Desmosomes exhibit site-specific features in human palm skin


Abstract: Hereditary skin disorders resulting from desmosome gene pathology may preferentially involve the palms and soles. Why this is so is not clear. Moreover, even in normal control skin it is unknown whether there are differences in desmosome number, size or structural organization in palmoplantar sites compared with skin from other body regions. Therefore, we sought evidence for such differences by examining desmosome expression in relation to epidermal differentiation in both epidermis and cultured keratinocytes from normal human palm and breast skin samples. Confocal microscopy of skin biopsy material showed relative differences in the expression profiles of several desmosomal proteins (desmogleins, desmocollins, desmoplakin, plakoglobin and plakophilin 1) between the two sites. Western blotting revealed a higher expression level of all five proteins in palm compared with breast cultured keratinocytes. Staining for the differentiation-associated component, involucrin, suggested an earlier onset of synthesis of this protein in palm epidermis, and a suspension-induced differentiation assay showed that involucrin synthesis began earlier in palm keratinocytes than in breast cells. At 4–8 h, the number of involucrin-positive cells in palm keratinocytes was almost twice that in breast. Morphometric analysis showed that, overall, desmosomes were larger but of similar population density in the palm compared with breast skin. These findings demonstrate differences in desmosome structure and protein expression between the two sites, possibly reflecting the needs of palms and soles to withstand constant mechanical stress. They may also help to explain the preferential involvement of this region in certain hereditary disorders (palmoplantar keratodermas), associated with mutations in desmoplakin or desmoglein 1.

Introduction

Desmosomes (DMs) are intercellular junctions that are abundantly expressed in a variety of epithelia including epidermis. They contain two types of cadherins, the desmogleins (Dsgs) and desmocollins (Dscs), and a number of cytoplasmic proteins including desmoplakin (Dp), plakoglobin (Pg), plakophilin (Pkp), desmocalmin, pinin, envoplakin, plectin and IFAP300/plectin (1–3). These molecules are assembled in highly organized complexes on the cell surface (4) and serve both in cell-cell adhesion and in the peripheral anchorage of keratin intermediate filaments (KIF). In conjunction with KIF, DMs create an integrated three-dimensional scaffold across the epithelial tissues responsible for withstanding physical stress. An important role of DMs as sensors responding to environmental and cellular cues has also been postulated (5).

It has been shown recently that mutations in genes that encode certain DM components may underlie a number of hereditary disorders of the skin, hair and nail (6,7). For example, mutations in the Pkp 1 gene, PKP1, were shown to cause an autosomal recessive skin disease, ectodermal dysplasia/skin fragility syndrome (OMIM 604536) (8–11). Inherited abnormalities in the Dp or Dsg 1 genes may result in the striate form of palmoplantar keratoderma (PPK) (OMIM 148700) associated with haploinsufficiency or other autosomal dominant mutations (12–14). Homozygous autosomal recessive mutations in Dp or Pg have been found to underlie more severe disorders manifesting woolly hair, cardiomyopathy and PPK (OMIM 601214; 605676).
(15,16). Marked thickening of the epidermis, particularly the stratum corneum, is a major histological feature of all forms of PPK.

Electron microscopy provided the first clues to an underlying structural desmosomal disorder, initially in patients with Pkp1 deficiency (8) and then in those with autosomal dominant and recessive mutations in Dp (12,16). In both of these disorders the DMs, particularly in the spinous layer, were much smaller than normal and were aberrantly formed, often lacking discernible inner plaques. Normal connections with KIF were reduced or entirely lacking, so that the KIF network appeared condensed in a perinuclear distribution. Further, the intercellular spaces were widened as a result of diminished cell-cell adhesion (8,12,14,16). These different reports all suggest that DMs play a fundamental role in the maintenance of structural integrity in the skin as well as other DM-bearing tissue such as the myocardium.

Palmoplantar skin has different structural and functional properties compared with thinner skin from other body sites. For example, the nucleated cell layers are increased and the rete ridges are more prominent (17). The stratum corneum is thicker in the palm, and may vary in thickness according to the degree of mechanical stress (18). In histological sections, the stratum lucidum is seen between the stratum granulosum and stratum corneum. Keratin expression is also more complex. For example, a unique differentiation-related cytokeratin, K9, is expressed in palmoplantar skin (19) and exhibits a specific vertical segmented mRNA expression pattern as depicted by in situ hybridization analysis (20). Furthermore, stereological analysis has shown that the relative density of KIF is greater in palm skin than in thin skin (21). Bearing in mind these observations, and clinical observations of preferential involvement of palms and soles in hereditary DM diseases, the question arises whether DMs are different in palmoplantar skin (thick skin) in comparison with thinner skin at other sites? We wished to test the hypothesis that DMs possess site-specific features which are integral to the specialized differentiation program characteristic of palmoplantar epidermis. In order to address this issue, we carried out a comparative study using a combination of morphological, immunohistochemical and biochemical approaches on the epidermis and cultured keratinocytes from palm and breast skin.

Materials and methods

Tissue section preparation and immunohistochemistry

With the approval of our hospital’s Ethics Committee, fresh samples of normal skin from breast (n = 5) and palm (n = 5) were obtained from 10 individuals, aged 20–50 years, undergoing plastic surgery operations. Skin was washed immediately in phosphate buffered saline (PBS) for 30 min before being embedded in OCT compound (TAAB, UK) and snap-frozen in isopentane cooled by liquid nitrogen. Cryosections (6 μm thick) were fixed in 1:1 acetone and methanol at −20°C for 20 min and rehydrated in PBS for 2 × 15 min before blocking of non-specific immunoabsorptive binding sites with 10% normal goat serum in PBS, containing 0.2% Tween 20, for 30 min. Antibody staining was performed on the same day. Sections were incubated in the primary antibodies (Table I) for 1 h at 37°C followed by 3 × 5-min washes in PBS/0.2% Tween 20. They were then incubated with secondary antibodies, either goat antimouse Alexa Fluore 488 conjugate or goat antirabbit Alexa Fluore 568 conjugate (Molecular Probes via Cambridge Biosciences, UK) for 1 h. After extensive washes in PBS/0.2% Tween 20 they were mounted with coverslips. The fluorescence antibody conjugates used in the study were stated by the supplier to have a high degree of cross-absorption, particularly for dual labelling to prevent cross-reactivity. The sections were stored at 4°C and confocal microscopic imaging was carried out within 48 h.

Laser scanning confocal microscopy

Single or dual immunofluorescence images were acquired with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Jena, Carl Zeiss Ltd, UK), using an Aron laser (458 nm) and a HeNe1 laser (543 nm) combined with band pass filters of 505–530 nm for Alexa Fluora 488 and 560–615 nm for Alexa Fluora 568, respectively. The dual labelling image stacks were obtained sequentially to prevent cross-talk between the two channels and each antibody staining was acquired using the same microscope setting. All images were compressed from the same number of slices to give equal thickness for comparison. Data were expressed as the mean ± SEM of five observations.

Determination of cell counts

As the cell size appeared to differ between the two types of skin, the total cell number in a given area, i.e. 4000 μm² in breast skin and 8000 μm² in palm skin including all the nucleated layers, was counted in sections with Dp and involucrin staining from 10 individual samples. The number of cell layers with positive Dp, but absent involucrin staining, was counted and the data were analyzed using Student’s t-test.

In situ hybridization

In order to confirm Dp expression at the mRNA level, in situ hybridization (ISH) was performed using 12-μm cryosections from both types of skin. We used a double FITC-labelled probe supplied as a kit including positive and negative randomer controls (Biognostik, Germany). In situ hybridization was performed following the protocol provided by the supplier but slightly modified. Briefly, the fresh frozen sections were fixed in 4% paraformaldehyde/PBS for 5 min, washed in PBS before enzyme digestion with 10 μg/ml protease K (Sigma, UK) for 15 min at 37°C and further fixation in 1% paraformaldehyde for 5 min. After washing in distilled water, the sections were treated in 0.2 N HCl for 10 min followed by acetylation in 0.25% acetic anhydride/0.1 M triethanolamine solution for 10 min. After further rinsing in distilled water, they were air-dried and incubated in prewarmed prehybridization buffer for 2–4 h at 30°C, hybridised with the probe over 16 h at 30°C followed by washing in 1% and then 0.1% sodium chloride and sodium citrate solution, respectively. The sections were incubated with alkaline
phosphatase-conjugated anti-FITC antibody (Roche, UK). Finally, for visualization of the signal, the sections were incubated with 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitroblue tetrazolium chloride (BCIP/NBT) (Roche, UK) containing 240 μg/ml levamisole in alkaline detection buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2, pH 9.5). A parallel set of ISH was also performed on the cultured palm keratinocytes. Two positive controls of Poly-d(T) and β-actin and three negative controls including randomer, RNAse digestion before hybridization and without probe were tested on both the sections and cells. Signals were visualized with an Olympus VANOX-S microscope (Olympus Optical Co (UK) Ltd) and photographs were taken within 48 h of hybridization.

**Human primary keratinocyte culture**

Primary keratinocyte cultures were generated following a standard procedure (22). Briefly, skin samples were collected in transport medium [Iscove medium (Gibco, UK) plus 10% fetal calf serum (FCS) (ICN Pharmaceuticals Inc.), 100 iu/ml penicillin and 100 μg/ml streptomycin (Sigma, UK), 2.5 μg/ml amphotericin B (Gibco)] and washed several times in an antimicrobial solution [PBS containing 100 μg/ml gentamicin and 250 μg/ml amphotericin B (Sigma)]. Subcutaneous tissue was trimmed from the samples, which were then minced before digestion in 0.25% trypsin at 37°C for 2 h. The resulting single-cell suspensions of keratinocytes were passed through filters with a 100-μm pore size, spun down and resuspended in keratinocyte culture medium [DMEM and Ham’s F12 medium in a ratio of 3:1 (v/v) (Gibco) supplemented with 10% FCS, 0.4 μg/ml hydrocortisone (Sigma), 10−10 M cholera toxin (Sigma), 10 ng/ml epidermal growth factor (Gibco), 5 μg/ml insulin (Sigma) and 1.8 × 10−4 M adenine (CALBIOCHEM, UK)]. The keratinocytes were plated on a 3T3 feeder layer lethally treated with 4 μg/ml mitomycin C (Sigma) in tissue culture flasks and propagated in an incubator with a humidified atmosphere containing 10% CO2. At approximately 60–70% confluence, the cells were harvested with 0.25% trypsin plus 0.02% EDTA following removal of 3T3 feeders, and the cell number was counted using a hemocytometer.

**Immunoblotting**

Whole-cell protein extracts from six individuals, three from breast and three from palm, were prepared by growing keratinocytes in vitro from the first passage, initially plated in Petri dishes coated with type I collagen (IBFB, Germany) at the same density (1.7 × 105 cells/35-mm Petri dish). When they reached monolayer confluence, the cells were washed with ice-cold PBS before lysis in boiling sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.001% bromophenol blue and 1.44 M 2-mercaptoethanol]. The cell lysate was transferred to microcentrifuge tubes and boiled for 8 min before being subjected to SDS-PAGE or stored or at −70°C. The total protein concentration was determined by a Coomassie protein assay (Pierce, UK). Thirty μg of total protein was loaded in each lane, resolved on 4–20% Tris-Glycine ready gel (Bio-Rad, UK), and transferred electrophoretically onto nitrocellulose (Amersham). Non-specific protein binding of the resolved protein bands was blocked overnight with 5% w/v non-fat milk and 0.1% v/v Tween-20 in Tris-buffered saline (TTBS). The membranes were subjected to sequential incubations in the primary antibody (Table 1) and secondary peroxidase-conjugated antimouse or antirabbit IgG (Chemicon International Inc., UK). Following 3 × 10-min washes in TTBS, detection was performed by an enhanced chemiluminescence (ECL), (Amersham, UK). An internal control of heat shock protein HSC-70 was re-probed with antibody sc-7298 (Table 1) in each blot to demonstrate equivalent protein loading. All blots were subjected to densitometry analysis using the NIH (National Institutes of Health, MD) Image 1.6.1 software Gel Plotting macro. Finally, band densities from three individual samples were presented as the mean ± SEM.

**Suspension-induced differentiation assay**

A cell suspension assay was performed as described previously (23). Briefly, preconfluent cultures of breast and palm keratinocytes were disaggregated in trypsin/EDTA and resuspended at a concentration of 107/ml in medium supplemented with 10% fibronectin-free FCS and 1.45% methylcellulose (4000 centipoises, Sigma). Aliquots (0.2 ml) of triplicates were plated in 15-ml Falcon tubes coated with 0.4% polyHEMA to prevent cell-substratum adhesion. After incubation for a time course of 0, 2, 4, 8 and 24 h at 37°C, the methylcellulose was diluted with PBS and the cells recovered by centrifugation. Following three extensive washes, the cells were plated onto the coverslip in 70-μl keratinocyte medium and dried in a hybridization oven at 37°C for 1 h before fixation in 3.7% formaldehyde for 20 min and permeabilization in ice-cold methanol for 5 min. The cells were immunostained for involucrin using DHI (Table 1) and counterstained with DAPI (Molecular Probes via Cambridge BioScience), and the ratio of involucrin-positive cells to total DAPI staining cells was counted. The data were plotted as the average of triplicate counts at each time point.

**Electron microscopy and morphometry**

Skin specimens from four individuals, two from breast and two from palm, were fixed in 2% formaldehyde with 2.5% glutaraldehyde in Sörensen’s phosphate buffer pH 7.4 followed by further fixation in 1.3% aqueous osmium tetroxide and en-block staining with 2% uranyl acetate in 50% ethanol. The samples were then processed using standard techniques and embedded in TAAB 812 (medium hardness) epoxy resin (24). Ultrathin sections (60–90 nm) were stained with 2% uranyl acetate (50% ethanol) and Reynold’s lead citrate before observation in a JEOL 100CX transmission electron microscope. Desmosome size and population density were estimated in four preassigned epidermal zones (1, basal; 2, the first and second suprabasal; 3, all of the spinous layer except zone 2; and 4, granular layer). To measure the DM size, electron micrographs were taken at 13,000× magnification of several arbitrary fields covering each zone in ultrathin sections from each individual. All the negatives were scanned into a personal computer using Adobe Photoshop image software (Adobe Systems Inc., CA). Only DMs with a clear midline structure were included. Measurement of the DM midline length was taken using the Photoshop measurement tool. In total, we analyzed 612 DMS in this study. For DM volume density measurement, the electron micrographs were taken at 5000× magnification and two arbitrary fields within each zone were chosen. The negatives were scanned into the computer using the same image software. To perform point counting for relative estimation of DM volume density, a coherent double square lattice test system (25) was applied using a coarse test lattice (at a distance of 0.5 cm, corresponding to 1 μm on the section) with four subdivisions, giving a total of 252 heavy test points and 4032 (252 × 16) light test points for each negative. In total, 35 negatives were analyzed. As it was noted in our immunofluorescence studies that the cells were larger in palm than breast, particularly in the spinous layers, we chose the cytoplasm as the reference space. We counted the light test points falling on the extracellular core domain of DMS (Pext) and heavy points on keratinocyte cytoplasm (Pcy). The stereological parameter was determined as the ratio of DM counts to cytoplasm counts [VDMcy−PDMcy/Pcy × 16] and referred to the DM volume density.
Statistical analysis

Data from the EM analysis were checked for normal distribution before group comparison. One-way analysis of variance was used to compare the means of DM midline length and volume density between the zones, as well as between the two skin types. Data were expressed as the mean ± SEM.

Results

Desmosomal cadherins desmocollins and desmogleins show a graded distribution, but distinct expression profiles, across the epidermis of thin and thick skins

Dual immunofluorescent staining of skin sections from normal human breast and palm samples using pan-antibodies 52–3D and 919 directed against Dscs and Dsgs (Table 1), respectively, showed a graded overlapping distribution of DM cadherins (Fig. 1). Both cadherins showed mostly peripheral staining in the epidermal keratinocytes. Desmogleins were more prominently expressed in the basal and immediate suprabasal layers whereas Dsc expression increased from the first suprabasal layer into the spinous layer. In thick skin, Dsc expression decreased in the upper spinous layer but in thin skin its expression increased steadily in ascending layers and finally reached its maximum in the upper spinous layer or granular layer. On the other hand, Dsgs appeared more evenly expressed across the epidermis (Fig. 1g). Strong expression of both Dsgs and Dscs was seen only in the lower epidermis in thick skin, i.e. the rete ridges. In a broad zone from the immediate suprabasal layer in the suprapapillary epidermis to the upper spinous or granular layer, staining for Dsgs and Dscs decreased. In the granular layer Dsg staining remained very weak or disappeared, but Dsc expression increased again (second expression peak) and the staining pattern became punctate (Fig. 1d, →). These distinct expression profiles suggest that DM assembly and organization might differ between thin and thick skins, and the DM appear to undergo transitional changes within the upper epidermis of thick skin.

Desmocollins 1 and 3 isoforms also show distinct expression profiles between thin and thick skin

In order to explore this difference further, we examined the expression of Dsc1 and 3 isoforms...
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Figure 2. Desmocollin (Dsc) 1 and 3 show distinct expression patterns in breast and palm skin. Confocal images of immunostaining (a, b, d, e) for Dsc1 (a, d) and Dsc3 (b, e) with U100 and U114, respectively, in breast (a, b) and palm (d, e). The profiles of fluorescence intensity from the basal to outermost layer in epidermis (c, f and lines in a, b, d, e). In breast skin, Dsc1 is expressed in all suprabasal layers and increases steadily in ascending layers while Dsc3 is evenly expressed across the epidermis. In palm skin, the fluorescent intensities for both proteins are weaker and appear to be much lower in the upper epidermis. The staining pattern of Dsc1 appears to be punctate in the outermost layers (d, → and insert) whereas Dsc3 staining almost disappears. Scale bar, 20 μm.

Figure 3. Dual labelling plakoglobin (Pg) and desmoglein (Dsg) and single labelling Dsg3 demonstrates differences between breast and palm skin. Immunostaining for Pg (green, c, d) and Dsg (red, e, f) with antibodies PG5.1 and AHP321, respectively, and single staining for Dsg3 (g, h) with Dsg-G194 in breast (a, c, e, g) and palm (b, d, f, h). Merged images of Pg and Dsg staining (a, b). Both proteins are strongly expressed in the spinous layers but are not always colocalized. In palm, staining for both proteins is relatively uniform in lower and middle spinous layers but slightly reduced in upper spinous layers (b, →). In contrast, in breast skin, strong fluorescence is seen in the upper spinous layers (a, →). Desmoglein changes its expression pattern to punctate in the upper spinous cells in palm (f, insert). Desmoglein 3 staining appears bright in the basal layer in breast skin while the bright staining extends into the spinous layers in the rete ridges of palm (g, h). Scale bar, 20 μm.

Evidence for distinct terminal differentiation processes in thick skin vs. thin skin

In order to clarify the putative transitional changes in DM expression, we examined the expression of involucrin, an early terminal differentiation marker (26), together with the major DM plaque protein, Dp, using antibodies DH1 and 115F using the monoclonal antibodies U100 and U114 (Table 1). We observed that Dsc1 was expressed exclusively in the suprabasal layers in both skin types while Dsc3 was present evenly across the living layers of epidermis in thin skin (Fig. 2a–c). However, in thick skin, the fluorescence intensities for both Dsc1 and Dsc3 were much weaker (Fig. 2f). As for Dsc staining in Fig. 1, staining for both isoforms in the upper epidermis was attenuated or absent. Again the staining pattern for Dsc1 in the granular layer appeared to be punctate (Fig. 2d, → and insert), resembling that of 52–3D antibody staining (Fig. 1d). The Dsc3 expression was strongest in the first suprabasal layer and gradually decreased in ascending layers upwards, and finally disappeared in the upper spinous or granular layers (Figs 2e,f). Moreover, staining for Pg and Dsgs with PG5.1 and AHP321 antibodies (Table 1), respectively, was attenuated in the upper spinous cells in palm but not in breast skin (Figs 3a,b, →). This finding further confirmed our observation of distinct DM expression profiles in thin and thick skins, suggesting that DMs might possess different assembly and functional properties associated with the degree of epidermal differentiation. To investigate whether the expression of Dsg3, a basal cell associated isoform, was different in the two types of skin, we used Dsg-G194 antibody (Table 1) and found that Dsg3 expression was present in the basal layer in breast skin but extended into the suprabasal layer in palm skin (Figs 3g,h). These different expression profiles in desmosomal cadherin isoforms further suggest possible distinct keratinocyte properties between thin and thick skins.
Desmosomes in palm skin (Table 1), respectively. Desmoplakin staining was abundant and uniform at the cell periphery in the typical punctate pattern. Cytoplasmic staining also appeared to be present in several sections. Strong expression was confined to the rete ridges in palm and began to decline and shift to a peripheral linear pattern in the upper epidermis (Fig. 4a,b). Correspondingly, staining for involucrin in palm occurred from the middle spinous layer in the rete ridges and the first suprabasal layer above the dermal papilla (Fig. 4d, →), indicating that the cells with weak Dp staining were undergoing terminal differentiation (Fig. 4d). In contrast, expression of involucrin in breast skin was limited to the stratum granulosum or the outermost layers close to the skin surface (Fig. 4c), suggesting the later onset of terminal differentiation in thin skin. H&E staining showed that the stratum granulosum contained 2–3 cell layers in breast and 4–6 layers in palm (data not shown). The number of cellular layers with Dp-positive and involucrin-negative staining in thick skin was noted to be twice that in thin skin (Fig. 4f). To see whether there were any differences in terminal differentiation in cultured primary keratinocytes from thin and thick skins, we conducted an in vitro suspension-induced terminal differentiation assay. We found that keratinocytes from thick skin underwent terminal differentiation earlier than those from thin skin (Table 2). At 4 h, the percentage of involucrin-positive cells was approximately 35% in palm cells but only 15.2% in breast cells. At 8 h this rose to approximately 95% and 55%, respectively. To examine further the reduction in Dp expression in the upper spinous layer, we used ISH to investigate Dp mRNA expression in the skin. Consistent with our immunohistochemical findings, the Dp message increased steadily from the first suprabasal layer upwards in thin skin (Fig. 4g) but appeared most abundantly in the middle spinous layer within the rete ridges of thick skin (Fig. 4h). A cluster of cells located at the bottom of the deep rete ridge in palm showed a very low level of Dp message (Fig. 4h, → and insert).

**Figure 4.** A distinct expression profile of desmoplakin (Dp) and involucrin is seen in breast and palm skin. Desmoplakin staining (a, b) (115F) shows abundant expression in breast skin (BS) (a) and palm skin (PS) (b) but is more evenly distributed in breast skin. The expression in palm predominates within the rete ridges but is attenuated from the middle spinous layer upwards where the staining pattern changes to a fine linear appearance. Merged images of Dp (green) and involucrin (red) with 115F and DH1 (c, d). The onset of involucrin synthesis begins in the outermost layer in breast skin but in the middle spinous layer in the rete ridge and in the first suprabasal layer above the dermal papilla (d, →) in palm skin where reduced Dp staining is seen. Bar chart shows that there are fewer cells in the unit area (e) in palm (P) vs. breast (B) (*P < 0.05), indicating keratinocyte size is larger in palm. Bar chart shows that there is almost twice the number of cell layers with Dp-positive/involucrin-negative staining (f) in palm (P) vs. breast (B). In situ hybridization with double FITC-labelled probe for Dp mRNA expression in breast, palm and cultured human palm keratinocytes (g,i,j), respectively. Desmoplakin is abundantly expressed in the upper spinous layer in breast. Detection is specific, cf. the random probe (g, insert). In palm, a cluster of cells located at the bottom of the deep rete ridge express low level of Dp mRNA (h, → and insert). Cells in the suprabasal layer express a higher level and those in the upper spinous layer show reduced expression. Detection is also seen in cultured human palm keratinocytes (j). Cultured palm keratinocytes immunostained for Dp (115F; green) with nuclear counterstain (DAPI, blue) (i). Scale bar (a–h), 20μm; (i, j), 5μm.

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*Mean ± SEM of three observations at each time point of one independent experiment.

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BS = breast (thin) skin; PS = palm (thick) skin.
Figure 5. Cultured primary keratinocytes from palm show greater desmosome protein expression than cells from breast skin. Western blotting and densitometry analysis for Dsc3 with U114, Dsg3 with AIHP319, Dp with 115F, Pkp1 with 5C2, Pg with 11E4, respectively. Internal control HSC-70 with sc-7298 is displayed to demonstrate equal loading. All five desmosomal proteins are expressed at a higher level in palm (PS) vs. breast skin (BS), particularly desmocollin 3 (Dsc3) and Dsg3 which show an approximately 30% increased level in palm.

Desmosome protein expression is greater in cultured keratinocytes from thick skin than thin skin

In order to determine whether there were any quantitative differences in DM expression between thin and thick skin, we further assessed expression of five major DM proteins on whole cell protein extracts generated from six individual keratinocyte cultures by Western blotting in conjunction with densitometry analysis. Equal amounts of protein samples were resolved by SDS-PAGE, blotted on nitrocellulose membranes, and probed for Dsc3, Dsg3, Dp, Pkp1 and Pg using specific antibodies (Table 1). Heat shock protein HSP-70 was probed in each blot to confirm equivalent loading of protein samples and used as a reference in later densitometry analysis. The blots shown in Fig. 5 are representative data from four individual samples, two from each skin type. The bar chart under each blot represents the mean ± SEM from three individuals. The results clearly indicated that the expression of all five DM proteins was constantly greater in thick skin cells than those in thin skin, particularly for Dsc3 and Dsg3, where the difference was approximately 30%.

Desmosomes are larger in size, rather than greater in density, in thick skin vs. thin skin

In order to elucidate whether the increased expression of DM proteins in thick skin resulted from differences in either DM size or number, or both, we undertook quantitative EM morphometry studies. To assess DM size, the midline length of a total of 612 DMs was measured (Table 3) in four preassigned zones in the epidermis for both thin and thick skin. We found that DMs in thick skin were generally larger (cross-sectional measurement) than in thin skin \((P < 0.05)\) (Fig. 6b) although they did not appear to vary in size throughout the suprabasal layers of either thick or thin skin \((P > 0.05)\) (Fig. 6c). Desmosome size in the basal layer of thick skin was equivalent to that in the suprabasal layer in thin skin \((P > 0.05)\) (Fig. 6c). Moreover, systemic point counting analysis showed that there was no statistically significant difference in relative volume density of DMs between thin and thick skin. However, the number of DMs was greater in all suprabasal layers in both types of skin \((P < 0.05)\) (Fig. 6d). No differences were found between the two individual samples of
either breast skin or palm skin on both DM midline length and volume density assessments. This result clearly suggested that the higher DM protein expression in the thick skin cells was because the DMs were larger, and not because their relative volume density was greater.

Discussion

In this study we set out to test the hypothesis that DMs are different in thick (palm) skin compared with thin (breast) skin. Our first goal was to investigate the gross expression of two desmosomal cadherins, Dsgs and Dscs, using two pan-antibodies (Table 1) directed against each desmosomal cadherin family. We found that Dsgs and Dscs exhibited a graded distribution across the epidermis (Fig. 1). However, the expression profile differed in detail between the two types of skin. In thin skin, Dsgs were more evenly expressed across the epidermis while Dsc increased suprabasally. By contrast, in thick skin, we found two peaks in the profile of Dsc expression, the first occurring in the middle spinous layer, and the second in the granular layer. In a broad zone in the upper epidermis, staining for both cadherin families was decreased, suggesting a site-specific desmosomal constitutive assembly and function in this zone. However, the implication of this altered distribution is still not clear. It is possible that the increase in Dsc expression in the suprabasal layers might reflect an additional requirement for cell-cell adhesion in the keratinocytes. Other studies have proposed that this might underscore a necessary adhesive gradient across the epidermis and a specific protein profile for DM signalling important in regulating epidermal differentiation within the epidermis (27). However, a recent in vivo study has shown that misexpression of differentiation-specific human Dsc1a isoform in basal keratinocytes of mouse skin, driven by a K14 promoter, failed to induce any abnormalities in the skin or hair follicles (28), suggesting that this differentiation-related Dsc1 isoform alone was not able to regulate keratinocyte differentiation, at least in the basal layer.

In order to explore the comparative expression of DM cadherin isoforms as well as other DM plaque proteins in both skin types, we examined expression of Dsc1 and 3, Dsg3, Dp, Pg and Pkp1 using a panel of specific antibodies against these proteins (Table 1). Interestingly, all these antibodies consistently showed reduced fluorescent staining in the upper epidermis in thick skin but not in thin skin (Figs 2–4). Not only was very weak staining observed, but also changes in the staining pattern occurred with certain proteins, such as Dp and Dsgs, in the upper epidermis. Desmoplakin was most abundantly expressed in a characteristic punctate pattern in both the periphery and putative cytoplasm of keratinocytes, and this expression pattern occurred consistently in all living layers across the epidermis in thin skin but was restricted to only the rete ridges in thick skin. There were twice as many layers with this staining pattern in thick skin than in thin skin (Fig. 4f). This might simply be a result of the effect of the actual skin thickness. When the cells moved to the middle spinous layer in the rete ridges and to the immediate suprabasal layer above the dermal papilla, in thick skin the staining for Dp declined and changed to a fine linear staining pattern at the cell boundary. This attenuated expression was further confirmed at the mRNA level by ISH analysis, and again a similar distinct expression profile was observed between thin and thick skin. Interestingly, our ISH analysis of Dp message showed a low expression level in basal cells in thin skin, but this was defined to a cluster of cells residing at the bottom of deep rete ridges in thick skin, where putative stem cells are located (17). This distinct expression profile suggests phenotypic variation between these two types of skin. Our observation of Dsc1 and Dsc3 expression pattern in thin skin resemble those previously reported in human fetal and adult scalp epidermis, using the same antibodies (29). Our study also found that Dsc1 was exclusively expressed in the suprabasal layers while Dsc3 was present in all living epidermal layers (Fig. 2). However, the expression profile of these two isoforms was different in thick skin and thin skin. The staining intensities for both Dsc1 and Dsc3 were markedly reduced or absent in a broad zone in the upper epidermis. These findings strongly support our view of contrasting cell biologic properties of keratinocytes in thin and thick skin.

An intriguing question is why the expression of DM proteins was reduced in the upper layers of thick skin? To answer this question, we investigated the expression of the terminal differentiation-associated protein, involucrin (26) (Fig. 4). We found evidence for a distinct differentiation process between the two types of skin. The onset of involucrin synthesis in thick skin occurred in the middle spinous layer where Dp staining was reduced, indicating that DMs changed their expression and organization once the cells withdrew from the cell cycle and underwent terminal differentiation. In contrast, in thin skin involucrin expression was restricted to the granular layer (Fig. 4). This difference was supported by our in vitro kinetic studies using a suspension-induced differentiation assay which showed that a higher percentage of palm cells stained for involucrin compared with breast cells (Table 2), suggesting earlier terminal differen-
process of epidermal terminal differentiation. We also found that the onset of involucrin synthesis in thick skin was followed by sequential expression of cornified cell envelope (CE) proteins including transglutaminase, filaggrin, and finally loricrin (data not shown), whereas in thin skin their expression was restricted to a narrow band close to the skin surface. It is possible that this distinct profile might be a consequence of the different relative thickness of the skin. However, it is more likely that the alterations reflect different requirements for structural properties as an adaptation to the varied degree of site-responsive mechanical stress. Nevertheless, this study demonstrates that the expression profiles of some DM proteins undergo marked changes during the process of epidermal terminal differentiation.

Our Western blotting analysis clearly showed greater DM protein expression in the cultured basal keratinocytes from thick skin vs. thin skin (Fig. 5). Although the differences appeared to be moderate (only up to 30%), the findings of greater relative expression in thick skin were constantly demonstrated. This suggests that DM expression might occur in a physical stress-dependent manner, an interpretation consistent with our observations on cultured sole keratinocytes that revealed higher expression levels of DM proteins compared with nonacral cultured keratinocytes (data not shown). In other words, this moderate but fundamentally significant increase of DM protein expression might be an advantageous or evolutionary response in trauma-prone skin sites such as the palm. Nevertheless, further studies on the expression of differentiation-related DM proteins such as Dsg1 and Dsc1 in epidermal organ culture (i.e. differentiated keratinocytes) might be more revealing with regard to quantitative, site-specific differential protein expression.

However, at the outset it was unclear whether this greater expression in thick skin resulted from a larger DM size or a greater DM number, or both. To address this issue, we applied stereological and morphometric methods to analyze DMs in skin from two body sites. We found that DMs were larger in thick skin vs. thin skin, and that those in all suprabasal layers were larger overall than DMs in the basal layer in both thin and thick skin. By contrast, we did not find any differences in our relative estimation of DM volume density between the two types of skin, although DM volume density in all suprabasal layers was twice that in the basal layer in either thin or thick skin. Correspondingly, this finding is consistent with earlier studies on the KIF network (21), which showed that the volume density of KIF was significantly higher in all suprabasal layers in thick skin vs. thin (arm) skin. Other similar studies have also shown an increase in DM number associated with epithelial differentiation (30). Our Western blotting results reflected changes only in the basal keratinocytes, and therefore the morphometric studies provide evidence that the greater expression of DM proteins in the palm keratinocytes is the result of the larger DM size rather than the greater DM number. In agreement with others (31), this study has provided important original clues that keratinocytes in thin and thick skin exhibit different intrinsic characteristics in their differentiation and in several other structural and functional properties.

It is still unclear whether or not the DM protein expression is down-regulated in the committed cells in the upper epidermis in thick skin. Our limited immunofluorescence and ISH analyses support the notion of down-regulation, but more detailed analysis is necessary. Our ISH study was confined only to Dp mRNA, and therefore the findings cannot necessarily be extrapolated to changes in DM protein expression as a whole. For example, an alternative explanation might be access limitation of the antibodies to antigenic proteins when the event of CE protein cross-linking takes place (32). This could account for our immunofluorescence microscopy observations of punctate staining for Dsc1 and Dsgs in the upper spinous and granular layers. To explore this in more detail, several attempts were made to try to unmask epitopes of the antigens by using either trypsin or proteinase K digestion of skin sections or paraformaldehyde fixation. However, none of these additional preincubation measures provided substantially different results (data not shown). It is known that additional differentiation-related DM proteins such as envoplakin and periplakin are expressed in the CE complexes (33–35). This view is supported by our EM observation, which indicated that DM size was consistent in all the suprabasal layers (Fig. 6). Further studies using biochemical analysis and immuno-EM for a broad range of DM proteins in the committed cells may be able to resolve this dilemma.

Overall, this study provides new findings of distinct site-specific properties for DMs in thick or thin skin with regard to various structural, histochemical and biochemical criteria. This variability in DM parameters reflects differing patterns of terminal differentiation programs in the epidermis at different body regions according to the functional needs at particular sites (e.g. response to trauma or weight-bearing). These discoveries may have significant implications for a better understanding of the pathogenic consequences of mutations in striate PPK and other inherited disorders of DMs.
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References


Desmosomes in palm skin


