted from the epidermis of several biopsy samples, displayed on SDS polyacrylamide gels, blotted onto nitrocellulose paper, and stained with antibodies to keratin and filaggrin proteins. No differences were found compared with samples from a normal age-matched fetus [19].

**Skin Autopsy Samples** Coomassie blue-stained gels and immunostained Western blots of extracted epidermal proteins revealed strong bands corresponding to the distribution of 40, 48, 50, 56/56.5, 58, 64, and 67 kDa keratins. The 40 kDa keratin is typical for fetal epidermis [32], the 64 kDa keratin is normally present in palm and sole epidermis [33], and the 50, 56/56.6, 58, and 67 kDa keratins are present in normal adult epidermis [29]. Keratins with molecular weights of 48 and 65 were present in variable quantities depending upon the region (Fig 5 and Table II). The 48-kDa keratin (along with the 56-kDa keratin) is a marker of hyperproliferation [9] in pathologic adult epidermis, but it is also present normally in developing human epidermis. Thus the significance of its presence in the autopsy samples cannot necessarily be attributed to in utero expression of disease. The 65-kDa keratin is the post-translationally modified 67-kDa that is characteristic of neonatal and adult keratinized epidermis [32].

Palm and sole had very minor quantities of the 48-kDa keratin. The epidermis from regions that were more similar to normal fetal skin (chest, arm, thigh, and leg) had little evidence of the 65-kDa keratin in comparison with the more keratinized regions (palm, sole, face, scalp, and back). The quantity of filaggrin (37 kDa) also correlated with the state of keratinization, thus appeared prominent in the extracts from the palm sole, scalp and face, and, to a lesser extent, the back. Immunostained blot preparations of the gels emphasized the specific differences in keratin polypeptides among the regions (Fig 5). These studies demonstrated that the expression of keratin and filaggrin proteins correlated with the extent of keratinization of the region and did not reveal protein abnormalities specific for CIE.

### Table I: Status of Epidermal Keratinization as Determined by Morphology and Biochemistry of Multiple Body Regions Sampled from Fetus 2 at Autopsy

<table>
<thead>
<tr>
<th>Region</th>
<th>Thickness/ Superficial Layers of Incompletely Keratinized Cells</th>
<th>Presence of More Typically Cornified Cells</th>
<th>Extracts of Epidermal Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 kDa 56.6/67 kDa 65 kDa Filaggrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sole</td>
<td>++</td>
<td>++</td>
<td>++      ++</td>
</tr>
<tr>
<td>Palm</td>
<td>++</td>
<td>++</td>
<td>++      ++</td>
</tr>
<tr>
<td>Scalp</td>
<td>++</td>
<td>++</td>
<td>++      ++</td>
</tr>
<tr>
<td>Face</td>
<td>++</td>
<td>++</td>
<td>++      ++</td>
</tr>
<tr>
<td>Upper back</td>
<td>++</td>
<td>++</td>
<td>++      ++</td>
</tr>
<tr>
<td>Chest</td>
<td>++</td>
<td>++</td>
<td>++      ++</td>
</tr>
<tr>
<td>Upper arm</td>
<td>++</td>
<td>++</td>
<td>++      ++</td>
</tr>
<tr>
<td>Lateral thigh</td>
<td>++</td>
<td>++</td>
<td>++      ++</td>
</tr>
<tr>
<td>Lateral &amp; medial leg</td>
<td>++</td>
<td>++</td>
<td>++      ++</td>
</tr>
</tbody>
</table>

*The incompletely keratinized cells are located superficial to the more normally keratinized cells.*
Figure 4. Light (a-c) and electron (d) micrographs of skin samples obtained from fetus 2 at autopsy. a: Thickened interfollicular and follicular epidermis from the face. Note the stacks of incompletely keratinized cells (arrows). b: The epidermis of the chest shows more normal numbers of cell layers in the interfollicular epidermis but marked hyperkeratinization of the hair canals (HC). c: Epidermis from the palm shows superficial layers of incompletely keratinized cells and an excessive number of cell layers that appear to be more typically keratinized located proximal to the viable epidermis. c: Incompletely keratinized cells compared with more completely keratinized cells. The latter cells contain profiles (arrows) suggestive of lipid droplets. These are characteristic of ichthyotic epidermis. a-c: X 400; d: X 25,660.

Immunocytochemistry Paraffin sections of skin were stained with the AE1, AE2, AE3, and 40-Kd antikeratin antibodies, anti-involucrin and AKH1. The epidermal layers and structures normally stained by these antibodies in adult and fetal epidermis have been reported elsewhere [29,30,32] and are summarized on Table II.

In all of the autopsy samples, AE1 labeled basal cells intensely and suprabasal cells with variable intensity (Fig 6a,b). AE1-positive suprabasal cells are characteristic of hyperplastic epithelia. The viable, suprabasal cells, and the incompletely keratinized, superficial cells, stained with the AE2 antibody (Fig 6c). The more normally keratinized cells of the palm, sole, face, and scalp did not react with the antibody. AE3 stained basal and suprabasal layers prominently, incompletely keratinized cells variably, and failed to stain keratinized cells of the stratum corneum and the thickened lining of the hair canals (Fig 6d). The outer layers of the follicle and the intraepidermal and dermal portions of the sweat duct stained positively.

The anti-40-kDa keratin stained basal cells of all regions except palm and sole. Staining ended abruptly at sites where interfollicular basal cells merged with outer cells of the hair follicle (Fig 6e). The sebaceous gland, cells of the outer root sheath distal to the sebaceous gland, and sweat ducts also stained positively.

Two to three layers of suprabasal cells and the inner cells of the sweat duct of the palm and sole stained with a faintly positive reaction.

Keratinized cells of the hyperkeratotic hair canals, the incompletely keratinized epidermal cells, and a variable number of uppermost, suprabasal cell layers stained with the anti-involucrin antibody (Fig 6f). The keratinized layers of the palm and sole showed a negative reaction as is typical for normal adult stratum corneum.

The AKH1 antibody recognized keratohyaline granules in the several granular layers of the scalp, face, palm, and sole (Fig 6g) and in granular layers surrounding hair canals (Fig 6h) of the chest, upper arm, upper back, and thigh.

The antibodies that are considered markers of keratinization (AE2, anti-involucrin and AKH1) were expressed most consistently in the regions of thickened, superficial layers of abnormally or incompletely keratinized cells (face, scalp). Skin of the palm and sole have a number of similarities with these regions even though the epidermis in these regions in the adult normally stains with a different pattern than that of the remainder of the body.

Lipid Biochemistry Lipid Biochemistry was performed on selected autopsy samples (Table III). The lipid composition of epidermis from normal fetal skin after 130d EGA is characterized by a large contribution from sterol esters and triglycerides [34]. These fractions are most prominent in older fetal material and in cephalad body sites [34] (Table III) in parallel with the development of sebaceous glands and follicular keratinization [34]. The lipid compositi-
tion of fetus 2 deviated somewhat from the normal pattern in that, while the sterol ester contribution of arm and back epidermis was higher or comparable to normal fetuses [38.7% and 41.8% vs. 12.5% and 17% (19 wk normal fetus) and 44.1% and 52.1% (20 wk normal fetus)], the triglyceride content was somewhat lower than expected [8.5% and 6.3% vs. 11.8% and 20.1% (19 wk normal fetus) and 15.1% and 15.3% (20 wk normal fetus)] and the free sterol content was somewhat higher than expected [19.8% and 23.3% vs. 11.4% and 14.9% (19 wk normal fetus) and 13.1% and

13.0% (20 wk normal fetus)]. Thus only the triglyceride data reflect what might be expected of a fetus affected with CIE [1,2].

**Fetus 3: Light and Electron Microscopy**

**Fetal Skin Biopsy Samples** Four biopsy samples from the ventral and lateral surfaces of the trunk were obtained from the fetus at risk. All of the samples revealed a five-layered epidermis covered by periderm; none showed evidence of hyperkeratinization or thickening of the superficial epidermal layers (Fig 7a, b). The linings of the hair canals varied in thickness. On the basis of epidermal morphology it was concluded that the skin of this fetus was consistent with that of an unaffected fetus and the pregnancy was continued. An affected male was born at 33 wk EGA.

**Amniotic Fluid Cells** Amniotic fluid was sampled prior to biopsy. Sections through the cell pellet revealed some stacks of cells, but they were not as abundant as noted in the sample from fetus 2. As part of the re-evaluation (see below) the blocks containing amniotic fluid pellets were reoriented for sectioning and a morphometric study of the different cell populations was performed (data to be reported elsewhere). From the more in-depth, retrospective study, it was clear that the amniotic fluid reflected epidermal hyperkeratosis.

**Affected Newborn** The skin of the head was strikingly abnormal at birth, described by the clinicians as similar to harlequin ichthyosis in severity; the body was covered with a mild, white scale. The marked clinical differences in the skin from the two regions were reflected primarily by the thickness of the stratum corneum (Fig 8a, b). In both regions, the cornified layers were electron dense. The extent of keratinization varied from multiple layers of incompletely cornified cells to keratinized cells that were electron dense (Fig 8c, d). Profiles of nuclei and other organelles were easily recognized in the first type of cells, but were indistinct in the more cornified cells. Both types of cornified cells contained vacuoles suggestive of lipid droplets (Fig 8c, d). The viable epidermis was acanthotic in both regions. Normal lamellar granules were absent, although small, granule-like structures were common in the granular cells. Hair canals were markedly hyperkeratotic.

Other studies of the newborn were not undertaken because the tissue was not available.

**DISCUSSION**

Examination of fetal skin biopsy samples is rarely undertaken without prior studies of skin from affected family members. This is particularly important when there has been no previous experience in recognizing the disorder in utero. In this family, neither parent was affected and the affected infant was unavailable. Thus the skin biopsy samples from fetus 1 were evaluated in 1981 with the clear understanding of the disorder at risk or the criteria which might identify the disease in utero. Nonetheless, because of the experience in recognizing other keratinization disorders in utero using skin biopsy samples [12-17], our understanding of normal fetal skin morphology and biochemistry [18], and the parent’s strong desire for prenatal diagnosis, it was thought that LI or CIE could be recognized before birth. It was hypothesized that an affected fetus might be revealed by early onset hyperkeratinization, as is characteristic for harlequin ichthyosis [15,16]. It was appreciated, however, that normal-appearing skin would not rule out the possibility of an affected fetus, because the onset of the disease could be concurrent with the onset of keratinization at approximately 24 wk EGA, and the parents were so counseled.

The morphologic and biochemical studies of the skin samples from fetus 2 revealed several features that increase our understanding of the expression of this disorder in utero and provide guidelines for prenatal diagnosis: 1) There is regional variation in the onset of the disorder that appears to correlate with the regional rate of differentiation of the epidermis [35]. This suggests that several fetal skin biopsies should be obtained from varied regions (to include the scalp) to optimize chances of recognizing the disorder. 2) The epidermis in affected regions has multiple layers of flattened, superfili...
Morphologic evidence of abnormal keratinization in this fetus, such as observed in this case, can be used diagnostically in the fetus. An enlarged database of normal, fetal, epidermal lipid composition will be required before variations in epidermal lipid composition, that reported for children and adults affected with CIE [1,2], is explained. Because living, affected offspring or adult family members were not available for study, this remains unanswered. An enlarged database of normal, fetal, epidermal lipid composition will be required before variations in epidermal lipid composition, such as observed in this case, can be used diagnostically in the fetus. The more normal keratinization of the follicles, compared with the interfollicular epidermis, may correlate with the earlier onset of follicular keratinization compared with interfollicular keratinization. The hair cone and hair canal keratinize around 15 wk, approximately 8-9 wk ahead of interfollicular keratinization [36]. The first keratinized cells that form in normal fetal epidermis around 22-24 wk EGA are incompletely keratinized, and the next several layers are similar to adult cornified cells [18]. The thickened but incompletely keratinized cells in the abnormal fetal skin samples appeared even less differentiated than the first keratinized cells in normal fetal epidermis. With extended time, the abnormal epidermal cells will keratinize in a more complete manner. The observations support an interpretation that there is early onset hyperproliferation but not keratinization. It is possible that the rapid proliferation in the epidermis of the embryo/early fetus [37] is sustained in an unregulated manner in fetuses affected with CIE rather than slowed to an adult-like rate late in the first trimester [37].

Because of the discrepancies in the morphology of the biopsies from fetus 2, it was important to document regional variation in the expression of CIE in utero. Studies of skin from multiple regions of the abortus revealed excessively thickened epidermis on the palm, sole, scalp, face, and to a lesser extent, the back, and hyperkeratotic follicles that bulged from the skin surface and were filled with keratogenous debris. The epidermis of the palms, soles, scalp, and face differentiated ahead of the trunk normally [35].

### Table II. Immunolabeling Characteristics of the Epidermis from Multiple Regions of the Body Sampled from Fetus 2 at Autopsy

<table>
<thead>
<tr>
<th>Cell Layer or Structure Normally Labeled In:</th>
<th>Structure/and or Layers Stained in Autopsy Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Adult Epidermis</td>
</tr>
<tr>
<td>AE1 Basal cells of normal epidermis; suprabasal cells of hyperplastic epidermis</td>
<td>Basal and periderm cells</td>
</tr>
<tr>
<td>AE2 Suprabasal cells</td>
<td>All suprabasal cells of fetal epidermis &gt; 60 d EGA, except periderm</td>
</tr>
<tr>
<td>AE3 All viable adult layers</td>
<td>All layers of the fetal epidermis including periderm</td>
</tr>
<tr>
<td>40 kDa Not expressed in adult epidermis; marker of simple epithelium</td>
<td>Basal and periderm cells</td>
</tr>
<tr>
<td>Anti-involucrin Granular cells</td>
<td>(data not reported)</td>
</tr>
<tr>
<td>AKH1 Granular cells</td>
<td>Granular cells</td>
</tr>
</tbody>
</table>

* Data from Ref 29.

b Data from Ref 32.

cial cells that are poorly differentiated; granular layer cells lack normal lamellar granules, and periderm covers the thickened epidermis. 3) The follicles are hyperkeratotic; the cornified cells lining the hair canals are more completely keratinized than interfollicular epidermal keratinocytes but contain abundant vacuoles and lipid droplets. 4) Aggregations of cells in the amniotic fluid may reflect the condition of the skin. The differences in quantity of keratinization-specific proteins reflect the state of differentiation of the epidermis in that region.

The profile of epidermal lipids from fetus 2 was not identical to that reported for children and adults affected with CIE [1,2]. Although triglycerides were somewhat reduced compared with two normal fetuses, an elevation in hydrocarbons was not observed. Morphologic evidence of abnormal keratinization in this fetus suggests that the defect in cornification may be several steps removed from a primary lipid defect in CIE, that this property is not expressed in adult epidermis.

### Table III. Epidermal Lipid Composition of Selected Autopsy Sites from Fetus 2 Compared with Normal Fetal Skin

<table>
<thead>
<tr>
<th>Site: Lipid Fraction</th>
<th>Fetus 2 (22 WWKS EGA)</th>
<th>Normal Fetus 1&lt;sup&gt;b&lt;/sup&gt; (19 WKS EGA)</th>
<th>Normal Fetus 2&lt;sup&gt;b&lt;/sup&gt; (20 WKS EGA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upper Arm</td>
<td>Lower Back</td>
<td>Arm</td>
</tr>
<tr>
<td>Hydrocarbon</td>
<td>N.D.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N.D.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>Sterol/Wax Esters</td>
<td>38.7</td>
<td>41.8</td>
<td>12.5</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>8.5</td>
<td>6.3</td>
<td>11.8</td>
</tr>
<tr>
<td>Free Fatty Acids</td>
<td>6.6</td>
<td>5.4</td>
<td>8.6</td>
</tr>
<tr>
<td>Free Sterols</td>
<td>19.8</td>
<td>23.3</td>
<td>11.4</td>
</tr>
<tr>
<td>Sphingolipids</td>
<td>14.7</td>
<td>14.6</td>
<td>16</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>9.5</td>
<td>8.5</td>
<td>29.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Normals from Williams et al [34]

<sup>b</sup> The epidermis was separated from dermis using EDTA; lipids were extracted by liquid scintillation (see Methods). The data represent the mean of three rods each and are expressed as % total lipid.

<sup>c</sup> N.D. = none detected
Figure 6. Immunohistochemical labeling of paraffin sections of skin from various regions obtained from the affected fetus at autopsy. a: Basal cells, external root sheath, and outer layers cells of the sweat duct (arrows) of the cheek are labeled with AE1, but the chest (b) shows suprabasal labeling with the antibody characteristic for hyperplastic epithelia. c: AE2 labels the viable epidermal layers and thickened layers of cells surrounding the hair canals as illustrated by staining of the chest. d: AE3 stains keratin in all viable layers of the epidermis, the nonkeratinized layers of the hair follicle, and both layers of the sweat duct. The keratinized epidermal cells and cells lining the hair canal do not stain with this antibody. The example is from the face. e: The anti-40 kDa keratin labels only basal epidermal cells. Note the absence of staining of cells of the follicle. The region illustrated is the back. f and h: The AKH1 antibody stained granular and incompletely and completely keratinized cells of the epidermis and hair canals. The region illustrated is the back. g and h: The AKH1 antibody stained granular and some of the cornified layers of the most highly keratinized regions of the fetus (face, scalp, palm, and sole). In regions where the superficial cells were incompletely keratinized (e.g., chest) AKH1 staining was restricted to the more completely keratinized cells lining the hair canals. a–h: ×300.

and immunohistochemical data are consistent with differences in the extent of keratinization. It is important to note, however, that this pattern of expression may be characteristic only for this family. Moreover, it is recognized that disorders of keratinization demonstrate significant clinical heterogeneity [2].

Difficulty in identifying an affected fetus is apparent from the outcome of the prenatal diagnosis of fetus 3. The four biopsy samples examined from this fetus appeared normal. The structure of the skin was believed to be consistent with that of the normal fetus in the same family, thus the birth of an affected infant at 33 wk EGA was entirely unanticipated. Review of the biopsy samples was undertaken and new sections were cut deeper into the tissue remaining in the blocks. No new data that would have reflected an affected fetus were revealed. The scars from the biopsy were all identified on the trunk, which appeared at birth to be mildly involved. Although the biopsy samples did not show signs of the disorder at 19 wk EGA, the epidermis of this mildly affected skin at birth was characteristic of CIE (see Fig 8b).

Several prenatal diagnoses of fetuses at risk for IL and CIE have been attempted [38]. In all cases the results of the fetal skin biopsy suggested a normal fetus. In one case of CIE (1 of the 9 cases of congenital ichthyosis), however, a false-negative judgement was made. The findings were identical to ours. The skin appeared normal at 20 wk (menstrual age). The interfollicular epidermis was not keratinized and there was no evidence of lipid accumulation in epidermal cells as was characteristic for the affected sib. Hyperkeratinization of the follicular orifices was noted, but the investigators felt that this was insufficient evidence on which to base a positive
diagnosis. Similar difficulties in the prenatal diagnosis of lamellar ichthyosis are unpublished. Fetal skin biopsy samples, examined independently by two laboratories, were read as normal, but the fetus was born mildly affected.

From these studies it can be concluded that the onset of LI and CIE in utero may coincide with the onset of keratinization and that prenatal diagnosis of these disorders must be undertaken with extreme caution. Multiple biopsy sites should be sampled, probably to include the scalp, and several methods of evaluation should be employed. Lipid analysis may be a sensitive measure of disease expression, but further studies of normal and affected fetal skin are needed to determine whether this will be reliable. Aggregates of cells in the amniotic fluid may reflect the disorder, but this is a time-consuming evaluation; cell pellets must be prepared in a manner that assures appropriate sampling of cells of various sizes and density. The findings in the amniotic fluid cells apply only to those fluids obtained

Figure 7. Light micrographs of sections through two different skin biopsy samples from fetus 3. The thickness of the epidermis at all sites around the periphery of the specimens appears normal. Some of the hair canals appear hyperkeratotic (arrows), others do not (arrowheads). a, b: ×40.

Figure 8. Light (a and b) and electron (c and d) micrographs of skin from the head and trunk of the affected newborn infant. Epidermis from both the head (a) and trunk (b) shows a thick, compact stratum corneum and acanthosis of the viable epidermis. Hyperkeratosis of the hair canal is evident in a. Cells of the stratum corneum vary from incompletely keratinized cells characteristic of the fetal condition (c) to the more completely keratinized cells typical of fetal palm, sole, face, scalp, and hair canals. Note the lipid droplets (arrows) present in both types of stratum corneum. a, b: ×400; c, d: ×6860.
pre-biopsy at 19–20 wk EGA and cannot be extrapolated to cells obtained at amniocentesis, which is normally performed 3–5 wk earlier.

Morphology of the follicular and interfollicular epidermis may provide the most useful evidence of an affected fetus. The kinds of findings that are characteristic for CIE in utero are never characteristic for a normal fetus at 19–20 wk EGA, but the observation of normal-appearing epidermis in a fetus at risk may not indicate a normal fetus, and regional variation in expression of the disease is a serious problem. It would be advantageous to delay prenatal diagnosis of this disorder until the latest possible fetal age within the limits of the law and safety for the mother, and to obtain as many samples as is reasonably possible. Moreover, it needs to be cautioned that at present there is experience in prenatal diagnosis of CIE only from this family. Finally, owing to the limitations of morphology and biochemistry as a basis for prenatal diagnosis, efforts should be directed toward the development of molecular markers, such as restriction fragment length polymorphisms (RFLPs), for these disorders in specific families. Such markers will permit prenatal diagnosis to be moved back to 10–12 weeks EGA and to be accomplished by chorionic villi sampling.

The authors acknowledge the expert assistance of Mr. Robert Underwood for the preparation of photographs and of Mrs. Janet Kimball for the biochemistry and immunohistochemical staining. This work was supported by grants HD 17664, AR 21537, and DE 04660 from the National Institutes of Health.

REFERENCES


27. Harvey HR, Rigler MW, Patton JS: The use of the Iatroscan TH-10 analyzer to quantify total lipids in a variety of sample types and lipid classes in human gall bladder bile. Lipids 20:542–545, 1985


