Callus formation is associated with hyperproliferation and incomplete differentiation of keratinocytes, and increased expression of adhesion molecules

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Accepted for publication
25 April 2010

Key words
adhesion molecules, callus, desquamation, hyperproliferation, keratinocyte differentiation

Conflicts of interest
None declared.

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DOI 10.1111/j.1365-2133.2010.09842.x

Summary

Background A callus is a local thickening of skin, characterized by accelerated keratinization and a reduced rate of desquamation. However, the mechanism of callus formation is not fully understood.

Objectives To evaluate the expression patterns, in callused skin, of genes that are implicated in keratinization and adhesion/desquamation.

Methods Samples of skin from the dorsum of the foot (DF), centre of the plantar arch (CP) and anterior aspect of the heel (AH) were obtained from fresh cadavers, and protein and gene expression were determined by immunohistochemistry and reverse transcription–polymerase chain reaction, respectively.

Results The stratum corneum in the DF showed a splitting phenotype by conventional haematoxylin and eosin staining, while the stratum corneum was normal in the AH. Cells of the stratum corneum in the AH were nonsquamous. Expression of cornification-related molecules including involucrin, filaggrin, caspase 14 and calcium-sensing receptor was higher in the AH. Similarly, expression of adhesive proteins such as corneodesmosin, desmoglein 1 and desmocollin 1 was increased in the AH. However, protease-activated receptor 2 expression was reduced in the stratum granulosum in the AH. The number of proliferating cells in the stratum basale was significantly increased in the AH, compared with the DF and CP.

Conclusions Our data suggest that calluses form as a result of hyperproliferation and incomplete differentiation of epidermal keratinocytes, and increased expression of adhesion molecules.

A callus is a localized, firm thickening of the outer layer of skin as a result of repetitive friction or pressure. It is commonly observed in areas of skin such as the palms and soles that are subjected to continual rubbing. The physiological mechanisms underpinning the development of calluses are not fully understood. Only a few studies have examined the biomechanics and biochemical composition of the stratum corneum.[1,2] In response to repetitive friction or pressure, normal healthy skin undergoes accelerated keratinization. Combined with a lower rate of desquamation, this results in an increase in the thickness of the stratum corneum. This process, sometimes referred to as physiological hyperkeratosis, is considered to be a protective mechanism that prevents damage to the deeper tissues by dispersing the applied force over a large area and volume of skin.[3]

Histological studies have revealed several notable changes in callused skin, including an increase in the thicknesses of the stratum corneum and stratum granulosum, a decrease in keratinocyte density (which may indicate that the corneocytes obtained from calluses may be less differentiated than normal plantar corneocytes), and abnormal rete peg patterns.[3,4]

Hyperkeratotic lesions (calluses and corns) collectively represent one of the most prevalent foot problems in older people. Plantar pressure is significantly increased in callused regions of skin in older people. Raised pressure may play a role in the development of plantar calluses by accelerating the turnover rate of epidermal keratinocytes.[5]

Although calluses are one of the most prevalent foot problems in older people, the precise mechanisms underpinning

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callus formation remain unknown. Furthermore, callus gene and/or protein expression patterns have not yet been thoroughly studied. In this study, we examined the mechanisms behind callus formation by investigating the expression in callused skin of genes and proteins associated with keratinization and adhesion/desquamation.

Materials and methods

Skin specimens

Skin was dissected from the dorsum of the foot (DF), the centre of the plantar arch (CP) and the anterior aspect of the heel (AH) from five fresh cadavers (aged 59–76 years, mean 69·2), donated for medical research and education to the Department of Anatomy, School of Medicine, Chungnam National University, Korea. Half of each piece of tissue was fixed in 4% paraformaldehyde solution for haematoxylin and eosin (H&E) staining, Nile red staining and immunohistochemistry. Fixed tissue was embedded in paraffin prior to H&E and immunohistochemical analyses. For Nile red staining, tissue was embedded in optimal cutting temperature compound (Leica Microsystems, Bannockburn, IL, U.S.A.) and cryosectioned. The remaining half of each piece of tissue was frozen for subsequent analysis by reverse transcription–polymerase chain reaction (RT-PCR).

Immunohistochemistry and immunofluorescence

Paraffin-embedded tissue was sectioned at 4 μm. In immunohistochemical analyses, tissue sections were deparaffinized, and antigen retrieval was then performed by heating for 4 min in 10 mmol L^{-1} citrate buffer (pH 6·0) in a pressure cooker. Subsequent procedures were conducted at room temperature. Sections were pretreated with 1% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity. They were then sequentially treated with appropriate primary antibodies for 1 h, and biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, U.S.A.). Immunoreactivity was visualized by incubation for 1 h in an avidin-biotin-peroxidase complex (Vectastain ABC system; Vector Laboratories) in phosphate-buffered saline (PBS), and then for 5–10 min in 0·05% 3,3′-diaminobenzidine, 0·01% H_2O_2, in 0·1 mol L^{-1} PBS. The immunolabelled sections were then dehydrated though graded ethanol solutions, cleared in xylene, and mounted. As a negative control, sections were treated as described above, but with the omission of the primary antibody treatment.

In immunofluorescence analyses, paraffin sections were treated with 3% normal rabbit serum (NRS) in 0·1 mol L^{-1} PBS for 30 min, and were then incubated with appropriate primary antibody. Sections were next rinsed twice in 0·1 mol L^{-1} PBS containing 1% NRS and incubated for 1 h in the same buffer containing Cy2-labelled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.). Sections were finally rinsed in 0·1 mol L^{-1} PBS, mounted in fluorescence mounting medium (Dako, Carpinteria, CA, U.S.A.), and visualized using a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

The following primary antibodies were used: mouse monoclonal anti-involucrin, goat polyclonal antifilaggrin and goat antiprotease-activated receptor 2 (PAR2) (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.), sheep polyclonal anti-corneodesmosin (CDSN) (R&D Systems, Minneapolis, MN, U.S.A.) and rabbit polyclonal anti-Ki67 (Vector Laboratories).

Table 1 List of the primers used in reverse transcription–polymerase chain reaction

Gene name	Primer sequence	
CaSR	Forward	5′-GCAACACACCCATTGTCAAG-3′
	Reverse	5′-GGCAAAGAAGAAGCAGATGG-3′
Caspase 14	Forward	5′-CGAAGAAGACCTGGATGCTC-3′
	Reverse	5′-TCATGTCGGTAGGCGATGTA-3′
KRT9	Forward	5′-AGGCTCTAGAGGAGGCCAAC-3′
	Reverse	5′-GCAGCTCAATCTCCAACTCC-3′
KRT14	Forward	5′-CAGTTCACCTCCTCCAGCTC-3′
	Reverse	5′-TCCTCAGGTCCTCAATGGTC-3′
Wnt5A	Forward	5′-GGACCACATGCAGTACATCG-3′
	Reverse	5′-CACTCTCGTAGGAGCCCTTG-3′
β-catenin	Forward	5′-CCCACTAATGTCCAGCGTTT-3′
	Reverse	5′-AATCCACTGGTGAACCAAGC-3′
CDSN	Forward	5′-TCCCCCAATCACCTCTGTAG-3′
	Reverse	5′-CAACCTTGGGGTAGTGGAGA-3′
DSG1	Forward	5′-AAGGTGTAGGTTCCCCTGCT-3′
	Reverse	5′-ATCTCGCAAGTCAGGCATCT-3′
DSC1	Forward	5′-GCAACAACTGCAGATGGCTA-3′
	Reverse	5′-TGTGAGCTCGCTGCTTTAGA-3′
E-cadherin	Forward	5′-TGGAGAGACACTGCCAACTG-3′
	Reverse	5′-AGGCTGTGCCTTCCTACAGA-3′
KLK5	Forward	5′-TGATCACAGCCTTGCTTCTG-3′
	Reverse	5′-TCCTCGCACCTTTTCTGACT-3′
KLK7	Forward	5′-ATGGCAAGATCCCTTCTCCT-3′
	Reverse	5′-GGCAGCCTGACTTTCTTCAC-3′
KLK14	Forward	5′-ACGCACCCCAACTACAACTC-3′
	Reverse	5′-CGTTTCCTCAATCCAGCTTC-3′
α2ML1	Forward	5′-CCACCACTGGAACATCACAG-3′
	Reverse	5′-CTGCCTTCTCCAGGTACTGC-3′
LEKTI	Forward	5′-TGGATCAGAATCAGGGAAGG-3′
	Reverse	5′-TCACTGCACTCATCCTTTGC-3′
LEKTI2	Forward	5′-GAATGTGCCAAACAGACGAA-3′
	Reverse	5′-TGGTGATGAGTAGGCAATGTG-3′
GAPDH	Forward	5′-GAAGGTGAAGGTCGGAGT-3′
	Reverse	5′-GAAGATGGTGATGGGATTTC-3′

CaSR, calcium-sensing receptor; KRT9, keratin 9; KRT14, keratin 14; CDSN, corneodesmosin; DSG1, desmoglein 1; DSC1, desmocollin 1; KLK5, kallikrein-related peptidase 5; KLK7, kallikrein-related peptidase 7; KLK14, kallikrein-related peptidase 14; α2ML1, α2-macroglobulin-like 1; LEKTI, lymphoepithelial Kazal-type 5 serine protease inhibitor; LEKTI2, lymphoepithelial Kazal-type 5 serine protease inhibitor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Nile red staining

Nile red solution (4 mmol L^{-1} in 75% glycerol) was applied to cryosections (10 μm thick). Stained sections were examined using a fluorescence microscope. The excitation and emission wavelengths were 488 and 514 nm, respectively.[6]

Reverse transcription–polymerase chain reaction

Skin samples were incubated in preheated PBS (60 °C) for 45 s, and then cooled in ice-cold PBS for 1 min. The epidermis and dermis were mechanically separated using forceps.[7] The epidermis was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.), and total RNA was isolated according to the manufacturer's instructions. Approximately 10 μg of total epidermal RNA was used to generate cDNA in a 40-μL reaction using 200 U of SuperScript II (Invitrogen) reverse transcriptase and oligo (dT) primers. Subsequently, 2 μL of cDNA was subjected to PCR amplification to measure the expression of genes associated with epidermal keratinization and adhesion/desquamation. The forward and reverse primers used to amplify each target gene are listed in Table 1. To normalize for differences in cDNA loading, primers designed to amplify glyceraldehyde-3-phosphate dehydrogenase were used.

Results

Morphological characteristics of callused skin

To investigate the morphological features of callused skin, we compared the skin tissues obtained from three different sites: the DF, the CP and the AH. Histological analysis revealed that the epidermis was thicker in the AH than in the DF and CP. Notably, the stratum corneum was 3–4 times thicker in the AH than in the CP (Fig. 1a–c). Interestingly, most of the stratum corneum in the DF and the superficial layer of the stratum corneum in the CP displayed a splitting phenotype in conventional H&E staining. The stratum corneum in the AH showed no splitting phenotype. These results suggest that the texture of the stratum corneum differs between normal and callused areas of skin.

In Nile red-stained cryosections, the cell margins of the stratum corneum could not be identified in the DF, suggesting that the layers of the stratum corneum were very thin. In contrast, the lipid envelopes and intracellular spaces were distinguishable in the CP and AH. Furthermore, cells of the stratum corneum in the AH were significantly thicker than those in the CP, with characteristic features of less flattened, nonsquamous-type cells (Fig. 1d–f).

Altered expression of molecules associated with cornification in callused skin

The stratum corneum consists of multiple layers of dead, terminally differentiated keratinocytes, which provide a physiological barrier against environmental insults. Because morphological analyses revealed that the stratum corneum was markedly thicker in callused regions of skin, we examined the expression of cornification-related genes.

Involucrin and filaggrin are the major components of cornified cell envelope, the expression of which is increased in the upper layers of the epidermis. Involucrin immunoreactivity was detected in the suprabasal layers of the epidermis in the DF. Similarly, involucrin immunoreactivity was detected in both the stratum granulosum and stratum spinosum in the CP and AH. However, the thickness of the involucrin-positive layers was markedly increased in the AH, compared with the CP (Fig. 2a).

Filaggrin immunoreactivity was detected in the stratum granulosum in the DF, CP and AH. Of the three sites tested, the filaggrin-positive layer of the epidermis was thickest in the AH (Fig. 2a).

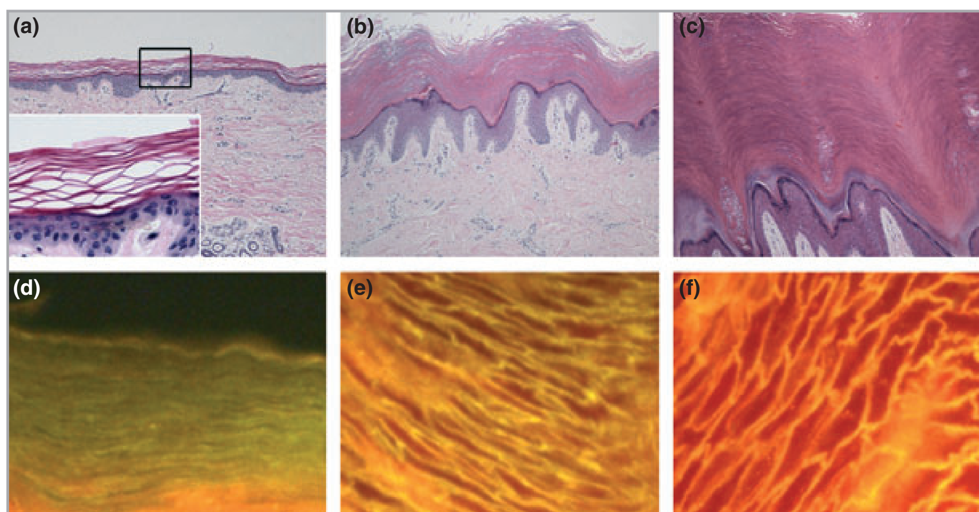

Fig 1. Morphological characteristics of callused skin. (a–c) Haematoxylin and eosin stain (original magnification × 100) [panel in lower left area of (a), enlargement of highlighted region in upper area of (a)]. (d–f) Nile red stain (original magnification × 400). (a, d) Dorsum of the foot; (b, e) centre of the plantar arch; (c, f) anterior aspect of the heel.

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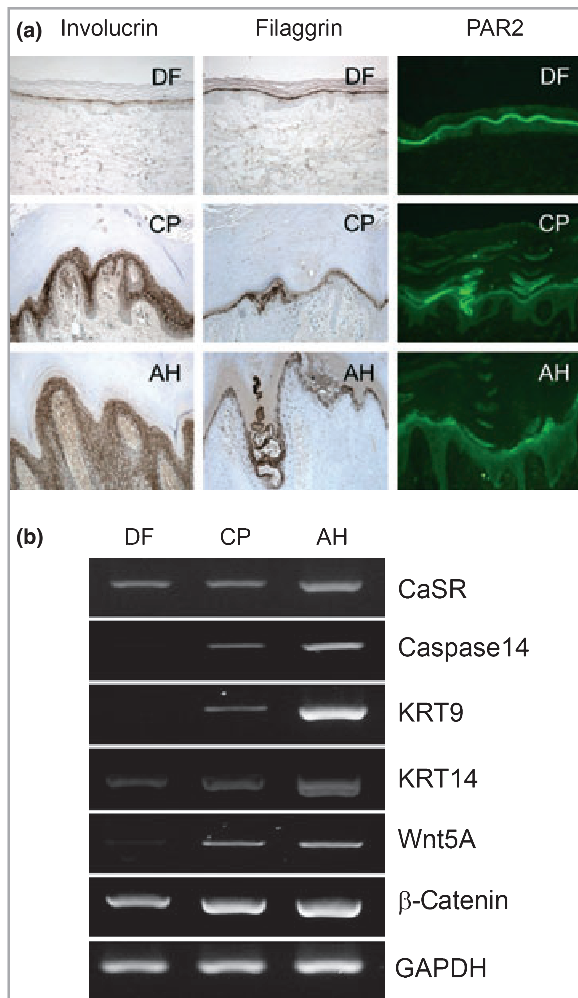

Fig 2. Altered expression of molecules associated with cornification in callused skin. DF, dorsum of the foot; CP, centre of the plantar arch; AH, anterior aspect of the heel. (a) Involucrin, filaggrin and protease-activated receptor 2 (PAR2) immunoreactivity in the epidermis of the DF, CP and AH (original magnification × 200). (b) Expression of calcium-sensing receptor (CaSR), caspase 14, keratin (KRT) 9, KRT14, Wnt5A and β-catenin mRNA in the epidermis of the DF, CP and AH. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PAR2 is involved in epidermal homeostasis and barrier function.[6,8] In the DF, strong PAR2 immunoreactivity was detected in the stratum granulosum. Similarly, PAR2 immunoreactivity was detected in the stratum granulosum and acrosyringia in the CP. In contrast, PAR2 immunoreactivity was reduced in the stratum granulosum in the AH (Fig. 2a).

Calcium-sensing receptor (CaSR),[9,10] Wnt5A, β-catenin[11,12] and caspase 14[8,13] are known to be involved in epidermal differentiation. Keratin 14 is expressed in the stratum basale of the epidermis in all anatomical sites. Keratin 9 is a suprabasal keratin of palmoplantar skin.[11] Expression of caspase 14, keratin 9 and Wnt5A was increased in the CP, relative to the DF. Expression of CaSR, caspase 14, keratin 9 and keratin 14 was markedly higher in the AH than in the CP (Fig. 2b).

Altered expression of molecules associated with adhesion/desquamation in callused skin

Based on the morphological differences in the stratum corneum detected through histological analysis (Fig. 1), we considered that its adhesive power would be stronger in the AH than in the DF. To test this, we analysed the expression of genes associated with adhesion and desquamation of the stratum corneum.

First, we detected the expression of CDSN immunohistochemically. CDSN, together with desmoglein 1 (DSG1) and desmocollin 1 (DSC1), participates in the formation of corneodesmosomes, the junctional structures that mediate corneocyte adhesion.[14,15] As shown in Figure 3a, CDSN was found to be expressed in the stratum granulosum in the DF, CP and AH. However, the CDSN-positive layers were markedly thicker in the AH than in the DF and CP (Fig. 3a).

Next, we further examined the expression of adhesion/desquamation-related genes by RT-PCR. It has previously been established that E-cadherin is involved in corneocyte adhesion.[9] Serine proteases, such as kallikrein-related peptidase (KLK) 5 and KLK7, can cleave several components of the corneodesmosome. KLK5 autoactivates and/or is activated by KLK14. Lymphoepithelial Kazal-type 5 serine protease inhibitor (LEKTI) fragment and α2-macroglobulin-like 1 (α2ML1) inhibit the activity of KLK species.[16] The expression of mRNA encoding the adhesive molecules CDSN, DSG1 and DSC1 was increased in the CP, compared with the DF. Expression of

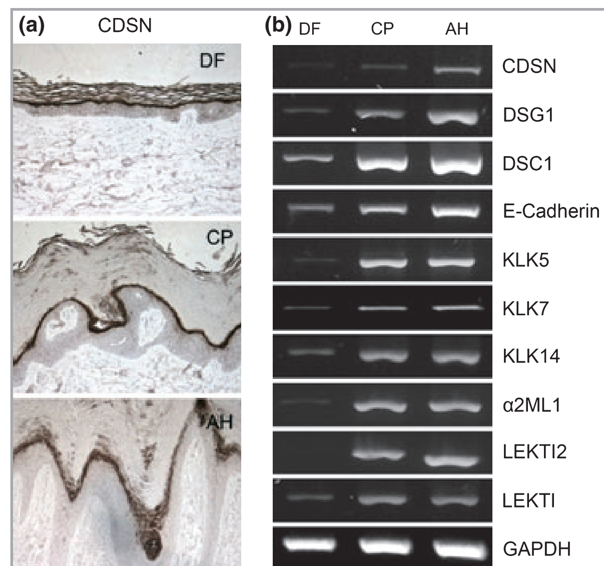

Fig 3. Altered expression of molecules associated with adhesion/desquamation in callused skin. DF, dorsum of the foot; CP, centre of the plantar arch; AH, anterior aspect of the heel. (a) Corneodesmosin (CDSN) immunoreactivity in the epidermis of the DF, CP and AH (original magnification × 200). (b) Expression of CDSN, desmoglein 1 (DSG1), desmocollin 1 (DSC1), E-cadherin, kallikrein-related peptidase (KLK) 5, KLK7, KLK14, α2-macroglobulin-like 1 (α2ML1), lymphoepithelial Kazal-type 5 serine protease inhibitor (LEKTI) and LEKTI2 mRNA in the epidermis of the DF, CP and AH. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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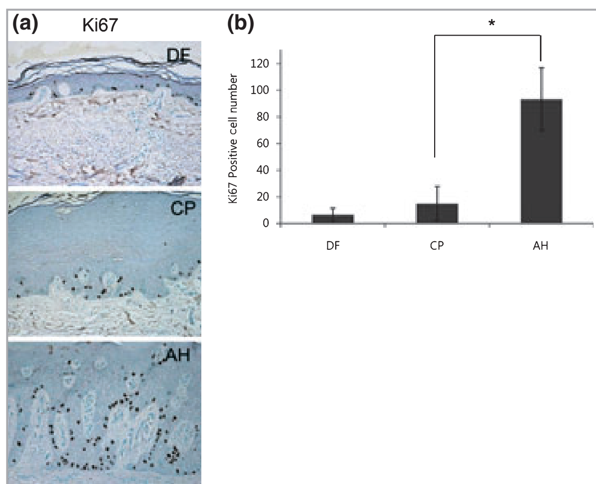

Fig 4. Ki67 immunoreactivity in callused skin. DF, dorsum of the foot; CP, centre of the plantar arch; AH, anterior aspect of the heel. (a) Ki67 immunoreactivity in the epidermis of the DF, CP and AH (original magnification × 200). (b) Numbers of Ki67-positive cells (mean ± SD, $n = 5$) in the epidermis of the DF, CP and AH (counted at × 200 magnification in specific areas). *Statistically significant difference ($P < 0{\cdot}05$).

these same adhesive molecules was also higher in the AH than in the CP. Expression of the serine proteases KLK5, KLK7 and KLK14 was higher in the CP than in the DF, and comparable between the CP and AH. Expression of the KLK inhibitors LEKTI, LEKTI2 and α2ML1 was upregulated in the CP, relative to the DF. As with the serine proteases we examined, expression of these KLK inhibitors was similar in the CP and AH (Fig. 3b).

Changes in keratinocyte proliferation in callused skin

The epidermal thickness was increased significantly in the AH, compared with the DF and CP. This finding suggested that the epidermal keratinocytes in the AH might be hyperproliferative. We thus examined the proliferative status of the keratinocytes through immunohistochemical staining for Ki67. As shown in Figure 4a, the number of Ki67-positive cells was greatly increased in the AH, compared with the DF and CP. Quantification confirmed that cell proliferation was significantly increased in the AH, compared with the DF and CP, implicating the high proliferative rate of epidermal keratinocytes in callus formation (Fig. 4b).

Discussion

Although it has been recognized that the histological texture of the epidermis is markedly different in normal and callused skin, the relative expression patterns of genes involved in cornification, adhesion and desquamation are unknown. In this study, we demonstrated, using human foot skin tissue, altered expression of molecules related to the general features of callused skin.

Plantar tissue is not homogeneous in the context of callused skin. For example, there is a clear difference in epidermal thickness between the CP and AH. It is likely that this difference is linked to the alteration of foot function.[17] The CP is not usually subjected to pressure in the standing position, whereas the AH receives heavy pressure. The morphological features of the CP are similar to those of the palm (data not shown). In our study, the stratum corneum of the DF, but not the AH, was shown through conventional H&E staining, to be split. Considering the processes that this core histological technique involves, including the use of solvents such as ethanol and xylene, the differences in stratum corneum splitting pattern and thickness between the AH and CP indicate that callus formation is dependent on intrinsic adhesiveness within the stratum corneum.

In this study, we evaluated, although the level of transcripts and proteins are not always proportional, the expression of the adhesive proteins CDSN, DSG1 and DSC1, the serine proteases KLK5, KLK7 and KLK14, and the KLK inhibitors LEKTI, LEKTI2 and α2ML1 in callused skin using RT-PCR. These molecules were found to be more highly expressed in the CP and AH than in the DF. Interestingly, some of the adhesion molecules (but not the serine proteases or KLK inhibitors) were increased only in the AH, reflecting the fact that, among the three sites tested, adhesiveness within the stratum corneum was highest in the AH.

A previous study[3] showed that corneocyte surface area was essentially identical in normal and callused skin. However, the mean volume of corneocytes from callused skin was increased, indicating that corneocytes from callused skin were thicker (or rounder) than those of normal plantar skin. Our data also showed that the epidermal cells in the AH were less flattened than those of the DF and CP. Cornification is the process by which stratified squamous epithelia are formed, or the formation of the so-called 'horny layer' of the skin. Thus, incomplete cornification or differentiation may occur in the stratum corneum in the callused skin of the AH.

PAR2 and caspase 14 are involved in corneocyte formation.[8] Keratin 14 is expressed in the basal layer of the epidermis in human skin from all anatomical sites. Keratin 9 is a suprabasal keratin of palmoplantar skin.[11] While strong PAR2 immunoreactivity was detected in the stratum granulosum in the DF, it appeared that expression of PAR2 was slightly decreased in the AH. This altered pattern of PAR2 expression may be linked to incomplete cornification or differentiation in callused skin.

Wnt5A is known to be an upstream regulator of keratin 9 expression.[11] Expression of Wnt5A and keratin 9 was upregulated in the CP, compared with the DF. Moreover, while keratin 9 expression was higher in the AH than in the CP, Wnt5A expression was similar in these two sites. These data suggest that other proteins, in addition to Wnt5A, may regulate keratin 9 expression in callused skin.

Extracellular Ca^{2+} is a key promoter of epidermal keratinocyte differentiation. CaSR is essential for mediating Ca^{2+}

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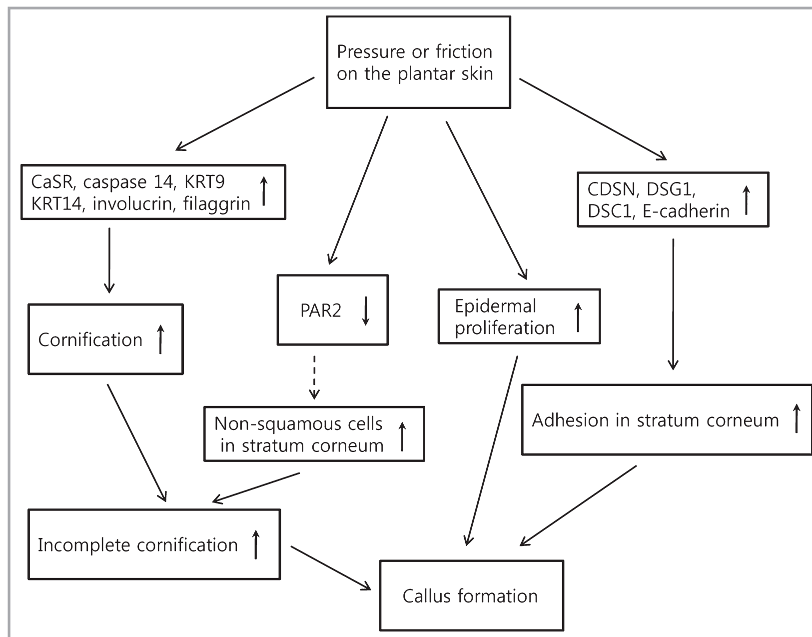

Fig 5. Diagram for mechanism of callus formation. Calluses form as a result of hyperproliferation and incomplete differentiation of epidermal keratinocytes, and increased expression of adhesion molecules in the epidermis. CaSR, calcium-sensing receptor; KRT, keratin; CDSN, corneodesmosin; DSG1, desmoglein 1; DSC1, desmocollin 1; PAR2, protease-activated receptor 2.

signalling during extracellular Ca^{2+}-induced differentiation.[9] Although expression of the CaSR-encoding *CASR* gene was similar in the DF and CP, levels of CaSR mRNA were higher in the AH than in the CP. These results suggest that increased calcium signalling, mediated by CaSR, may be involved in callus formation.

Mackenzie[18] proposed that the cells of the stratum corneum were less differentiated after mechanical stimulation due to an increase in the rate of keratinocyte proliferation. The increases in the suprabasal nucleated cell population and number of cell layers in the stratum corneum suggest that keratinocyte turnover time may be shortened. Thymidine autoradiographic labelling indices were also found to be increased in calluses, suggesting that the epidermis has a higher rate of cell division than normal skin and that the cells generated do not remain within the epidermis for a sufficient length of time to mature and differentiate fully.[3] Consistent with this, in our study, the number of Ki67-positive cells in the stratum basale was significantly increased in callused plantar skin compared with normal plantar skin. Furthermore, cells of the stratum corneum were thicker and not flattened, and PAR2 expression in the stratum granulosum reduced, in the callused skin, indicating incomplete maturation and differentiation of epidermal keratinocytes in callus formation in plantar skin. Taken together, our data suggest that calluses form as a result of hyperproliferation and incomplete differentiation of epidermal keratinocytes, and increased expression of adhesion molecules (Fig. 5).

What's already known about this topic?

- General features of callused skin are accelerated keratinization and a lower rate of desquamation.

What does this study add?

- This study provides novel information regarding the differential patterns of expression of molecules associated with keratinocyte differentiation and desquamation in callus tissues.
- Our findings suggest that callus formation is associated with the hyperproliferation and incomplete differentiation of keratinocytes, and increased expression of adhesion molecules.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0086846).

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