THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 84:191–194, 1985

Vol. 84, No. 3
Printed in U.S.A.

# Ichthyosis Vulgaris: Identification of a Defect in Synthesis of Filaggrin Correlated with an Absence of Keratohyaline Granules*

VIRGINIA P. SYBERT, M.D., BEVERLY A. DALE, PH.D., AND KAREN A. HOLBROOK, PH.D.

*Departments of Medicine (Dermatology), Pediatrics (Medical Genetics), Periodontics, Oral Biology, and Biological Structure, University of Washington School of Medicine and Children's Orthopedic Hospital and Medical Center, Seattle, Washington, U.S.A.*

**Ichthyosis vulgaris is an autosomal dominant disorder of keratinization characterized histologically by absent or reduced keratohyaline granules in the epidermis and mild hyperkeratosis. The basic defect in ichthyosis vulgaris is unknown. We have tested for the presence of filaggrin and its precursor, profilaggrin, in the epidermis of affected and unaffected individuals from 2 families with ichthyosis vulgaris and correlated its presence and relative quantity with ultrastructure findings in the same individuals.**

**Filaggrin was present on stained sodium dodecyl sulfate gels and immunoblots of epidermal proteins from controls and unaffected family members. It was absent from the more severely affected individuals in each family and reduced in intensity in the less severely affected family members. Immunohistology in controls showed localization of filaggrin-related protein in the stratum corneum and within the granular layer. In contrast, tissue from affected individuals showed little or no reaction. Electron microscopic studies showed that keratohyaline granules were absent in 3 severely affected individuals, and reduced in number in the others. The relative amount of keratohyalin by electron microscopy correlated with the amount of filaggrin detectable on immunoblots. The stratum corneum was thicker than in normals but showed the typical "keratin pattern" staining suggesting that filaggrin is not essential for keratin filament aggregation and may have another function in vivo.**

**We have demonstrated that the structural proteins, profilaggrin and filaggrin, are reduced or absent in 5 patients from 2 pedigrees with ichthyosis vulgaris. This biochemical abnormality correlates with the morphologic reduction in the amount of keratohyalin, and with the clinical severity of the disorder.**

Ichthyosis vulgaris is a common autosomal dominant disorder of keratinization characterized clinically by scaling, keratosis pilaris, hyperlinearity of the palms, and an association with atopy [1–3]. The biochemical defect in this disorder is unknown. Histologically, there is a decrease in, or absence of, keratohyaline granules in the epidermis, mild to moderate hyperkeratosis, and generally a normal rete ridge pattern [4]. In some cases, small keratohyaline granules can be resolved by electron microscopy, but these are abnormal in structure and have been described as "crumbly" [5].

One of the main components of keratohyalin is the precursor form of filaggrin, a histidine-rich protein [6–8]. Human epidermal filaggrin has recently been isolated [9] and an antiserum prepared. We have used this antiserum to test for the presence of filaggrin and profilaggrin in the epidermis of patients from 2 families affected with ichthyosis vulgaris, unaffected family members, and unrelated normal adults. The immunologic and biochemical studies are correlated with the histology and ultrastructure of the epidermis in the same individuals.

## MATERIALS AND METHODS

### *Patients*

The families were identified by clinical examination and the diagnosis confirmed by electron microscopy. All subjects were examined and family histories were obtained (Fig 1). All affected individuals had classic signs of ichthyosis vulgaris on at least one examination. These included fine scale which spared the flexures and involved the face, keratosis pilaris, and increased palmar markings. Several patients reported improvement in warm weather and/or had associated atopy. One affected individual was clinically normal at the time of the biopsy. She had been examined the previous winter and had classic signs of ichthyosis vulgaris. No affected individual had active eczema, and none of the patients had been using any topical agents for at least 1 month prior to the time of biopsy. An unrelated normal adult was also biopsied as a control. Informed consent was obtained from each subject; all subjects were adults.

Two 3-mm punch biopsies were taken from the posterior aspect of the nondominant arm using a Baker punch under anesthesia with 1% lidocaine with epinephrine injected lateral to the biopsy sites. Samples were transported in normal saline. One biopsy was used for extraction of epidermal proteins. The other sample was bisected; one half was placed in ½-strength Karnovsky's fixative [10] for light and electron microscopic studies. The other half was placed in Carnoy's fixative for immunoperoxidase staining.

### *Light and Electron Microscopy*

Tissues were fixed for 2–4 h in the cold, washed in 0.1 M cacodylate buffer, and postfixed in 1% $OsO_4$ in distilled water for an additional hour. They were washed again in buffer, then flooded with 1% aqueous uranyl acetate for 1 h to stain en bloc. Dehydration was carried out through a graded series of alcohols, into propylene oxide, and embedded in Epon 812 [11]. One-micron sections were cut for light microscopy and stained by the method of Richardson et al [12]. Thin sections were cut at approximately 80 nm in thickness, stained with uranyl acetate and lead citrate [13], and viewed in a Philips 201 transmission electron microscope.

### *Immunoperoxidase Localization*

Biopsy samples were fixed in Carnoy's fixative for 2 h, washed in absolute ethanol for 1 h, 95% ethanol for 1 h, and stored in 70% ethanol until processed for paraffin embedding. Five-micron sections were cut, deparaffinized, and then rehydrated. Sections were preincubated with normal goat serum, then incubated with rabbit antifilaggrin (1:200 to 1:1000 dilution). Binding was localized by sequential incubation in biotin-conjugated goat antirabbit IgG, avidin-biotin-peroxidase complex, and peroxidase substrate [14]. Sections were then dehydrated, permanently mounted, and photographed using a blue filter.

Manuscript received May 7, 1984; accepted for publication September 27, 1984.

This work was supported by United States Public Health Service NIH Grants No. AM21557, HD17664, and GM15253.

* This work was presented in part in abstract form at the National Meeting of The Society for Investigative Dermatology, Washington, D.C., May 8–11, 1984.

Reprint requests to: Virginia P. Sybert, M.D., Division of Medical Genetics, Children's Orthopedic Hospital and Medical Center, 4800 Sand Point Way N.E., Box C-5371, Seattle, Washington 98105.

Abbreviations:
PBS: phosphate-buffered saline
SDS: sodium dodecyl sulfate

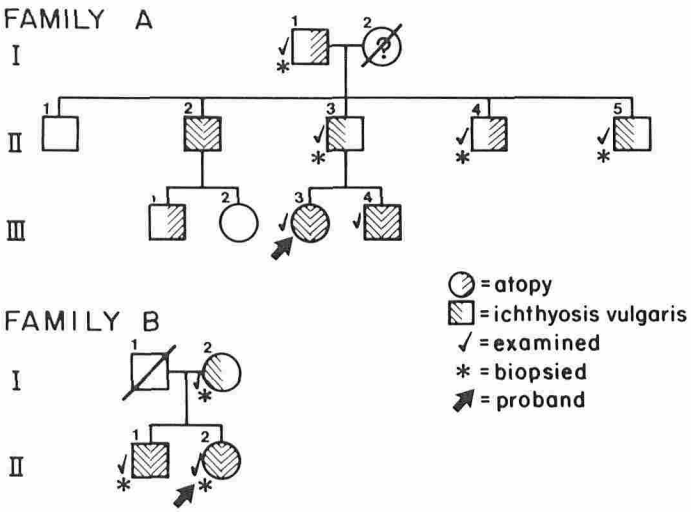


FIG 1. Pedigrees of the two families in this study affected with ichthyosis vulgaris. Males are indicated by *squares*, females by *circles*; / = deceased.

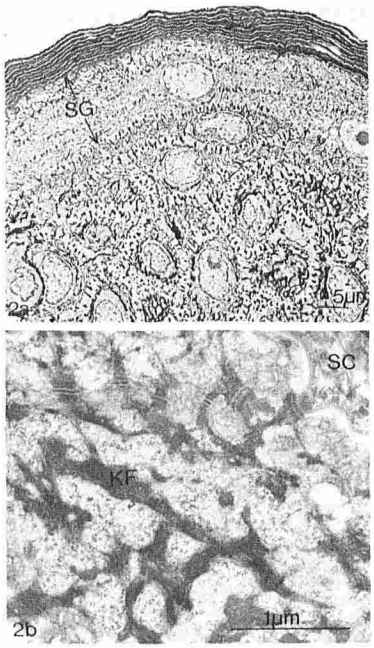


FIG 2. Electron microscopy of affected individual B.II.1. *a*, Note the apparent absence of keratohyaline granules in the stratum granulosum (*SG*). *b*, Aligned keratin filaments (*KF*) but no keratohyaline granules. *SC* = stratum corneum.

*Epidermal Protein Extraction*

The epidermis was incubated in 5 mM EDTA in phosphate-buffered saline (PBS) for 3 min at 50°C and approximately 2 min in cold PBS, then separated from the dermis by gentle dissection. The epidermis was immediately homogenized in 8 M urea/50 mM Tris-HCl (pH 7.6)/0.1 M 2-mercaptoethanol/1 mM dithiothreitol/100 µg/ml phenylmethylsulfonyl fluoride. The particulate matter was removed by centrifugation at 10,000 *g* for 4 min. Protein concentration was assayed by the BioRad method.

*Sodium Dodecyl Sulfate-(SDS) Polyacrylamide Gel Electrophoresis and Detection of Antigen*

The discontinuous buffer system of Laemmli [15] was used in polyacrylamide gradient gels (7.5–15%). The samples were boiled for 3–5 min with 2% SDS and 3% 2-mercaptoethanol prior to the loading of 10–14 µg protein on each lane for electrophoresis.

Proteins from unstained polyacrylamide gels were transferred electrophoretically to a nitrocellulose membrane using a BioRad Transblot apparatus (overnight at 60 V in Tris-glycine buffer, pH 7.5, with 20% methanol). The blots were incubated for 1–2 h in 3% bovine serum albumin to block additional protein binding sites, then incubated sequentially in rabbit antiserum to human filaggrin, goat antirabbit IgG, and rabbit peroxidase-antiperoxidase complex (Miles-Yeda, Inc. Elkhart, Indiana, 1:80 dilution). Incubations were done at room temperature on a rocker platform with buffer washes between each step. Color was developed by incubation in freshly prepared substrate solution containing 50 mM Tris-HCl (pH 7.6)/0.05 mg/ml 3,3-diaminobenzidine-HCl, 0.01% $H_2O_2$, at room temperature for 30 s to 3 min.

*Antiserum*

Polyclonal antiserum to human filaggrin was elicited in rabbits using the 37 kD doublet from the final stage of human filaggrin purification [9]. It was absorbed twice with human dermis prior to use.

## RESULTS

*Light and Electron Microscopy (Fig 2)*

The epidermis of skin biopsy samples obtained from affected and normal individuals of both families was studied by light and electron microscopy. At the histologic level the epidermis from affected individuals: (1) was slightly thickened and had exaggerated rete ridges; thickening seemed to be a consequence of additional layers in the spinous region; (2) had a thickened, compacted stratum corneum compared with the basketweave appearance of the stratum corneum normally seen in sectioned specimens; (3) usually lacked keratohyaline granules in cells of the granular zone; when keratohyalin was present (in less severely affected patients) it was neither as large nor as dense as in normal individuals; and (4) had a different staining pattern of cells in the granular region compared with cells of the spinous and basal layers.

The epidermis of the more severely affected individuals in both families was remarkable for variation in density of cells among different epidermal layers and absence of keratohyalin. The 3 layers of cells that would normally form the stratum granulosum were more electron-lucent than the cells of the stratum spinosum and stratum germinativum (Fig 2*a*). The difference in density between abnormal lucent and normal dense zones was caused by variations in cytoplasmic density and in the amount and organization of keratin filaments. Smaller and less intensely stained bundles of filaments were present in granular layer cells compared with spinous and basal cells (Fig 2*a*). The filaments in the granular layer cells were aligned closely into bundles of a similar size, but there was no visible "matrix" material associated with them (Fig 2*b*). Other organelles in the granular layer cells appeared to be normal in number and structure. There was no evidence of an increased number of "T" (transitional) cells and the cells of the stratum

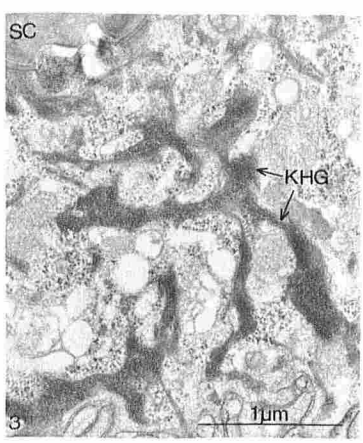


FIG 3. Electron microsocpy of less severely affected individual B.I.2. A small amount of electron-dense material is associated with keratin filaments forming abnormal keratohyaline granules (*KHG*). *SC* = stratum corneum.

corneum did not appear to be altered in the extent of differentiation, keratin pattern, shape, or organization.

Small keratohyaline granules were present in granular cells of less severely affected individuals, but compared with normal keratohyalin, they appeared as finely granular deposits along material that joined adjacent bundles of keratin filaments (Fig 3). This form of keratohyalin may be equivalent to the "crumbly" appearing keratohyalin described by others [5]. The stratum corneum in the more mildly involved patients was also normal in structure and the morphologic state of differentiation.

*Immunoperoxidase Localization of Filaggrin (Fig 4)*

Immunohistologic localization of filaggrin in the normal control (Fig 4*a*) showed positive reaction in the stratum corneum and in granules within the granular layer when compared with the preimmune serum control (Fig 4*b*). The 3 more severely affected subjects showed little or no reaction with the antifilaggrin antibody, either in the stratum corneum or in the granular layer (Fig 4*c*). The less severely affected individuals (Fig 4*d*) showed variable reaction in the granular layer, consistent with the variable quantity of keratohyaline granules demonstrated by light and electron microscopy. It should be noted that the immunohistologic staining cannot be interpreted in a strictly quantitative manner because of possible masking of antigenic sites.

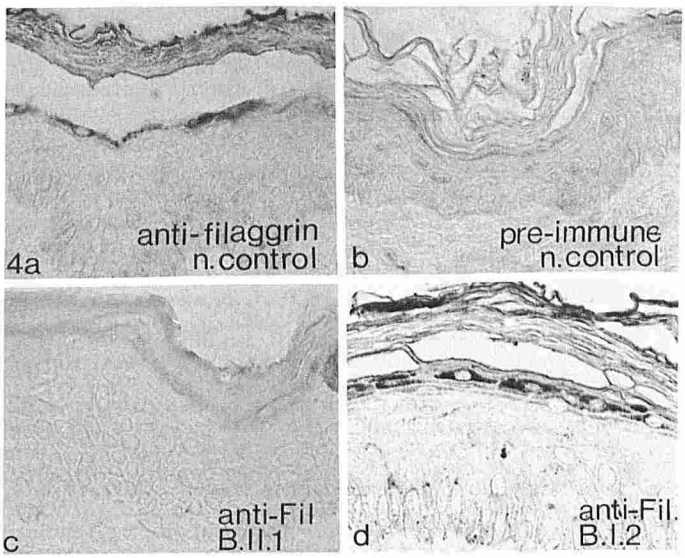


FIG 4. Filaggrin localization by ABC immunoperoxidase stain (× 350). *a*, Normal control subject. Note staining in granular and cornified layers. *b*, Normal control subject; preimmune serum control. *c*, Ichthyosis vulgaris patient (B.II.1). Note the negative reaction. *d*, Less severely affected ichthyosis vulgaris patient (B.I.2) showing a positive reaction in granular and cornified cells.

*Epidermal Protein Studies*

SDS gel electrophoresis and immunoblot identification of filaggrin in individuals from 2 families with ichthyosis vulgaris and an unrelated normal control are shown in Fig 5 (see also Fig 1). The major structure proteins of epidermis, the 40–70 kD keratins, were similar in all affected and normal individuals (Fig 5, lanes 1–8). Filaggrin, a diffuse band of approximately 37 kD, was identified on the stained SDS gel (Fig 5*A*) and the immunoblot (Fig 5*B*), in the normal control (lane 1) and unaffected family members A.II.4 and A.I.1. It was absent in subject A.II.5, and marginally detectable in A.II.3 and B.II.1, all severely affected. Subjects B.I.2 and B.II.2 had reduced amounts of filaggrin compared with the normal control. B.I.2 was mildly affected and B.II.2 was free of clinical signs at the time of the biopsy.

## DISCUSSION

The underlying biochemical abnormality in ichthyosis vulgaris is not known. Transit time [16], lipid synthesis [17], and keratins [18] all appear to be normal. Baden et al [19] demonstrated a decrease in the incorporation of histidine in the uppermost layers of the epidermis of patients with ichthyosis vulgaris. This decrease in incorporation corresponded with the absence of keratohyaline granules at the light microscope level.

Keratohyalin contains a histidine-rich protein [20–22] which is the precursor form (profilaggrin) of the stratum corneum histidine-rich protein, filaggrin, a keratin filament aggregating protein [6–8,23,24]. The present study has demonstrated an absence or reduction in the amount of filaggrin in individuals with ichthyosis vulgaris which appears to be correlated with clinical severity. Protein analysis, immunoperoxidase tissue localization, light and electron microscopic studies are all consistent with a reduction in filaggrin in the stratum corneum and with its precursor in the keratohyaline granules of the

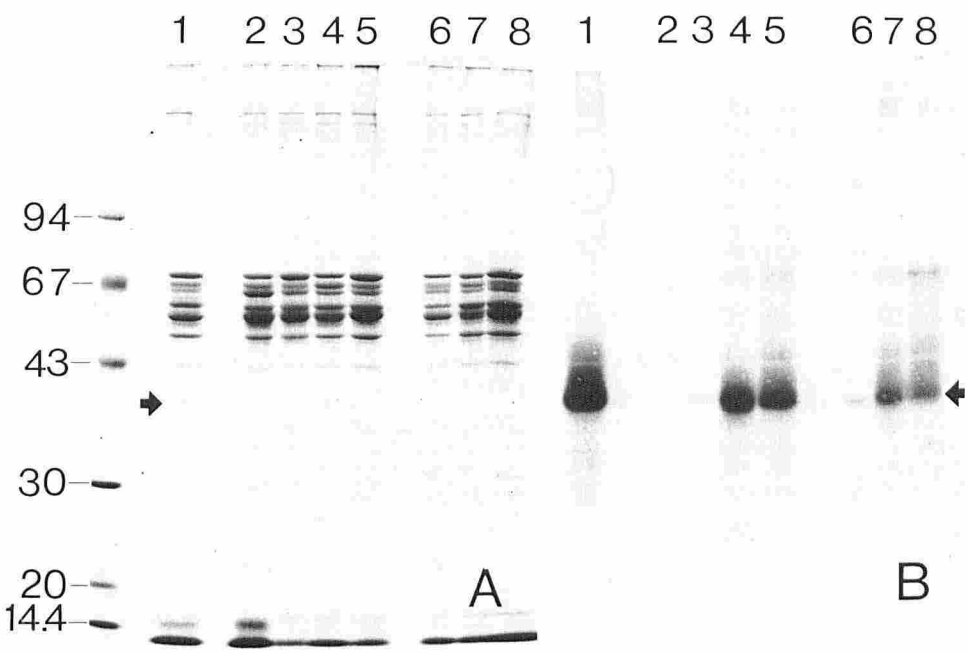


FIG 5. SDS-polyacrylamide gel electrophoresis of epidermal proteins and filaggrin immunoblot. Epidermal proteins extracted from biopsies were separated on 7.5–15% polyacrylamide SDS gels and stained with Coomassie Brilliant Blue (*A*) or transferred to nitrocellulose and stained by the peroxidase-antiperoxidase reaction after incubation with antiserum to human filaggrin (*B*). The position of standard proteins is shown on the left with molecular weight $\times 10^{-3}$ indicated. Lane 1, normal control; lane 2–5, Family A, II.5, II.3, I.1, and II.4; lane 6–8, Family B, II.1, II.2 and I.2. *Arrows* indicate position of filaggrin.

granular layer of the epidermis. Voorhees et al [25] demonstrated a similar lack of histidine-rich protein associated with an absence of keratohyaline granules in psoriasis.

The reduction in filaggrin was also shown by the immunoblots of SDS gels; however, the antibody was less effective in reproducibly staining a discrete profilaggrin band. Profilaggrin of rodents and humans [26–28], is known to be an extremely large protein. Weak high-molecular-weight staining is detectable in Fig 5 and in other blots (not shown), and represents profilaggrin. [$^3$H]Histidine is readily incorporated into this band in organ culture (Fleckman, Dale, Holbrook, manuscript in preparation), confirming its identity as profilaggrin. Its staining intensity follows the same general pattern as the filaggrin reaction (negative or weak in affected individuals and stronger in unaffected family members and normal controls). This is, in general, consistent with the number of keratohyaline granules seen by light and electron microscopy and with the immunoperoxidase tissue staining. Thus, the absence of filaggrin is probably due to an absence of profilaggrin from which it is derived, rather than an accumulation of precursor due to a block in its conversion to filaggrin, in which case increased amounts of profilaggrin and keratohyaline granules would be seen.

It has been previously suggested that filaggrin functions as the keratin matrix protein within cells of the stratum corneum [29]. In our subjects lacking filaggrin, the stratum corneum was more compact and thicker than in normals but did show the keratin pattern originally described by Brody [30], in which lightly staining filaments are surrounded by a darkly staining matrix. The hypothesis that filaggrin functions as the matrix was based on the rapid aggregation of filaggrin and keratin filaments in vitro to form bundles of filaments (macrofibrils). Our results suggest that other epidermal components may contribute to this role in vivo which may not be the primary function of filaggrin. Other roles suggested for filaggrin include the production of free amino acids, urocanic acid, and pyrogentamic acid, whose function in the stratum corneum is not yet understood [31].

Genetic considerations suggest that the absence of filaggrin may not be the primary genetic defect in ichthyosis vulgaris. This is an autosomal dominant disorder in which affected individuals are heterozygous for a normal and an abnormal allele. As such, one should see both gene products, a normal and an abnormal protein. The complete absence of profilaggrin and filaggrin in the severely affected subjects and the reduction in the mildly affected individuals found in this study suggest that the abnormality in profilaggrin and filaggrin production may be secondary to an abnormality in a factor(s) which modulates profilaggrin/filaggrin synthesis. Similarly, the complete absence of keratohyaline granules in the most severely affected individuals suggests that the ultrastructural abnormality in keratohyalin is also not the primary abnormality. A variety of extrinsic factors may modulate the expression of keratohyalin, profilaggrin and filaggrin causing variation in the clinical severity of the disorder.

The authors wish to thank Janet Kimball, Julie Scofield, and Mary Hoff for technical assistance, and Bonnie McLoughlin for help in the preparation of the manuscript.

## REFERENCES

1. Goldsmith LA: The ichthyoses. Prog Med Genet 1:185–210, 1976
2. Wells RS, Kerr CB: Genetic classification of ichthyosis. Arch Dermatol 92:1–6, 1965
3. Williams ML: The ichthyoses—pathogenesis and prenatal diagnosis: a review of recent advances. Pediatr Dermatol 1:1–24, 1983
4. Feinstein A, Ackerman AB: Histology of autosomal dominant ichthyosis vulgaris and X-linked ichthyosis. Arch Dermatol 101:524–527, 1970
5. Anton-Lamprecht I, Hofbrauer M: Ultrastructural distinction of autosomal dominant ichthyosis vulgaris and X-linked recessive ichthyosis. Hum Genet 15:261–264, 1972
6. Dale BA, Ling SY: Immunologic cross-reaction of stratum corneum basic protein and a keratohyalin granule protein. J Invest Dermatol 72:257–261, 1979
7. Dale BA, Ling SY: Evidence of a precursor form of stratum corneum basic protein in rat epidermis. Biochemistry 18:3539–3546, 1979
8. Ball RD, Walker GK, Bernstein IA: Histidine-rich proteins as molecular markers of epidermal differentiation. J Biol Chem 253:5861–5868, 1978
9. Lynley AM, Dale BA: The characterization of human epidermal filaggrin—a histidine rich keratin filament aggregating protein. Biochim Biophys Acta 744:28–35, 1983
10. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 27:137a, 1965
11. Luft JH: Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9:409–414, 1961
12. Richardson KC, Jarrett L, Finke EH: Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol 35:313–323, 1960
13. Reynolds ES: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–213, 1963
14. Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577–580, 1981
15. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970
16. Frost P, Weinstein GD, Van Scott EJ: Autoradiographic studies of epidermal proliferation. J Invest Deramatol 47:561–569, 1966
17. Summerly R, Yardley HS: Cholesterol synthesis in ichthyosis vulgaris. Br J Dermatol 79:378–385, 1967
18. Baden HP, Goldsmith LA, Lee LD: The fibrous proteins in various types of ichthyosis. J Invest Dermatol 65:228–230, 1975
19. Baden HP, Roth SI, Goldsmith LA, Baden SB, Lee LD: Keratohyalin protein in disorders of keratinization. J Invest Dermatol 62:411–414, 1974
20. Fukuyama KF, Nakamura T, Bernstein IA: Differentially localized incorporation of amino acid in relation to epidermal keratinization in the newborn rat. Anat Rec 152:525–535, 1965
21. Fukuyama K, Epstein WL: A comparative autoradiographic study of keratohyalin granules containing cystine and histidine. J Ultrastruct Res 51:314–325, 1975
22. Sibrack LA, Gray RH, Bernstein IA: Localization of the histidine-rich protein in keratohyalin: a morphologic and macromolecular marker in epidermal differentiation. J Invest Dermatol 62:394–405, 1974
23. Lonsdale-Eccles JD, Haugen JA, Dale BA: A phosphorylated keratohyalin-derived precursor of epidermal stratum corneum basic protein. J Biol Chem 255:2235–2238, 1980
24. Dale BA, Lonsdale-Eccles JD, Holbrook KA: Stratum corneum basic protein: an interfilamentous matrix protein of epidermal keratin, Biochemistry of Normal and Abnormal Epidermal Differentiation. Edited by IA Bernstein, M Seiji. Tokyo, Tokyo Univ Press, 1980, pp 311–325
25. Voorhees JJ, Chakrabarti SG, Berstein IA: The metabolism of "histidine-rich" protein in normal and psoriatic keratinization. J Invest Dermatol 51:344–354, 1968
26. Lonsdale-Eccles JD, Teller DC, Dale BA: Characterization of a phosphorylated form of the intermediate filament-aggregating protein filaggrin. Biochemistry 21:5940–5948, 1982
27. Ramsden M, Loehren D, Balmain A: Identification of a rapidly labeled 350K histidine-rich protein in neonatal mouse epidermis. Differentiation 23:243–249, 1983
28. Harding CR, Scott IR: Histidine-rich proteins (filaggrins): structural and functional heterogeneity during epidermal differentiation. J Mol Biol 170:651–673, 1983
29. Dale BA, Holbrook KA, Steinert PM: Assembly of stratum corneum basic protein and keratin filaments in macrofibrils. Nature 276:729–731, 1978
30. Brody I: An ultrastructural study on the role of the keratohyaline granules in the keratinization process. J Ultrastruct Res 3:84–104, 1959
31. Scott IR, Harding CR, Barrett JG: Histidine-rich protein of the keratohyalin granules. Source of the free amino-acids, urocanic acid and pyrrolidone carboxylic acid in the stratum corneum. Biochim Biophys Acta 719:110–117, 1982