Modification of human prekeratin during epidermal differentiation

Paul E. BOWDEN

Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

and William J. CUNLIFFE

Department of Dermatology, The General Infirmary, Leeds LS1 3EX, U.K.

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The polypeptide-chain components of human epidermal prekeratin and keratin were analysed by high-resolution SDS (sodium dodecyl sulphate)/polyacrylamide-gradient-gel electrophoresis. Size heterogeneity existed amongst prekeratin components and at least ten polypeptides, in the molecular-weight range 46 000–70 000, were observed in 0.1 M-citric acid/sodium citrate buffer (pH 2.65) extracts of scalp epidermis. Prekeratin from scalp pilosebaceous ducts was identical with that from the contiguous epidermis, and no prekeratin was found in extracts of scalp dermis. Prekeratin from plantar epidermis contained additional polypeptide chains, but only slight anatomical variation existed between the non-callus sites examined. Keratin differed from prekeratin in at least two major respects: (a) many major components did not co-electrophorese on high-resolution SDS/polyacrylamide slab gels, and (b) keratin, but not prekeratin, required denaturing and reducing conditions for extraction. Keratin extracted from scalp epidermis after complete removal of prekeratin was identical with forearm stratum-corneum keratin. Palmar and plantar keratin contained additional polypeptide chains and had a different size distribution compared with forearm and scalp keratin components. Modification of prekeratin components to produce the keratin polypeptide profile occurred during epidermal differentiation, and these changes appeared to take place in the granular-layer region of the epidermis.

The surface of mammalian skin is composed of a flexible layer of dead cornified cells, the stratum corneum, which forms a barrier to the external environment. This horny layer is produced by terminal differentiation of the living epidermal cells below, a process called keratinization [for review, see Matoltsy (1976)]. The living cells synthesize, modify and accumulate the structural proteins required to form the cell envelope and the stable fibrous/amorphous matrix characteristic of the fully differentiated corneocyte. These structural proteins are organized within the cells of the granular layer, and during, or just before transition (loss of cellular organelles) the proteins undergo a stabilization process that renders them highly insoluble. The exact molecular nature of the stabilization is unknown, but the cell-envelope proteins are covalently cross-linked (Rice & Green, 1977, 1979) and disulphide bridging is involved in the maturation of the keratin filament matrix (Steinert & Idler, 1979; Sun & Green, 1978).

Denaturing and reducing conditions are required to solubilize the fibrous/amorphous matrix of the corneocyte, and such extracts of bovine, rabbit and human stratum corneum have been analysed by SDS/polyacrylamide-gel electrophoresis (Steinert & Idler, 1975; Skerrow & Hunter, 1978; Baden & Lee, 1978; Bowden & Cunliffe, 1980; Fuchs & Green, 1980). The filament-forming protein, keratin, is a major component of these extracts, and appears to consist of several polypeptide chains with molecular weights in the range 50 000–70 000. Evidence suggests that not all the polypeptide chains are necessary for filament formation (Steinert *et al.*, 1976, 1980; Sun & Green, 1978), and the polymerizing unit is thought to be a three-chain α -helical structure (Skerrow, 1974; Steinert, 1978).

There is evidence that the tonofilaments, a class of intermediate filaments located in the basal, spinous and granular layers of the epidermis, are precursors of the stratum-corneum keratin filaments (Matoltsy, 1975; Steinert, 1975; Baden & Lee, 1978; Brody, 1979). The tonofilament protein, prekeratin, can be extracted from the living epidermal cells with CASC

Abbreviations used: SDS, sodium dodecyl sulphate; CASC buffer, 0.1 M-citric acid/sodium citrate, pH 2.65.

buffer at pH 2.65, a relatively gentle procedure (Matoltsy, 1964). Prekeratin extracted from bovine (Skerrow, 1974; Steinert, 1975; Lee *et al.*, 1979) and human (Skerrow, 1977; Baden & Lee, 1978; Bowden & Cunliffe, 1980) epidermis demonstrates some interspecies heterogeneity and appears to consist of several polypeptide chains within the molecular-weight range 45 000–70 000 as determined by SDS/polyacrylamide-gel electrophoresis. Intermediate filaments have been found in many cell types, but in cells of epithelial origin they appear to be composed of prekeratin-like polypeptides (Franke *et al.*, 1978; 1979; Sun *et al.*, 1979).

Intraspecies heterogeneity (anatomical-site variation) has been demonstrated for bovine, rabbit and human keratins (Skerrow & Hunter, 1978; Lee *et al.*, 1979; Bowden & Cunliffe, 1980; Fuchs & Green, 1980; Steinert *et al.*, 1980) and for bovine prekeratin (Lee *et al.*, 1979). In the present work, high-resolution SDS/polyacrylamide-gradient-slab-gel electrophoresis has been used to examine size heterogeneity amongst prekeratin and keratin polypeptides from human epidermis and to investigate the modification of prekeratin to mature keratin during epidermal differentiation. We also report that human prekeratin demonstrates anatomical-site variation, providing an explanation for the atypical polypeptide profile of callus keratin.

Materials and methods

Human scalp skin was obtained from a local hair-transplant clinic, and both breast and foreskin were obtained during routine surgical procedures, all subjects having no history of disorders of the epidermis. Heel skin and scalp skin were taken from cadavers less than 24 h after death by cardiac failure. All samples were taken with the donors' consent or that of the next of kin. Stratum corneum was obtained by scraping the forearms of normal volunteers, and heel callus was removed from similar subjects by electrokeratome (supplied by Altomed Health Care Products, Gateshead, Tyne & Wear, U.K.). All samples of living skin were used immediately, but some stratum corneum and callus samples were stored in sterile containers at -20°C .

Enzymes used as molecular-weight markers, and fluram for protein assays, were obtained from Sigma, Poole, Dorset, U.K. SDS, specially purified for electrophoresis, was obtained from Pierce Chemical Co., Rockford, IL, U.S.A., whereas specially pure acrylamide, bisacrylamide and urea were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Preparation of skin

Skin samples were rinsed in ice-cold distilled water and the epidermis removed by a single stroke of the electrokeratome set to cut at 0.1 mm. In the case of

heel skin, serial slices were taken parallel to the skin surface with the electrokeratome (0.1 mm) until the dermis was reached, and each layer extracted separately.

Pilosebaceous ducts were removed from human scalp skin by a modification of Kellum's (1966) technique. Epidermis was removed by electrokeratome (0.1 mm) and then a further slice was taken at 0.3 mm, which consisted of dermis traversed by many pilosebaceous ducts. After immersion in 1 M-CaCl₂ for 30 min at 4°C, the loosened ducts were removed from the surrounding dermis under a dissecting microscope. Pure preparations of dermis and pilosebaceous ducts were obtained and pure epidermis was obtained by blunt dissection after soaking a 0.3 mm slice in 0.5% (v/v) acetic acid for 30 min at 4°C. All tissue samples were checked for normal histological appearance, and both histology and macroscopic observations were used to define the purity of the skin component preparations.

Extraction of prekeratin

Minced epidermis was extracted in 10 vol. of CASC buffer, pH 2.65, made by mixing 0.1 M-citric acid and 0.1 M-trisodium citrate in the ratio of 23:2. After 5 min at 4°C, the extract was homogenized for 1 min with a Polytron model PT 10 instrument, set at maximum speed (supplied by Northern Media Supply Ltd., North Cove, Brough, N. Humberside, U.K.), followed by sonication for 30 s at 50 W with an Ultrasound model 180 sonicator fitted with a 3 mm probe (supplied by Ultrasonics Ltd., Shipley, W. Yorks., U.K.), the samples being kept on ice during both procedures. The resulting homogenate was centrifuged at 50 000 *g* (r_{av} 6 cm) for 30 min at 4°C, and the supernatant containing the total acid-soluble proteins removed. Occasionally, the high-speed pellet (CASC residue) was further extracted with CASC buffer or with Tris/urea or Tris/urea/mercaptoethanol buffers (see below).

Prekeratin was partially purified from the high-speed supernatant by serial isoelectric precipitation at pH 4 (Skerrow, 1977) and solubilized by sonication in a small volume of CASC buffer. The pH 4 supernatants were pooled and concentrated by freeze-drying. Samples of pilosebaceous duct material, heel-skin epidermis and pure dermis were extracted by the same procedure.

Extraction of keratin

Minced stratum-corneum samples (forearm, heel callus) and samples of scalp epidermis after removal of prekeratin (high-speed pellet after several extractions) were extracted with 50 vol. of 0.05 M-Tris/HCl buffer, pH 7.2, containing 6 M-urea and 2% (v/v) 2-mercaptoethanol for 16–24 h at 4°C (Skerrow, 1977). The samples were homogenized and sonicated on ice, and centrifuged at 50 000 *g* as detailed

above. The high-speed supernatants were dialysed against CASC buffer (100 vol., 4°C) and the keratin partially purified by serial isoelectric precipitation at pH4 (as for prekeratin).

Extraction of whole epidermis

Samples of scalp-skin epidermis were divided into four pieces and each extracted with a different buffer: (1) CASC, as described above under 'Extraction of prekeratin'; (2) 0.05 M-Tris/HCl, pH7.2; (3) 0.05 M-Tris/HCl, pH7.2 containing 6 M-urea; and (4) Tris/urea/mercaptoethanol, as described above (under 'Extraction of keratin'). All samples were extracted for 5 min at 4°C and homogenized, sonicated and centrifuged as described above. The supernatant from the Tris buffer extract was concentrated by freeze-drying, whereas the supernatants from extracts with Tris/urea and Tris/urea/mercaptoethanol buffers were dialysed against CASC buffer and subjected to serial isoelectric precipitation at pH4.

A preparation of scalp epidermis was also serially extracted with the four buffers detailed above. After initial extraction with Tris buffer, the residue (high-speed pellet) was then extracted twice with CASC buffer, twice with Tris/urea buffer (16–24 h each) and twice with Tris/urea/mercaptoethanol buffer (16–24 h each). The urea-containing buffer extracts were dialysed against CASC buffer and the proteins fractionated by serial isoelectric precipitation at pH4.

Protein assay

The absorption spectrum of epidermal prekeratin was measured in CASC buffer over the wavelength range of 240–260 nm by using a Pye-Unicam SP.1800 spectrophotometer, and the maximal absorbance used to quantify the prekeratin and keratin samples. They were also quantified by fluorimetric assay with a Perkin-Elmer MPF-44 fluorimeter. The assay was based on the reaction between fluorescamine (30 mg/100 ml of acetone) and the protein in a sodium borate buffer (0.2 M, pH9.2); 0.5 ml of reagent solution was added to 1.5 ml of protein solution and the fluorescence at 475 nm was read after excitation at 390 nm.

Polyacrylamide-gel electrophoresis

All samples were analysed by SDS/polyacrylamide-slab-gel electrophoresis in a discontinuous Tris/glycine buffer system (Laemmli, 1970). A linear polyacrylamide gradient was used (7.5–17.5%, w/v; acrylamide/bisacrylamide ratio, 75:2) with a 4% (w/v) stacking gel (modified from Lambin, 1978). For comparative purposes, some samples were separated on gels of uniform polyacrylamide composition (7.5, 10 and 12.5%) by using both discontinuous Tris/glycine-buffered slab and tube gels

(Lemmli, 1970) and continuous phosphate-buffered slab and tube gels (Weber & Osborn, 1969).

The gels were calibrated with a mixture of the following proteins of known molecular weight (thyroglobulin, 330 000; β -galactosidase, 130 000; phosphorylase *a*, 94 000; bovine serum albumin, 68 000; pyruvate kinase, 57 000; L-glutamate dehydrogenase, 53 000; fumarase, 49 000; alcohol dehydrogenase, 41 000; aldolase, 40 000; carbonic anhydrase, 29 000; lysozyme, 14 300). The molecular weight of the prekeratin and keratin polypeptides were calculated from a linear-regression plot of log (mol.wt.) against the relative mobility of the standard proteins.

Fig. 1. SDS/polyacrylamide-gradient-gel electrophoresis of human scalp epidermal prekeratin

Extracts of epidermal prekeratin from human scalp skin were analysed on SDS/polyacrylamide gradient (7.5–17.5%) slab gels calibrated over the molecular-weight range 14 300–130 000. Ten polypeptides in the molecular-weight range 46 000–70 000 were observed in prekeratin extracts, which were divided into four groups (A–D) for identification purposes. Average molecular-weight values (shown throughout as $10^{-3} \times$ molecular weight) are given for the major chains, together with our nomenclature (a_1 , b_1 , etc.).

Results

Characterization of prekeratin

The approximate yield of prekeratin from human scalp epidermis was 15–35 mg per g wet weight for a single CASC buffer extract, which represents about 60–70% of the CASC buffer-extractable material. Prekeratin in CASC solution absorbed maximally at 278 nm ($A_{278}^{1\%} = 13.5$), and the absorbance was linear with respect to prekeratin concentration over the range 0.2–1.2 mg/ml. The relative fluorescence obtained in borate buffer (0.2 M, pH 9.2) was also linear with respect to prekeratin concentration over this range, and 0.1% SDS had no effect on the assay. Thus it would appear that, at this concentration, prekeratin was soluble in 0.2 M-sodium borate, pH 9.2, in contrast with the absolute necessity for either SDS or urea to obtain a solution of prekeratin in buffers at near neutral pH (e.g. 0.05 M-Tris/HCl, pH 7.2).

Good resolution of the component polypeptide chains in extracts of human scalp epidermis was achieved with SDS/polyacrylamide-slab-gel electrophoresis using a linear gradient of polyacrylamide from 7.5 to 17.5% (w/v). In a typical separation (Fig. 1, molecular weights and 'our nomenclature' given for most chains; see Table 1 for full details),

prekeratin resolved into three major bands (a_1 , b_4 , c_3), five moderate bands (a_3 , b_1 , b_3 , c_2 , d_1) and three minor bands (a_2 , b_2 , c_1) over the molecular-weight range 45 000–70 000. Variability in the resolution of these bands was observed from gel to gel and comparisons were only made between samples run on the same gel. Slight variability in the relative quantities of the minor polypeptides existed between individuals, but the relative quantity of the major bands, based on the density of staining, was reproducible (chains a_1 , b_4 , c_3 in the approximate proportions of 1:1:1). Prekeratin extracts also contained a number of minor bands of molecular weight greater than 70 000 and in the range 10 000–40 000, regarded as co-purifying non-keratin proteins. However, a major band of mol.wt. 38 000 was observed in many extracts and further characterization of this chain is required.

Samples of prekeratin treated with SDS and electrophoresed in the absence of mercaptoethanol showed no appreciable differences in polypeptide-chain profile. Lower resolution was observed with Tris/glycine-containing gels of uniform acrylamide concentration, and several of the minor components were not visible. Uniform-acrylamide-percentage phosphate-buffered gels also gave poor resolution; in this system prekeratin extracts appeared to contain

Table 1. Comparison of prekeratin and keratin polypeptides

The relative abundance of the polypeptide chains of prekeratin and keratin are shown, together with the group and individual chain nomenclature, and the corresponding molecular weight (average of several determinations). Separation was achieved by SDS/polyacrylamide-slab-gel electrophoresis using a polyacrylamide gradient (7.5–17.5%) and a 4% stacking gel. Key for chain abundance: ****, major; ***, moderate; **, minor; *, very minor; ND, not detected.

Group	Chain	10 ⁻³ × Molecular weight	Prekeratin		Keratin	
			Scalp	Heel	Scalp, forearm	Heel
A	a_1	70	****	****	*	*
	a_2	67	**	**	**	***
	a_3	66	***	**	****	****
	a_4	65	ND	****	ND	ND
	a_5	64	ND	ND	ND	****
B	b_1	62	***	***	*	**
	b_2	60	**	**	**	*
	b_3	59	***	***	**	**
	b_4	57	****	****	****	****
	b_5	55	ND	ND	****	****
C	c_1	54.5	**	**	ND	ND
	c_2	54	***	**	**	*
	c_3	52	****	****	****	**
	c_4	51	ND	**	ND	*
	c_5	50	ND	****	ND	****
	c_6	48	ND	**	*	**
D	d_1	46	***	***	**	ND
	d_2	45	ND	ND	ND	**
	d_3	44	ND	***	ND	ND

Fig. 2. SDS/polyacrylamide-gel electrophoresis of (a) acid-soluble proteins from human scalp skin components and (b) epidermal prekeratin from different anatomical sites

(a) Human scalp skin was dissected into three components (Der, dermis; Epid, epidermis; Psd, pilosebaceous ducts), and the acid-soluble proteins extracted, fractionated and analysed on gradient slab gels (see the Materials and methods section). Prekeratin from pilosebaceous ducts was almost identical with that from the epidermis (lanes 3 and 2 respectively), but only high-molecular-weight bands ($>130\,000$) were observed in the dermal extract (lane 1). The calibration standards are shown (lane 4, molecular-weight values $\times 10^{-3}$) (b) Prekeratin was extracted from scalp, foreskin (F/Sk), breast (Br) and heel epidermis and analysed on gradient slab gels calibrated in the molecular-weight range 29 000–130 000 (see the Materials and methods section). Scalp, foreskin and breast prekeratin (lanes

only three components, of mol.wts. 70 000 (A), 62 000 (B) and 56 000 (C). However, excision of these three bands followed by electrophoresis on gradient slab gels resulted in their further fractionation into the subgroups detailed in Fig. 1 and Table 1.

Frozen epidermis stored at -70°C gave satisfactory prekeratin extracts up to 6 months later, but the yield of prekeratin was consistently 50–65% lower than from fresh human skin.

Prekeratin from different skin components

Preparations of pure epidermis and pure pilosebaceous-duct material from human-scalp skin were extracted with CASC buffer and the prekeratin from each compared by SDS/polyacrylamide-slab-gel electrophoresis (Fig. 2a). Pilosebaceous-duct prekeratin was almost identical with that from the contiguous epidermis, but a preparation of pure dermis contained no prekeratin. However, four polypeptides of high molecular weights (150 000–350 000) were found in CASC buffer extracts of dermal tissue, but these remained soluble at pH 4.

Anatomical-site variation

Epidermal prekeratin extracts from scalp skin, foreskin and breast skin were all similar in polypeptide profile (Fig. 2b). Slight differences were observed in the minor chains, but this could represent variation between individuals. However, heel epidermal prekeratin was atypical in profile and contained three polypeptides of mol.wts. 65 000 (a_4), 50 000 (c_3) and 44 000 (d_3) in addition to those found in prekeratin from other anatomical sites. These differences were highly reproducible and present when tissues from the same individual were compared. The extra bands were not present in surgically excised scalp skin that had been stored at 4°C for 4 days before prekeratin extraction.

Comparison of prekeratin and keratin

The polypeptide-chain components of scalp prekeratin and forearm keratin were compared by SDS/polyacrylamide-slab-gel electrophoresis (Fig. 3a). Both types of extract contained a group of polypeptides in the molecular-weight range 45 000–70 000 with similar isoelectric properties. However, differences in size distribution of the individual chains were apparent.

1–3) were all of similar electrophoretic profile and consisted of several polypeptides ranging in molecular weight from 40 000 to 70 000 (values $\times 10^{-3}$ given for the major bands in each group). However, heel prekeratin contained three additional polypeptides (mol.wts. 65 000, 50 000 and 44 000). Standard proteins are shown in lane 5.

Fig. 3. Comparison of the electrophoretic profiles of epidermal prekeratin and keratin

Prekeratin was extracted from scalp (a) and heel (b) epidermis with CASC buffer, pH 2.65. Keratin was extracted from forearm (a) and heel (b) stratum corneum with 6M-urea and 2% (v/v) mercaptoethanol in 0.05M-Tris/HCl buffer, pH 7.2, and then dialysed against CASC buffer. The isoelectric precipitates (pH 4) from all four samples were analysed on gradient slab gels (7.5–17.5%) calibrated in the mol.wt. range 14 300–130 000. In each case, the keratin differed from the corresponding prekeratin in that the major polypeptides showed a different size distribution (values $\times 10^{-3}$ given for the major bands). In addition, it was apparent that the heel 'keratins' (b) were atypical.

Keratin from forearm stratum corneum contained four major bands, of mol.wts. 66 000 (a_3), 57 000 (b_4), 55 000 (b_5) and 52 000 (c_3), and a number of minor bands in this region. Two of the major keratin bands co-electrophoresed with major prekeratin chains (b_4 , c_3) and another (a_3) with a minor prekeratin chain, whereas the fourth band (b_5) appeared to be unique to keratin. The nomenclature used to label the keratin polypeptides is that established for prekeratin (see Fig. 1 and Table 1); this grouping is purely descriptive and should not be taken to mean that such polypeptides are identical in respects other than molecular weight.

Tris/urea/mercaptoethanol buffer extracts of forearm stratum corneum contained polypeptides of lower molecular weight (10 000–18 000) in addition to the keratin chains. When such an extract was dialysed against CASC buffer, treated with SDS and electrophoresed, in the absence of mercaptoethanol, only a smear was observed on the gel (cf. prekeratin). However, after isoelectric fractionation, the same SDS/polyacrylamide-gel profiles were observed in the presence and absence of mercaptoethanol for both the supernatant (low-molecular-weight chains) and the pellet (keratin chains).

Fig. 3(b) shows a similar comparison for heel-skin prekeratin and keratin, again each consisting of several polypeptides in the same molecular-weight range but differing in size distribution. Heel stratum-corneum (callus) keratin separated into six major bands, of mol.wts. 67 000 (a_2), 66 000 (a_3), 64 000 (a_5), 57 000 (b_4), 55 000 (b_5) and 50 000 (c_5), together with several other minor bands in this region. Two of the major keratin bands (b_4 , c_5) co-electrophoresed with major prekeratin polypeptides, two (a_2 , a_3) with minor prekeratin chains, whereas the remaining bands (a_5 , b_5) appeared to be unique to keratin. In addition, two of the minor keratin bands (a_1 , c_3) co-electrophoresed with major prekeratin chains. Samples of palmar keratin were almost identical in polypeptide profile with heel-callus keratin.

Thus 'keratins' (i.e. both prekeratin and keratin) from plantar, and possibly palmar, epidermis have additional polypeptide chains compared with the 'keratins' from other anatomical sites. Furthermore, when the 'keratin' polypeptides common to both heel and scalp epidermis were considered (note that scalp and forearm keratins are identical), three groups became apparent: (a) chains considered major prekeratin components (a_1 , b_1 , b_3 , d_1); (b) chains considered major keratin components (a_3 , b_5); and (c) chains considered major components of both prekeratin and keratin (b_4 , c_3).

Modification of prekeratin during differentiation

The acid-soluble prekeratin from the lower living epidermis and the insoluble keratin from the dead

corneocytes at the skin surface represent two extremes. In order to investigate changes in polypeptide-chain profile between these extremes, serial slices parallel to the skin surface were made and the 'keratins' extracted from each portion of the epidermis. The skin on the heel was used for this purpose as it can be easily sliced at 0.1 mm intervals. Prekeratin was extracted from an adjacent piece of heel epidermis and all samples compared on SDS/polyacrylamide slab gels (Fig. 4).

The outermost slices were pure stratum corneum and contained keratin of identical polypeptide profile to the extracts of heel callus shown above (cf. Fig. 3b). 'Keratin' from slices containing predominantly spinous and granular cells was similar in its profile to heel prekeratin. The tissue between represented a mixture of upper living epidermis and lower stratum corneum and contained a 'keratin' of intermediate profile. This was characterized by a lack of chains a_1 , b_1/b_3 and c_3 , together with a relative increase in a_3/a_5 and b_5 . The 'keratin' from a mixture of lower living epidermis and dermis appeared deficient in the highest-molecular-weight polypeptide (a_1) and as the dermal content increased, so the polypeptides of mol.wt. 50 000–70 000 faded. All the 'keratin' extracts appeared deficient in the smaller polypeptides (d_1 , d_3) consistently found in heel prekeratin extracts.

Extraction of whole epidermis

In order to investigate changes in the solubility of the 'keratin' polypeptides as epidermal differentiation proceeds, and to assess the possibility of extracting keratin from epidermis containing pre-

keratin, samples of whole scalp epidermis were extracted in four different buffers (see the Materials and methods section). The resulting extracts were compared on a single slab gel (Fig. 5).

Many polypeptides over a wide range of molecular weight, including several in the 'keratin' region were observed in Tris buffer (0.05 M, pH 7.2) extracts of scalp epidermis. Polypeptides covering a greater range of molecular weight (10 000–350 000) were extracted with CASC buffer, and these were further fractionated by isoelectric precipitation at pH 4. The supernatant contained several major polypeptides, but only one (mol.wt. 55 000) was in the prekeratin region. The isoelectric precipitates from the urea buffer extracts ($\pm$ mercaptoethanol) were similar in polypeptide profile, but not identical with the CASC-extracted prekeratin nor with each other, the differences being in respect of chains a_2/a_3 and b_1/b_2 .

Although several polypeptides in the prekeratin molecular-weight region were removed by a prior extraction with Tris buffer, the prekeratin was not affected in gross terms. However, after examining several samples, it was noted that a relative reduction of certain chains (c_3 , d_1) often occurred after extraction with Tris buffer. Extraction of the CASC buffer residue with Tris/urea buffer released a group of polypeptides similar in profile to prekeratin, but with a relative increase in chain b_1 (mol.wt. 62 000). Further extraction of the insoluble residue with Tris/urea/mercaptoethanol buffer then released a different group of polypeptides that were almost identical in profile with those of forearm keratin.

Fig. 4. Changes in heel 'keratin' composition during epidermal differentiation

Heel skin was sliced parallel to the surface at 0.1 mm intervals and the Tris/urea/mercaptoethanol-soluble proteins were extracted from each slice. These extracts were dialysed against CASC buffer, pH 2.65, and the isoelectric precipitates (pH 4) were analysed on gradient slab gels (7.5–17.5%, see the Materials and methods section). Heel prekeratin from an adjacent piece of epidermis is shown (lane 1, values $\times 10^{-3}$ given for the major chains), as are the calibration proteins (lane 11, mol.wts. 29 000–130 000). Lanes 2–4 contain proteins from a mixture of dermis and lower epidermis (basal and spinous cells). Lanes 5 and 6 contain protein from mainly spinous and granular epidermal cells, the profile resembling that of prekeratin. Lanes 7 and 8 contain extracts of upper living epidermis and lower stratum corneum and the polypeptide profile is intermediate between that of prekeratin and keratin. Finally, lanes 9 and 10 contain protein from heel stratum corneum (callus), and a typical keratin polypeptide profile is observed.

Fig. 5. *Differential and serial extraction of human scalp epidermis*

Human scalp epidermis was extracted with four different buffers both individually (lanes 1–5) and as a serial extraction procedure (lanes 7–10). The extracts were compared by SDS/polyacrylamide-gradient-slab-gel electrophoresis (7.5–17.5%) and the gels were calibrated over the molecular weight range 14 300–130 000 (standards in lanes 6 and 12; see the Materials and methods section for details). An extract of forearm stratum-corneum keratin was run in lane 11. Many epidermal proteins were Tris (0.05 M, pH 7.2)-soluble (lane 1), including several in the same molecular-weight region as the 'keratins'. CASC buffer pH4 supernatants contained several polypeptides (lane 2) but only one (mol.wt. 55 000) was in the 'keratin region'. Isoelectric precipitates (pH4) from Tris/urea and Tris/urea/mercaptoethanol extracts (lanes 4 and 5 respectively) were similar, but not identical, in profile with epidermal prekeratin (lane 3). Prekeratin from a Tris-insoluble residue (lane 7) was similar to that from fresh epidermis (lane 8). Tris/urea-soluble protein from a CASC-insoluble residue (lane 9) was similar, but not identical, in profile to prekeratin (lane 8; also cf. lane 4). Finally, Tris/urea/mercaptoethanol-soluble material from a Tris/urea-insoluble residue (lane 10) differed from prekeratin (lane 8) but was almost identical with forearm keratin (lane 11) and therefore represents scalp keratin. Thus it is probable that the extract in lane 5 (Tris/urea/mercaptoethanol from whole epidermis) is a mixture of scalp prekeratin and keratin.

Discussion

A great deal of controversy exists with regard to the structure of epidermal keratin, the role of prekeratin as a precursor and the molecular details of the changes that occur during epidermal differentiation. There is still no general agreement on the number and molecular size of the polypeptide components of both keratin and prekeratin and the differences in structure, if any, between them (Steinert, 1975; Baden & Lee, 1978; Skerrow & Hunter, 1978; Bowden & Cunliffe, 1980; Fuchs &

Green, 1980), nor is there agreement on the mode of stabilization of the keratin filaments (Baden *et al.*, 1976; Sun & Green, 1978; Steinert & Idler, 1979). This situation has probably arisen owing to a combination of several factors that include the following: different extraction conditions, different electrophoretic systems, anatomical-site variation, species differences and the lack of a generally accepted definition as to what constitutes keratin and prekeratin. The present experiments on human epidermis have to some extent reconciled a number of these differences.

We have defined human epidermal prekeratin as a protein from living epidermal cells that is soluble in buffers between pH 2.65 and 3.5 and is composed of a family of polypeptide chains of variable size (mol.wts. 45 000–70 000). The composition of prekeratin is considerably more complex than previously appreciated (Skerrow, 1977; Baynes *et al.*, 1978; Baden & Lee, 1978), and the essentially crude acid buffer extracts contain many minor contaminating proteins. It would therefore be advantageous to extract and purify further the native tonofilaments before analysis and to examine the size/charge relationships between the individual prekeratin components by two-dimensional electrophoresis (e.g. O'Farrell, 1975; Ames & Nikaido, 1976).

The different number of polypeptide chain components, and the different molecular-weight values, reported for both bovine and human prekeratins may to some extent be a consequence of the gel system employed. We found a much greater variation in prekeratin composition indicated by the gel system than originated from anatomical-site variation (with the exception of the callus/non-callus differences) or the use of different extraction procedures. In fact our CASC buffer preparation of human scalp prekeratin resolved into three, seven or ten bands, in the molecular-weight range 45 000–70 000, in the three gel systems examined (Weber & Osborn, 1969; Laemmli, 1970; Lambin, 1978), and the molecular-weight values determined for prekeratin analysed in each system are in close agreement with the published values (e.g. Skerrow, 1977; Baden & Lee, 1978). The advantages of a discontinuous buffer system for the analysis of keratin components have already been discussed at some length (Steinert, 1975; Steinert & Idler, 1975) and those authors have more recently used high-resolution gradient gels for the analysis of bovine keratin (Steinert *et al.*, 1980).

However, the so-called 'chain d₁' (mol.wt. 46 000) presents a problem, as do the other polypeptides in this group. Baden & Lee (1978) have reported such a component of human prekeratin ('chain 2', mol.wt. 45 000), and the fact that this chain is not easily visible on gels prepared by the method of Weber &

Osborn (1969) may account for its absence in other reports. Keratin polypeptides of this molecular weight have been found in cultured cells and in human plantar epidermis (Fuchs & Green, 1980), but both of these tissues do appear to be atypical with regard to keratin content. Furthermore, in our hands the amounts of this chain were variable in different prekeratin extracts and this chain may therefore represent a major co-purifying protein. This could also be true of the smaller major polypeptide (mol.wt. 38000) often observed in prekeratin extracts, and such observations exemplify the need for better extraction and analytical techniques.

In common with Baden & Lee (1978) we observed slight variability between prekeratin extracts from different individuals and, in addition, have found slight variability in the composition of prekeratin taken from different anatomical sites (e.g. scalp, breast, foreskin). We have ruled out the possibility of contamination by dermal proteins, and as pilosebaceous-duct prekeratin is identical with that from the contiguous epidermis, the use of crude epidermal preparations in these studies is justified.

A major difference in the polypeptide-chain composition of prekeratin exists between plantar epidermis and the epidermis from non-callus areas (e.g. scalp, breast). The existence of three extra polypeptide chains in heel epidermal prekeratin provides an explanation for the anatomical-site variation reported for human stratum-corneum keratins (Skerrow & Hunter, 1978; Bowden & Cunliffe, 1980; Fuchs & Green, 1980). Thus it would appear that the callus of the hands and feet (palmar and plantar keratin profiles are almost identical) contain unique keratin polypeptides possibly derived from these additional prekeratin polypeptides. It seems unlikely that the different composition of callus keratin represents either a difference in modification of a common precursor or a more advanced stage of the keratin maturation process found in the stratum corneum at non-callus sites, these being explanations given in previous reports. Similar intraspecies heterogeneity has been demonstrated for bovine prekeratin and keratin (Lee *et al.*, 1979; Steinert *et al.*, 1980) and for rabbit, rat and guinea-pig keratins (Baden *et al.*, 1980; Fuchs & Green, 1980).

There has also been some dispute as to whether or not prekeratin and keratin differ in polypeptide composition and, if they do, exactly in what respects they differ (Steinert, 1975; Baden & Lee, 1978; Skerrow & Hunter, 1978). Our evidence that prekeratin differs from keratin in at least two respects, even though both have the same general properties, may be summarized as follows: (a) many of the individual polypeptide components do not co-electrophorese on high-resolution slab gels (see Fig. 3a and Table 1); (b) the extractability of

prekeratin and keratin vary, presumably because the keratin, but not the prekeratin, filaments are stabilized by disulphide links. Our evidence suggests that the keratin disulphide links are intermolecular and appear to involve a different class of protein, possibly the matrix proteins described by other investigators (Fraser *et al.*, 1972; Matoltsy, 1975; Dale *et al.*, 1978), which remain soluble in CASC buffer at pH4 and above. In our hands, keratin is not extractable from stratum corneum or from whole epidermis in the absence of a reducing agent, but once the keratin is removed from CASC solution by isoelectric precipitation at pH4, it behaves just like prekeratin, and there is no tendency for disulphide bridging to occur. Furthermore, there appears to be an intermediate between prekeratin and keratin that is not CASC-extractable but which can be removed from whole epidermis with a Tris/urea buffer. A similar observation has been reported by Baden & Lee (1978).

Finally, we wish to consider the changes that occur to the 'keratin' polypeptides during differentiation of the epidermis. The results obtained from examining the 'keratin' profile at different levels of heel epidermis (see Fig. 4) were very similar to those of Fuchs & Green (1980). The polypeptide profile of the 'keratin' from the living layers of the epidermis was that of prekeratin. However, extracts of slices containing large amounts of basal cells compared with spinous cells did appear to be deficient in the larger polypeptide chains (a_1 , mol.wt. 70000). This provides some explanation for the lack of these polypeptides in cultured epidermal basal cells (Sun & Green, 1978; Kubitil *et al.*, 1979) and supports the finding that basal-cell immunofluorescence could not be demonstrated with an antiserum prepared against a 67000-mol.wt. keratin polypeptide (Viac *et al.*, 1980). As tonofilaments are found in the basal cells, these observations raise a question as to the function of the higher-molecular-weight polypeptides (a_1 , a_2 , a_3 ; 70000–66000) in respect of filament formation.

At a later stage in keratinization, associated with the granular layer, the size and probably charge distribution of the prekeratin polypeptides are altered, which results in the formation of the keratin profile. Although alteration in the synthetic activity of the epidermal cells at a late stage during the differentiation process could explain this observation (Fuchs & Green, 1980), it is likely that the release of very active hydrolytic enzymes in the upper granular layer is involved (Lavker & Matoltsy, 1970). This would imply that at least some of the polypeptides in prekeratin and keratin extracts bear a product-precursor relationship. It is also at this stage that the disulphide stabilization occurs to complete the assembly of the fibrous/amorphous matrix of the mature corneocyte. In contrast with

Steinert & Idler (1979), we have no evidence for degradation of keratin near the skin surface. Examination of serial slices through callus showed that the polypeptide profile was identical throughout the outer stratum corneum. Deeper down, the only changes that were apparent was a tendency to approach the prekeratin profile, a highly specific event in differentiation rather than a generalized environmental effect.

Recent evidence suggests that several keratin genes exist in the epidermal cells, and that the synthesis of the gene products in the lower layers of the epidermis may be sequential, but it is not yet known whether control is exerted at the level of translation or transcription (Fuchs & Green, 1979; 1980; Bowden *et al.*, 1980; Schweizer & Goerttler, 1980). It appears that further size and charge heterogeneity is then introduced by post-translational modification of the gene products, which results in the complex polypeptide profile of epidermal prekeratin. This is at least in part due to phosphorylation (Sun & Green, 1978; Gilmartin *et al.*, 1980; P. E. Bowden, unpublished work), but the role of glycosylation has not yet been fully investigated. Thus, although the structure of the keratin filament appears to be based on a triple helix (Skerrow *et al.*, 1973; Steinert, 1978), the biosynthesis of this structural protein is complex and appears to involve several sequential molecular events during differentiation. Further understanding of epidermal keratinization, in normal and, perhaps more important, in pathological skin, and an appreciation of the control mechanisms involved, will require more detailed study of the biosynthesis of this fibrous protein.

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