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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Specialized keratin expression pattern in human ridged skin as an adaptation to high physical stress


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Accepted for publication 14 June 1998

Summary

We have analysed the expression of keratins in the epidermis of normal human palm and sole skin (ridged skin) using immunohistochemistry and in situ hybridization. The epidermis of human ridged skin expresses a more complex pattern of keratins than thin skin, which is probably due to the greater stress that ridged skin has to withstand. In addition to keratin K9, we document specific expression patterns of keratins K6, K16 and K17 which are suggestive of regional adaptations of this epidermis to a high cell turnover rate. In particular, the sequestered location of nests of K17-positive cells at the bottom of the deep primary epidermal ridges supports the notion of functional heterogeneity of basal cells and suggests that the K17-positive sites may include stem cells. Expression of K6 and K16 in some basal and most suprabasal keratinocytes is compatible with a constitutively high proliferative activity of normal ridged epidermis, but may also reflect different physical properties of the suprabasal cells, in contrast with regions expressing K9. The distinct labelling patterns observed in primary and secondary epidermal ridges as well as epidermal layers above dermal papillae suggest the existence of local microenvironmental niches leading to differences in keratinocyte differentiation.

Several genetic skin diseases show a striking predilection for palms and soles or manifest exclusively in these sites, e.g. the various forms of palmoplantar keratodermia. The ridged epidermis of palmoplantar skin is a unique tissue because of the amount of physical stress which it has to endure. To survive this stress, it has evolved structural specializations which bring their own hazards. The epidermis normally grows thicker in response to physical stress; palmoplantar skin also becomes abnormally thickened and keratotic in many skin disorders caused by mutations in keratin genes which weaken the cell cytoskeleton. Several studies have suggested that palm and sole skin is structurally different. Swensson and Eady observed that the keratinocytes in ridged skin have an unusually large amount of keratin filaments in their cytoplasm compared with thin skin, possibly due to the expression of a palmoplantar-specific keratin. To investigate the biological basis for the resistance of ridged skin to physical trauma and to understand the mechanism underlying the palmoplantar keratodermia that develops in people with epidermolysis bullosa simplex and related disorders, we undertook a study of keratin expression in ridged skin using immunohistochemistry and in situ hybridization. The results show that this tissue expresses several keratins additional to those in thin skin, and the spatial patterning of keratin expression provides insight into the extreme resistance to physical stress of palmoplantar skin.

Materials and methods

Skin samples

Skin samples were obtained from six healthy volunteers (two women and four men) aged 28–62 years. Xylocaine
Keratin  | Antibody | Dilution* | Supplier                  | Reference |
---------|----------|-----------|---------------------------|-----------|
K1       | LI017    |           | Authors' labs (L.M.E.E.R.) | 19        |
K2c      | IMP2     |           | Authors' labs (W.H.M.)    | unpublished|
K5       | RebK5    | 1:10      | Authors' labs (L.M.E.E.R.) | 20        |
K6       | KAI2     |           | Authors' labs (E.R.E.L.)  | unpublished|
K7       | LPIK     |           | Gift from J.Bartek        | 21        |
K7, K17  | C46      |           | Authors' labs (E.R.E.L.)  | 22        |
K8       | LBI41    |           | Authors' labs (E.R.E.L.)  | 23        |
K9       | AM1      | 1:200     | Authors' labs (L.L.)      | unpublished|
K9       | TY1      | 1:200     | Authors' labs (L.L.)      | unpublished|
K10      | LIHP2    | 1:10      | Authors' labs (L.M.E.E.R.) | 19        |
K14      | LI001    | 1:40      | Authors' labs (L.M.E.E.R.) | 20        |
K16      | LI025    |           | Authors' labs (L.M.E.E.R.) | unpublished|
K17      | E1       |           | Dako*                     | 24        |
K18      | RCX106   |           | Gift from J.Ramsaro       | 25        |
K19      | LP2K     |           | Authors' labs (E.R.E.L.)  | 26        |

*All monoclonal antibodies were used as undiluted tissue culture supernatants unless otherwise stated.
Dako Ltd, High Wycombe, U.K.

(2%) without adrenaline was used as local anaesthetic. Samples were obtained from the hypothenar region of the palms (n = 4) and soles (n = 4). One sample was obtained from a 6-month-old patient undergoing surgery for removal of an extra digit. Fetal tissue samples (n = 6) were obtained after elective termination of pregnancy with the approval of the Guy's and St Thomas' Hospital Trust Ethics Committee. The estimated gestational age of fetuses was based on maternal history (menstrual age minus 14 days) and measurements of crown-rump length. Fetal ridged skin of 12–16 weeks' gestational age was studied. Normal human skin from the inner aspect of the upper arm was used as a control. Specimens were divided and processed either for in situ hybridization or immunohistochemistry.

Immunohistochemistry

Specimens were immediately snap-frozen in liquid nitrogen-cooled isopentane using cryoembedding medium (Tissue-Tek OCT 4583 compound, Agar, Stansted, UK) and processed using standard immunofluorescence techniques. Briefly, 5-μm cryostat sections were collected on 3-aminopropyltriethoxysilane (APES)-coated multilwell slides and air-dried for 60 min. Incubation with primary antibodies (Table 1)19-26 was for 2 h at room temperature. After thorough rinsing in phosphate-buffered saline (PBS, pH 7.2), sections were incubated with either fluorescein isothiocyanate (FITC)-conjugated rabbit antirabbit antibody (1:50 in PBS) or FITC-conjugated swine antirabbit antibody (1:20 in PBS) (both from Dako Ltd, High Wycombe, U.K.). In the case of TY1 and AM1 (both anti-K9), rabbit anti-guinea-pig antibody (1:200 in PBS) was used as secondary and FITC-conjugated swine antirabbit antibody (1:100 in PBS) as tertiary antibody. Incubation was for 60 min in a humid chamber at room temperature. Sections were counterstained for 1 min with propidium iodide (50 μg/ml of 100 ml PBS), rinsed in distilled water and mounted in ‘antifade’ medium.27 Sections were viewed under a Nikon Optiphot-2 fluorescence microscope (Nikon, Kingston upon Thames, UK.). As negative controls, conditioned medium of the hybridoma parent myeloma cell line SP2/0-Ag14 or irrelevant antibodies were used instead of specific primary antibody. As positive controls, various human skin samples known to express the relevant keratin antigens28 were used.

In situ hybridization

Specific radioactive cRNA probes were made using in vitro transcription of human cDNA fragments from 3'-coding and non-coding regions of human keratins K1, K2c, K5, K9, K10 and K14. The cDNA fragments were cloned into Bluescript II KS+ (Stratagene, Heidelberg, Germany) using T3 and T7 polymerase (Boehringer Mannheim, Mannheim, Germany) for sense and antisense cRNA, respectively. Probes were labelled with 35S-rCTP and used in full length form (≈200–500 nucleotides) without hydrolysis. In situ hybridization was performed as described previously. Briefly, tissue specimens were snap-frozen in liquid nitrogen-cooled

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isopentane. Five-micrometre cryostat sections were collected on APES-covered glass slides, air dried, and fixed in 4% buffered paraformaldehyde for 20 min. Prehybridization was done for 3 h at 42 °C. For each tissue section 1–2 ng of denatured labelled probe were used for hybridization at 45 °C for 12 h in a humidified chamber. After hybridization, specimens were thoroughly rinsed with 2 × SSC/50% formamide at 55 °C, 1 × SSC/50% formamide at 50 °C, 0.1% sodium dodecyl sulphate at room temperature, 1 × SSC/20 μg/mL RNase A at 37 °C, 0.5 × SSC/50% formamide at 55 °C, and PBS. Slides were pre-exposed on X-ray films (X-omat, Kodak) for 24 h to calculate correct exposure times. Specimens were covered with photographic emulsion (NTB2, Kodak) and exposed for 2–3 days to detect radiolabelling. Sections were counterstained with haematoxylin and viewed using a confocal laser scanning microscope (LSM 410 UV, Zeiss, Oberkochen, Germany). Pictures were taken with a

Figure 1. Demonstration of mRNA synthesis of various keratin in plantar epidermis using radioactive in situ hybridization (hybridization signal in red combined with bright fields optics). (a) Keratin K14 mRNA is restricted to basal cells. It is also seen in basal keratinocytes of the eccrine duct. (b) K16 mRNA is present in the lower half of the epidermis, especially in secondary epidermal ridges. Most suprabasal cells show a strong signal for K1 mRNA, although there is less in the secondary ridges (where K16 is highest). In contrast, K12 mRNA (d) is predominantly localized in the upper spinous and granular layers. Scale bars = 25 μm.

Figure 2. Demonstration of keratin K9 mRNA in normal human plantar skin. (a) A discontinuous labelling pattern is seen in suprabasal cells with alternating vertical bands of K9-positive and K9-negative cells. (b) Primary epidermal ridge showing particularly strong labelling for K9 mRNA (left-hand side) next to secondary epidermal ridge with lower signal intensity (right-hand side). Scale bars = 50 μm.
Results

Keratins present in interappendageal epidermis of non-ridged skin are also expressed in ridged skin

Interappendageal epidermis of ridged and non-ridged skin showed immunoreactivity for keratins K5, K14, K1, K10, K2e, K8 and K18. In situ hybridization revealed the presence of K5, K14, K1, K10 and K2e mRNAs. In both sites the expression patterns were essentially similar for K14, K1 and K2e (Fig. 1a,c,d). One interesting detail was noted, regarding a different mRNA–protein relationship between K5 and K14. Immunostaining for K14 was observed in basal and lower suprabasal cells, whereas K14 mRNA labelling appeared to be confined to the basal layer. In epidermal ridges of palm and sole skin, however, K5 protein and mRNA appeared not to be restricted to the basal layer but were also detected in the first and sometimes second suprabasal cell layers. This suggests a different regulation mechanism for K5 vs. K14. Antibodies LE41 (K8) and RCK106 (K18), identifying keratin subtypes expressed in Merkel cells, labelled solitary cells in the basal layer in both skin types. In ridged skin, K18-positive cells were located at the bottom of epidermal rete ridges and appeared more numerous than in arm skin.

Ridged skin constitutively expresses keratins K9, K6 and K16

Ridged epidermis also expressed some additional keratins not detected in arm skin, i.e. K9 (Fig. 2) as reported by Knapp et al., Moll et al., and Langbein et al. and K6 and K16 (Fig. 3). In situ hybridization showed a discontinuous synthesis pattern for K9 mRNA. Alternating vertical segments of K9-positive and K9-negative cells were seen through the suprabasal compartment (Fig. 2a). Primary epidermal ridges usually showed strong expression of K9 mRNA whereas a low signal intensity was seen in secondary epidermal ridges (Fig. 2b). In the epidermis overlying the dermal papillae almost all suprabasal cell layers contained K9 mRNA. The immunolabelling patterns for K6 and K16 were not completely congruent. Antibody KA12 against K6 stained an epidermal band of cells in the lower half of ridged epidermis (Fig. 3c), and some individual basal cells were also positive.
for K6. K16 stained with LL025 was expressed in suprabasal cells, in most basal cells overlying the dermal papillae and in many basal cells located at the sides of epidermal ridges (Figs 1b and 3a,b). In some specimens K16 immunostaining was confined to distinct parts of ridged epidermis, producing a discontinuous labelling pattern of alternating vertical segments of K16-positive and K16-negative cells (Fig. 3a).

A subpopulation of basal cells in ridged skin strongly expresses keratin K17

A number of basal cells that were positive for RabK5 (K5) showed strong labelling with E3 (K17) and C46 (K7 and K17). Whereas some degree of C46 labelling was present in many basal cells, particularly strong immunostaining was detected in small groups of basal cells located either above dermal papillae or at the bottom of epidermal ridges (Fig. 4a). However, immunoreactivity with the monospecific K17 antibody E3 was confined to small numbers of basal cells arranged in clusters at the bottom of most primary epidermal ridges (Fig. 4b).

Site-characteristic keratins of ridged epidermis are present early in ontogenesis

At 12 weeks’ estimated gestational age, ridged epidermis comprised three cell layers, i.e. basal layer, intermediate layer and periderm. K17 immunoreactivity was observed in all cell layers but seemed particularly strong in cells of the developing primary epidermal ridges (Fig. 5a). At 15 weeks’ estimated gestational age, ridged epidermis comprised four to six cell layers, i.e. basal layer, two to four intermediate layers and periderm. Primary epidermal ridges and sweat duct anlagen were present. K9-positive cells were seen in the intermediate layers and periderm. In epidermal ridges cells of the lower intermediate layer were negative and cells of the upper intermediate layer weakly positive for K9 (Fig. 5c). A similar immunolabelling pattern was observed for K10. In contrast, K6 and K16 appeared to be expressed in all cell layers including periderm of ridged epidermis at this stage (Fig. 5b).

Discussion

Primary epidermal ridges contain the intraepidermal portion of the eccrine duct. They are present in fetal skin at about 14 weeks’ estimated gestational age. They are the site of eccrine bud formation and are therefore also known as ‘glandular ridges’. The formation of primary ridges and eccrine glands precedes the development of secondary epidermal ridges which form between primary ridges, producing a characteristic pattern of alternating primary and secondary ridges (Fig. 6). Finally, there are small epidermal septa that divide the papillary dermis transversely and interconnect primary and secondary ridges. The histological appearance of the epidermal ridges is dependent on the plane of

differentiation-specific suprabasal keratins is non-random and appears to be directly linked to the periodic pattern of epidermal ridges and dermal papillae. K9 is expressed strongly directly above the primary epidermal ridges, in the centre of the papillary ridge. K16 is complementary in its staining pattern, and is excluded from the primary epidermal ridge where K9 staining is strongest.

To interpret this complex pattern we should consider the physical constraints acting on the three-dimensional structure of palmoplantar skin. Of all the epidermis tissue of the body, palmoplantar epidermis is subjected to the most extreme and extensive physical stress. We know from studies of skin fragility disorders that the primary role of keratins in epidermal cells is to reinforce them so that they do not lyse upon physical pressure. It seems very likely that different keratins provide cells with subtly different properties of resistance and plasticity to equip the epithelial cells for the physical stresses of each particular body site. Figure 6 illustrates that the raised papillary ridges overlying the primary epidermal ridge take most of the compression stress on this skin. This is also the region where the palmoplantar-specific keratin K9 is most highly expressed, strongly suggesting that the function of K9 is to provide additional reinforcement in this stress-bearing epidermis.

The expression of K16 in a complementary pattern in the intervening secondary ridge zones may also be explainable in physical terms. Although K16 was originally thought to be primarily associated with hyperproliferation, this view is giving way to an interpretation of K16 as defining a pliable cytoskeleton because of its rapid appearance in wound healing and constitutive expression in non-cornified barrier epithelia. K16-expressing cells over the secondary ridges might provide periodic regions of plasticity or elasticity in between stiffer K9-expressing patches, an alternating 'hinge' region in between the papillary ridge 'plates' to provide this highly mobile skin with flexibility as well as strength to prevent it from being torn apart.

The variation in quantity and distribution of K16 vs. K17 in palmoplantar skin could also help to explain the different susceptibility to palmoplantar keratoderma seen in patients with the genetic disorder pachyonychia congeita. Jadassohn-Lewandowsky or type 1 pachyonychia congeita is associated with severe palmoplantar keratoderma and is caused by mutations in K16, while Jackson-Lawler or type 2 pachyonychia congeita is associated with mutations in K17 and has much less palmoplantar keratoderma. This stresses the greater structural role that K16 is likely to play in ridged skin, as opposed to K17 which is expressed by a smaller cell.
population largely situated in deep ridges and protected from physical damage.

K16 staining was observed to be unusually prominent in the basal layer in these experiments. K6 is usually assumed to be the polymerization partner of K16, but in these experiments K6 distribution was much more restricted than K16, although not excluded like K16 from the primary ridge. In this tissue, the staining pattern of K6 was actually closer to that of K17 than K16. The reason for these unconventional staining results is at present unclear but as yet we have no reason to doubt the specificity of the antibodies used in this study. K17, usually regarded as a suprabasal keratin, has been seen in basal cells of other tissues. Sequencing studies have uncovered multiple genes for K6 and possibly K17 and K16, although nothing is yet known about any differences in their relative expression patterns. The various probes used here would not have distinguished between different forms of K6 or K17. Further studies are required to clarify this.

The papillary ridge, or fingerprint line, takes the most stress as it is elevated above the skin surface. It will also therefore take the most abrasion and this will lead to a high cell loss rate, constant proliferation stimulus and high cell turnover. The greatest depth of live cells is also found here, between the deep primary epidermal ridges and the raised stratum corneum of the papillary ridges. This indicates that either cell proliferation takes place preferentially under the papillary ridges or that the region is populated by cells moving in from more lateral regions. The shape of the vertical sectors of positive or negative cells seen with keratin staining would suggest that there is not a substantial amount of lateral movement of differentiating keratinocytes. Such sectors reflect upward movement of cohorts of keratinocytes from the basal layer to the stratum corneum, and they will be most pronounced and straightest in body sites where the whole population of keratinocytes moves upwards at a similar rate. Thus the additional cells contributing to the papillary ridge are probably coming up from the K17-positive clusters.

Extrapolating from tissue sites where slow-cycling cells have been identified, stem cells are predicted to be located at the deepest point in the epidermis for protection, as they represent the tissue’s most valuable resource. It is possible therefore that the K17-positive cells at the base of the primary epidermal ridges represent a population of cells containing the progenitor or stem cells for palmoplantar epidermis. Further evidence for stem cells would require experiments to re-examine the distribution of integrin-positive cells in palmoplantar skin, reported by Jones et al., while discriminating between primary and secondary ridges, and/or analysis of the clonogenic potential of these cells taken from palmoplantar biopsies and/or in vivo labelling (with BrdU or radioactivity) to look for slow-cycling cells. These experiments cannot be readily done on human palmoplantar skin and substantial species differences mean
that a useful comparison with an animal model is also unlikely.

The striking localization of K17 to deep clusters of cells in what is probably the most proliferative part of this specialized epidermis highlights K17 as a useful marker for undifferentiated epithelial cells, possibly stem cells. It will be interesting to see if further analysis of systems more amenable to experimentation provide evidence to confirm or refute this association.

Acknowledgments

We thank Silke Prützel and Herbert Spring (both DKFZ) for their expert technical assistance with in situ hybridization and confocal scanning laser microscopy and Susanne Beissner-Schindler for her help in creating Figure 6. We are grateful to all volunteers participating in this study. This work was supported by The Wellcome Trust (grant 037444/A/93/Z to E.B.L. and R.A.J.E.), the Cancer Research Campaign Programme (grant SP 2060/0102 to E.B.L.) and the Dystrophic Epidermolysis Bullosa Research Association, DERRA U.K. (W.H.I.M. and E.B.L.). O.S. is a recipient of a Deutsche Forschungsgemeinschaft grant (Ch 58/7-2).

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