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Keratinocytes Cultured From Subjects With Ichthyosis Vulgaris Are Phenotypically Abnormal*

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Ichthyosis vulgaris (IV) is an autosomal dominant, scaling disorder in which keratohyaline granules and filaggrin are reduced in or absent from the epidermis of affected individuals. Morphologic and biochemical markers of epidermal differentiation were studied in keratinocytes cultured from clinically unaffected skin of patients with IV, from clinically unaffected skin of an obligate gene carrier, and from normal skin of unaffected family members and an adult volunteer. Cultured keratinocytes from affected subjects formed thickened layers of scaly cells that failed to react with monoclonal antibody to filaggrin. In contrast, normal cells contained many large, immunoreactive granules. Electron microscopy confirmed the absence of keratohyaline granules in affected cells and the presence of large keratohyaline granules in normal cells. Immunoblot analysis of keratinocyte extracts from subjects with ichthyosis showed that profilaggrin was absent, but no differences in keratins were detected between affected and control cells. For all parameters, findings in cells of the clinically unaffected obligate gene carrier were intermediate between those from affected patients and controls. We conclude that keratinocytes cultured from patients with IV maintain structural and biochemical phenotypic characteristics of the disease in vitro. J Invest Dermatol 88:640–645, 1987

The ichthyoses are a heterogeneous group of inherited diseases characterized clinically by scaling [1–5]. In all of the ichthyoses, epidermal differentiation is abnormal [5]. Transplantation studies using nude mice suggest that in some of the ichthyoses the gene defect for these abnormalities resides in the epidermis [6,7].

Of the ichthyoses, ichthyosis vulgaris (IV) is the most common, occurring with a frequency of approximately 1 in 250 [8]. The defect in IV is manifest histologically by mild hyperkeratosis and a decrease or absence of keratohyaline granules from the granular layer [2,9]. Biochemically, filaggrin and its high-molecular-weight precursor, profilaggrin, are absent or decreased in biopsies of epidermis from subjects with IV [10].

We have recently demonstrated unusually well-differentiated cultures of normal neonatal human foreskin keratinocytes in which profilaggrin is expressed but not processed to filaggrin [11]. Because of the known morphologic and biochemical abnormalities in IV and the extent of morphologic and biochemical differentiation now possible in cultured normal human epidermal keratinocytes, we hypothesized that epidermal keratinocytes cultured from subjects with IV would manifest the morphologic and biochemical abnormalities of the disease and could provide an in vitro system for the study of this disorder.

**MATERIALS AND METHODS**

**Patients** Proband were ascertained through the Dermatology Clinics of the University of Washington and Children’s Hospital and Medical Center. Diagnosis of the proband was made on the basis of pedigree, scaling typical of IV, keratosis pilaris, and hyperlinearity of the palms. Affected family members had two or more of the typical features associated with IV [8]. A total of 9 subjects from 3 families were analyzed (Fig 1). None of the subjects reported by Sybert et al [10] was cultured. One clinically normal carrier (A I-1) was identified on the basis of pedigree analysis. One affected individual (B I-2) had eczema and was under treatment for both eczema (topical 0.5% triaminolone acetonide and lubrication) and ichthyosis (lubricant containing keratolytic). When available, unaffected family members were used as intrainfamilial controls to avoid the potential confounding factor of multiple allelism. An unrelated 38-year-old healthy male was biopsied at the time of family A. For identification, subjects are labeled by family (A, B, and C), generation (I, II, III), and subject number (1, 2, 3, etc) (Fig 1).

After obtaining informed consent, both blisters (see below) and punch biopsies were obtained from the posterior aspect of the nondominant arm. All subjects had been instructed to avoid applying any therapeutic agents for 48 h prior to biopsy. Punch biopsies for electron microscopy, histochemical and biochemical analysis were taken after 1% lidocaine anesthesia.

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Abbreviations:

- DMEM: Dulbecco's modified Eagle's medium
- HBS: HEPES-buffered saline
- IV: ichthyosis vulgaris
- PMFS: phenylmethylsulfonyl fluoride

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Figure 1. Pedigree. Solid circles and solid squares indicate affected individuals. Open circle with dot in center indicates obligate gene carrier. Small solid triangles indicate probands. Small open circles denote patients examined and biopsied. Small closed circles indicate examination only.

Keratinocyte Culture Keratinocytes were obtained from suction blisters [12]. Blister roofs were rinsed in HEPES-buffered saline (HBS) [13], placed in 0.05% trypsin-0.02% EDTA (GIBCO Laboratories, Grand Island, New York) and incubated at 37°C for 25 min with periodic agitation. Cells were pelleted by centrifugation at 250 g, resuspended in Dulbecco’s modified Eagle’s medium (DMEM, low glucose, GIBCO) containing 20% fetal calf serum (Hy-Clone, Logan, Utah), 0.4 μg/ml hydrocortisone, 100 U penicillin-100 μg streptomycin/ml, 50 μg/ml gentamicin, and 10−10 M chola toxin and seeded onto mitomycin C-treated ST3 cells. Gentamicin was omitted after the first feeding, while 10 ng/ml epidermal growth factor (culture grade, Collaborative Research, Waltham, Massachusetts) was added 3–5 days after feeding. All other culture techniques followed modifications [11] of the methods of Rheinwald and Green [14,15] with the exception that serum concentration was maintained at 20% in all passages. Because profilagrin and the 67 kD keratin are not expressed in preconfluent keratinocytes [16], cultured cells were studied in the first or second passage 1–3 weeks after reaching confluence.

Morphology of Cell Cultures Cell cultures were prepared for light and electron microscopy and examined as previously described [11].

Immunolocalization of Filaggrin and Profilagrin in Biopsies and Cultured Cells Procedures paralleled those previously reported [11,17]. Cells were incubated with monoclonal anti-filaggrin antibody AKH1 [17] and binding detected with fluorescein-conjugated goat antirabbit antiserum. Coverslips were mounted and the cells observed and photographed on a Zeiss standard microscope equipped for epifluorescence.

Immunoperoxidase Localization Biopsies were processed as previously described [10], with the exception that reagents appropriate for mouse monoclonal IgG replaced those used for rabbit polyclonal IgG. Monoclonal anti-filaggrin antibody AKH1 (1/500–1/1000) binding was localized by sequential incubation in biotin-conjugated goat antirabbit IgG, avidin-biotin-peroxidase complex, and peroxidase substrate [18]. Sections were then dehydrated, permanently mounted, and photographed using a blue filter.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, Electrophoretic Transfer of Proteins to Nitrocellulose, and Immunoperoxidase Staining of Transferred Proteins Biopsies were washed in cold PBS (Ca2+ and Mg2+ free phosphate-buffered saline), epidermis was separated from dermis in 100 mM EDTA pH 7.3 at 50°C, and homogenized in 8 mM urea, 50 mM Tris pH 7.6, 100 mM dithiothreitol, 0.13 M 2-mercaptoethanol, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), and 100 μg/ml aprotinin (Boehringer Mannheim, Indianapolis, Indiana) (urea-Tris) [11]. Cultured cells were washed with cold HBS and harvested for biochemical studies by scraping from the dish in cold 1.5 M KCl, 10 mM NaCl, 2 mM dithiothreitol, 0.5% Triton X-100, 10 mM Tris pH 8.0, and 0.5 mM PMSF (high salt buffer) [11,19]. Cytoskeletal elements from the cultured cells were solubilized from the 16,800 g pellet of the high salt extract in urea-Tris.

Equal protein loadings [20] of extracts from biopsies or cultured cells were separated by electrophoresis in discontinuous slab polyacrylamide gels [21] with 3% stacking gels and acrylamide gradients of 7.5–15% for profilaggrin and filaggrin studies and 4–15% for keratin studies. Proteins were transferred onto nitrocellulose paper electrophoretically and visualized by the peroxidase-antiperoxidase method [22]. Either monoclonal (AKH1) or polyclonal rabbit antihuman filaggrin antiserum was used to visualize...
proflaggrin and filaggrin in immunoblots. Basic (type II) keratins were visualized using monoclonal antikeratin antibody AE3 (a generous gift of Dr. T.-T. Sun, New York University) [23].

RESULTS

Three families with IV were studied (Fig 1). One individual, I-1 in family A, was clinically unaffected but an obligate gene carrier for IV (the subject had affected siblings and offspring and an unaffected mate). In vitro abnormalities seen in this subject were intermediate in severity between those of control and affected subjects. Phase photomicroscopy, electron microscopy, indirect immunofluorescence, and keratin immunoblot analysis of cultures from the first family are presented. Cultured keratinocytes from the other families showed similar results. For the sake of brevity, only profilaggrin and filaggrin immunoblots from the other two families are shown.

Culture of Pathologic Cells Keratinocytes from patients with IV plated and grew as well as those from unaffected family members (data not shown). Cells have been carried through as many as 4 passages. However, in the later passages both pathologic and control keratinocytes appeared to senesce and did not reach confluence. Secondary cultures of keratinocytes from C III-2 did not reach confluence and were not studied.

Figure 3. Electron micrographs of cultured pathologic keratinocytes. Confluent tertiary cultures from Family A. Note the large, multiple keratohyaline granules (KHG) in the normal cultures (NC). Smaller and fewer keratohyaline granules are evident in I-1 cultures, whereas cultures from the clinically affected patients contain small, sparse KHG (II-1) or lack KHG (II-2, I-2). The less dense structure in the lower left-hand corner of II-2 is a phagocytic granule (PG), seen in all keratinocyte cultures. NC left, bar = 3 μm; right bar = 2 μm; I-1 and II-1, bar = 4 μm; II-2, bar = 3 μm; I-2, bar = 2 μm.

Morphology of Cultured Cells Keratinocytes from affected subjects formed "scaly"-appearing cultures at confluence. Under phase microscopy the cuboidal pattern of basal keratinocytes was obscured by multiple layers of randomly oriented, superficial cells (Fig 2).

Electron microscopy of normal cultured cells showed multiple, large, geometric keratohyaline granules with intermediate filaments coursing through them (Fig 3). In contrast, cultured ichthyotic cells contained small and sparse or no recognizable keratohyaline granules; however, intermediate filaments were easily identified (Fig 3).

Immunohistochemistry of Cultured Cells Immunolocalization of filaggrin with the monoclonal antibody AKH1 in normal cultures revealed large, immunoreactive granules (Fig 4, [11]). In pathologic cells fluorescence appeared as punctate, cytoplasmic fluorescence, was reduced, or was undetectable.
Immunoblot Analysis of Cultured Cells Extracts of keratinocytes cultured from normal subjects contained a broad, high-molecular-weight immunoreactive band of profilagrin but no filagrin, confirming previous reports [11,24]. In contrast, extracts of ichthyotic keratinocytes contained virtually no detectable immunoreactive profilagrin (Fig 5).

Keratin expression in normal and pathologic cultured cells was identical. The bulk of the basic keratins present in both normal and pathologic cultured cells was in the 58 kD keratin (keratin #5 [25]) and the 56 kD keratin (keratin #6 [25]). However, a small but reproducible amount of the basic, 67 kD differentiation-specific keratin [26] (keratin #1 [25]) was detectable in all cultures, as previously described for normal keratinocytes [16].

Skin Biopsies Electron microscopy of skin biopsies from affected subjects confirmed the decrease or absence of keratohyaline granules (data not shown). Little or no staining with monoclonal antihuman filagrin antibody AKH1 [17] or polyclonal rabbit antihuman filagrin antiserum [11] was seen in biopsies of ichthyotic skin (data not shown). Extracts of biopsies from normal subjects contained filagrin, whereas extracts of biopsies from ichthyotic skin did not (Fig 5). These findings confirm those previously published [10]. Keratin expression in biopsies of ichthyotic skin was identical to that in normal skin (Fig 6).

Figure 5. Polyacrylamide gel and immunoblots of profilagrin and filagrin from extracts of biopsies and cultured cells of patients with IV. Note that immunoreactive filagrin (F) is decreased in biopsies from A I-1 (1) and undetectable in A I-2 (2) when compared with normal foreskin (3). Likewise, immunoreactive profilagrin (PF) is decreased in extracts of cells cultured from A I-1 (5) and undetectable in A II-1, A II-2, B I-2, and C III-1 (6,8,9,11) when compared with cells cultured from normal control (4) or unaffected family members B I-1 (10) and C II-1 (12). 1-3, immunoblots of equal protein loadings [20] of extracts from skin biopsies; 4-12, immunoblots of equal protein loadings [20] of high salt extracts of confluent secondary (Family A) and tertiary (Families B and C) cultured cells; 13, Coomassie blue-stained gel of an extract of A I-1 (stained polyacrylamide gels of all culture extracts were identical). Numbers to the left are M, of standards (kD).

Figure 6. Immunoblots of keratins from extracts of biopsies and cultured cells stained with antikeratin antibody AE3. Confluent tertiary cultures from Family A. Note that the high-molecular-weight, differentiation-specific 67 kD keratin (keratin #1 [25]) and the 58 kD keratin (keratin #5 [25]), expressed by all stratified squamous epithelia [27] are expressed in both skin and cultured skin cells. The 56 kD keratin (#6 [25]) associated with hyperproliferative epithelia [28] is found only in cultured cells. The keratin profiles for affected and control skin biopsies are identical, as are those for cultured cells. c, immunoblots of equal protein loadings [20] of extracts from cultured cells; b, immunoblots of equal protein loadings [20] of extracts from biopsies. NC, tissue from normal control; I-1, II-1, II-2, and I-2, tissue from members of Family A. Numbers to the left are M, of standards (kD).
DISCUSSION

In this paper we show that epidermal keratinocytes cultured from subjects with IV manifest morphologic and biochemical abnormalities found in skin biopsies from the same and from other affected individuals. The absence of keratohyaline granules in cultured ichthyotic cells was confirmed morphologically, by immunohistochemistry using monoclonal antifilaggrin antibody, and by analysis of immunoblots of confluent cell extracts. The presence and distribution of another group of biochemical markers of epidermal differentiation, the keratins, was unchanged. Morphologic and biochemical abnormalities in cells cultured from a clinically normal obligate gene carrier were intermediate between those from clinically affected subjects and controls; thus the degree of abnormality in vitro correlated with clinical severity. While it is possible that a humoral factor from the original blisters is responsible for expression of the abnormal phenotype, secondary and tertiary cultures of dissociated keratinocytes maintained the abnormalities to the same extent as primary cultures, suggesting that the defect is intrinsic to the epidermal cells.

Studies of cells from subjects with the ichthyoses are limited. Fibroblasts cultured from patients with Refsrum's disease and X-linked ichthyosis display biochemical abnormalities [29,30]. Previous studies of pathologic epidermal cells have shown abnormalities in keratinocytes cultured from subjects with psoriasis [31] and X-linked ichthyosis [32]. With this report, IV can be added to the list.

In affected patients, the clinical severity of the disease and of the filaggrin and profilaggrin defects of IV fluctuate with environmental conditions, including humidity and temperature [4,8,10]. One might predict that in culture, where humidity and temperature are high, abnormalities in morphology and filaggrin expression would be less pronounced. Our observations of a clinically unaffected obligate gene carrier suggest that, if anything, the in vitro defects are more pronounced. One explanation for these observations is that culture somehow unmasks the potential for abnormal phenotype, overriding environmental regulatory influences that may exist.

The morphologic and biochemical abnormalities we have demonstrated in ichthyotic keratinocytes support and extend the findings demonstrated in skin biopsies of subjects with IV [10]. The decrease to absence of profilaggrin is a consistent finding. As IV is an autosomal dominant disorder in which one should see the gene products of both the normal and normal alleles, the complete absence of profilaggrin suggests that the primary abnormality resides in a factor that modulates profilaggrin expression, rather than in the gene for the structural protein itself.

How the profilaggrin defect is related to the clinical disease is unclear. In normal epidermis profilaggrin is processed to filaggrin [33]. In vitro, filaggrin combines with purified keratin filaments to form a macrofibril with ultrastructure similar to the keratin pattern seen in stratum corneum [34]. This observation has led to the hypothesis that filaggrin acts as a keratin matrix protein, acting as a scaffold for keratin filaments to organize in the stratum corneum and form the keratin pattern [33]. However, in affected IV epidermis filaggrin is absent or decreased and the keratin pattern is still observed [10], suggesting that filaggrin may not be the only factor responsible for keratin aggregation. In normal cultured human keratinocytes profilaggrin is not processed to filaggrin [11,24]; morphologic differentiation under these conditions is not as complete as that in normal epidermis, and the keratin pattern is not seen. In cultured ichthyotic keratinocytes, profilaggrin is virtually undetectable and yet, although keratohyaline granules are absent, other markers of epidermal differentiation do not differ from normal cultured cells. It has been proposed that an alternative or additional function of filaggrin is to act as a source for urocanic acid, pyrrolidone carboxylic acid, and free amino acids, which might maintain hydration in the upper layers of the stratum corneum [35,36]. Perhaps the absence of profilaggrin and filaggrin from the IV keratinocytes decreases hydration, which alters stratum corneum maturation and results in scaling.

In the ichthyoses associated with systemic, metabolic defects, e.g., X-linked ichthyosis and Refsrum's disease, skin manifestations are either secondary to systemic abnormalities or result from direct effects of the abnormal gene on the skin. In these diseases the defect can be demonstrated in tissues other than epidermis (e.g., fibroblasts, white cells, and serum) [29,30,37,38]. There remains, however, another group of ichthyoses in which the only manifestations are in the epidermis and no associated systemic defect has been defined. Thus, the concept of Briggaman [7] that the site of gene action in the ichthyoses can be at a systemic level, at the level of the dermis, or at the level of the epidermis, can be restated to include the concept of epidermal diseases, in which the primary defect is expressed in the epidermis and no associated dermal or systemic abnormalities exist. Included in this group are lamellar ichthyosis and nonbullous congenital ichthyosiform erythroderma [39,40], bullous congenital ichthyosiform erythroderma [1,41–43], and ichthyosis vulgaris [1,9,10]. The gene defect in one of these, lamellar ichthyosis, has been shown to be expressed by the epidermis [6]. If these are epidermal diseases, then the study of epidermal cells in these diseases may help to define the abnormalities. This paper supports that hypothesis.

The observations reported in this paper suggest that keratinocytes from individuals with epidermal diseases can be studied in vitro. The in vitro system described herein affords the opportunity for study of the gene defect and for testing therapeutic agents in ichthyosis vulgaris. Such a system may lend itself to the study of other epidermal diseases.

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