

Regulation of Keratin 9 in Nonpalmoplantar Keratinocytes by Palmoplantar Fibroblasts Through Epithelial–Mesenchymal Interactions

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Palms and soles differ from other body sites in terms of clinical and histologic appearance, response to mechanical stress, and the distribution of keratin 9. Because keratin 9 is exclusively expressed in the palmoplantar suprabasal keratinocyte layers, it is considered a differentiation marker of palms and soles. We studied palmoplantar mesenchymal influences on keratin 9 induction in nonpalmoplantar epidermis. Although palmoplantar keratinocytes when cultured alone continued to express keratin 9 mRNA in 12 (100%) of 12 cultures, nonpalmoplantar keratinocytes did not express it in 16 of 17 cultures. Although nonpalmoplantar keratinocytes did not express keratin 9 mRNA when cultured with nonpalmoplantar fibroblasts, they did express it within 2 h in cocultures with palmoplantar

tar fibroblasts derived from papillary dermis. Grafting of these coculture sheets on severe combined immunodeficient mice resulted in an epidermis, which histologically showed hyperkeratosis and acanthosis and immunohistochemically expressed keratin 9. Furthermore, pure epidermal sheets from nonpalmoplantar skin grafted on the human sole wounds due to burn, injury, and the resection of acral lentiginous melanoma, demonstrated adoption of palmoplantar phenotype and expressed keratin 9. Our report indicates extrinsic keratin 9 regulation by signals from dermal fibroblasts. This is also the first to suggest the possibility of treating palmoplantar wounds with nonpalmoplantar epidermis, which is much easier to obtain and harvest. Key words: heterogeneity/induction/palms and soles/SCID mouse. *J Invest Dermatol* 112:483–488, 1999

Palmar and plantar epidermis histologically has an extremely thick, compact stratum corneum and a well-defined stratum lucidum, as compared with the thin and hairy epidermis of other body sites. Rete ridges in the palm are deep or shallow, whereas those of other body sites are relatively uniform in size. Palmoplantar stem cells are located at the tips of the deep rete ridges, whereas nonpalmoplantar stem cells are above the papillary dermis (Lavker and Sun, 1982, 1983; Jones *et al*, 1995). Palmoplantar epidermis expresses keratin 9 (K9), which is a large and highly differentiated acidic type 1 keratin (63K) in the suprabasal layers of epidermis (Knapp *et al*, 1986; Moll *et al*, 1987; Schweizer *et al*, 1989; Langbein *et al*, 1993). Although K9 expression in palmoplantar keratinocytes is considered to be endogenously rather than exogenously regulated (Limat *et al*, 1996), keratinocyte differentiation is also regulated by extrinsic factors, such as Ca^{2+} concentrations (Hennings *et al*, 1980), vitamin A (Fuchs and Green, 1981), mechanical stress (Gormar *et al*, 1990), and mesenchymal factors (Bohnert *et al*, 1986). Using heterotypic recombinant experiments, Billingham and Silvers (1966) showed that the dermis determines the phenotypic expression of the transplanted epidermis and that sole dermis induces the sole type epidermis in ear and trunk epidermis. In this study, we report the

induction of highly differentiated keratin synthesis by heterotypic fibroblasts; both *in vitro* and *in vivo*. Because there is increasing evidence that dermal fibroblasts are heterogeneous (Harper and Grove, 1979; Schor and Schor, 1987; Goldring *et al*, 1990; Falanga *et al*, 1995), we also examined whether fibroblasts from papillary or reticular dermis could induce the same level of K9 expression in nonpalmoplantar keratinocytes. Considering the heterotypic mesenchymal–epithelial interaction, we also successfully treated with nonpalmoplantar epidermis plantar wounds due to burn, injury, and the resection of acral lentiginous melanoma, which is the most common melanoma in oriental races (Seiji *et al*, 1983).

MATERIALS AND METHODS

Keratinocyte culture Skin specimens were obtained from patients during plastic surgery. Human keratinocytes were cultured by the modified method of explant culture (Halprin *et al*, 1979; Itami *et al*, 1995). The dermis were carefully scraped to produce a thin piece of skin under a stereoscopic microscope. The skin was cut into 0.2–0.3 mm squares and single pieces were placed in 3.5 cm collagen type 1 coated dishes (Iwaki Glass, Tokyo, Japan). They were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) with 10% fetal bovine serum (FBS; JRH Biosciences, KS). After epidermal outgrowth was observed, cultures were supplemented with keratinocyte growth medium (KGM) (Boyce and Ham, 1983). Keratinocytes from third passage were principally used in these experiments.

Coculture system Four different systems were used for the cocultures. In the first system, fibroblasts and keratinocytes were simply mixed 50:50 at a density of 3×10^4 cells per cm^2 in KGM. Biocoat cell culture inserts (rat tail collagen type I, 6 well, 0.45 μm , Becton Dickinson, Bed Ford, MA) were used for the other three coculture systems. In the second system,

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Abbreviations: K9, keratin 9; KGM, keratinocyte growth medium.

keratinocytes were seeded onto the inserts in KGM. Fibroblasts were seeded onto the outer culture dish in 10% FBS/DMEM separately, and then the two were placed together after confluence in KGM. In the third system, the inserts were turned upside down and fibroblasts were seeded onto the bottom of the membranes. Two days later, the inserts were turned upright and keratinocytes were seeded onto the top of the membranes at a density of 5×10^4 cells per cm^2 in KGM. Finally, in a fourth system, fibroblasts were embedded in 2 ml of collagen lattices (Cellmatrix type I, Nitta Gelatin, Osaka, Japan) at a density of 1.5×10^6 cells per ml into the outer culture dish, and then washed with KGM three times. The inserts filled with keratinocytes in KGM were placed on the collagen gel. In certain experiments, papillary and reticular dermal fibroblasts were cultured individually under a stereoscopic microscope after dispase treatment (Harper and Grove, 1979).

Reverse transcriptase-polymerase chain reaction (PCR) Reverse transcriptase-PCR analyses were performed as previously described (Itami *et al*, 1995). The oligonucleotide primers for PCR were based on published mRNA sequences, which include the 1A rod domain of K9 (Langbein *et al*, 1993), and were as follows: cytokeratin 9 (accession number: z29074), 5'-ATA GTA GTT CTG GGG GTT TT-3' and 5'-GTG TCA TGC GAG TGT TGT CA-3' or 5'-TGG GGG TGG TTT TAG TG-3' and 5'-ACT GTC AGG TCC ACA ATC-3'; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control (Tso *et al*, 1985), 5'-CCC ATC ACC ATC TTC CAG-3' and 5'-CCT GCT TCA CCA CCT TCT-3'. PCR amplification times were 35 cycles (1 min at 94°C, 1 min at 62°C, 1 min at 72°C) for K9, and 20 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) for GAPDH. The sizes of products were compared with 100 bp molecular weight markers (BRA Life Technology, Gaithersburg, MD). We confirmed the identity of the cDNA products by the digestion with XbaI, i.e., the specific restriction enzyme.

DNA sequencing The cDNA products were purified by electrophoresis on 2% agarose gels, extracted with Quiaex (Qiagen, Hilden, Germany), and cloned in Bluescript vector (Stratagene, La Jolla, CA). The cloned plasmid DNA were isolated and purified using the Quiagen Matrix Columns (Qiagen). The recombinant plasmids were digested and analyzed for sequencing by 373 DNA sequencer STRETCH (PE Applied Biosystems, Norwalk, CT).

Immunohistochemical study Frozen sections (5 μm) were fixed with cold acetone and incubated with the primary antibodies at 4°C overnight. The primary antibodies used were Serum HK9-AM2, which was developed against K9 specific aminoterminal sequence and stained the foot sole epidermis suprabasally and cells around acrosyringia of eccrine sweat gland ducts and Serum HM-TY1, which was also made against human K9 specific rod end sequence (Langbein *et al*, 1993). These antibodies were very kind and generous gifts from Drs. W.W. Franke and L. Langbein. After washing with PBS containing 0.1% Tween 20, samples were incubated with fluorescein-5-isothiocyanate-conjugated goat affinity purified anti-guinea pig IgG antibody (Cappel, Westchester, PA) at room temperature for 60 min. The samples that were incubated with preimmune sera were used as negative control.

Transplantation of organotypic cultures Organotypic cocultures were grafted on the dorsal muscle fascia of SCID mice (Nomura *et al*, 1997). The transplants were excised 3 wk later and examined by anti-human HLA-Class I antigen antibody (DAKOPATTS, Glostrup, Denmark), hematoxylin and eosin, and anti-K9 antibodies as described above.

Transplantation of pure epidermal sheets All surgeries were done after written informed consent. Split-thickness skin was harvested from the anterior thigh of patients with plantar wounds due to burn, injury, and the resection of acral lentiginous melanoma. The skin was soaked into 500 U per ml of dispase (Godo Shusei, Tokyo, Japan) in DMEM at 37°C for 1 h to separate epidermis from dermis. The pure epidermal sheets were washed with DMEM three times and grafted on the plantar full-thickness skin defects.

RESULTS

K9 mRNA expression *in vitro* We first determined whether keratinocytes from soles and palms continue to express K9 mRNA *in vitro*, and whether keratinocytes from nonpalmoplantar sites do not. Keratinocytes were cultured to confluence with serum free medium without 3T3 feeder layers to avoid undefined mesenchymal effects in these studies (Rheinwald and Green, 1975; Boyce and

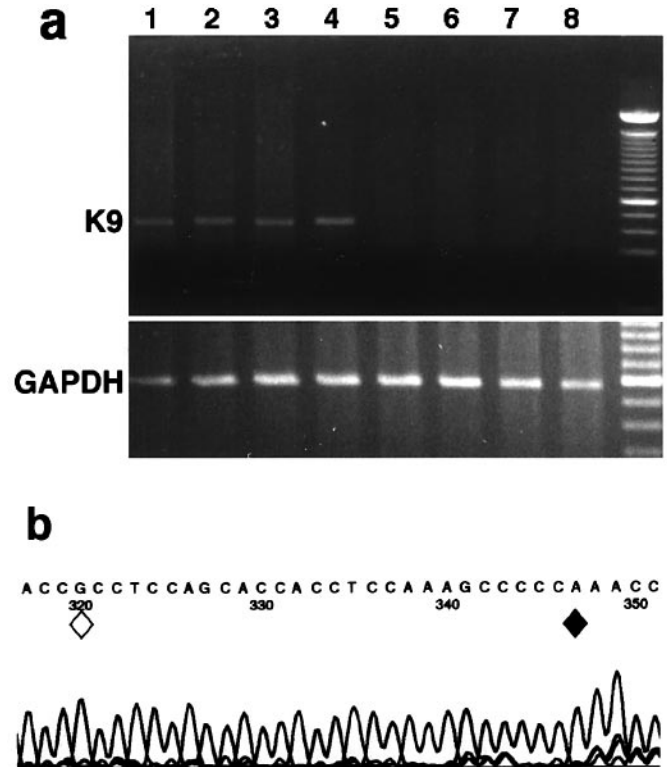


Figure 1. K9 mRNA expressions in palmoplantar and nonpalmoplantar keratinocytes from early to late passages. (a) Keratinocytes derived from palm, sole, trunk, and scalp in second passage (lanes 1, 3, 5, 7) and sixth passage (lanes 2, 4, 6, 8), respectively. Right margin indicates size markers (100 bp ladder). Expression of GAPDH mRNA was shown at the bottom. (b) DNA sequence analysis of PCR product. Two base pair differences were observed in the variant subdomain V1 of the head region between our sequence and the published sequence of K9. One was an A to T transition at position 468 (◆) and the other was an A to C transition at position 495 (◇).

Ham, 1983). The explant culture technique instead of a single cell suspension method was used to culture the keratinocytes, because the former method results in better palmoplantar keratinocyte growth. Explant cultures may have improved keratinocyte growth because stem cells from palmoplantar skin are known to be located at the tips of the rete ridges (Jones *et al*, 1995). Furthermore, trypsinization is not needed in primary explant cultures, thereby preventing damage to the stem cells. Both palmoplantar and nonpalmoplantar keratinocytes looked microscopically similar during cell culture (data not shown). The expressions of K9-specific mRNA in keratinocytes from various body sites were examined by reverse transcriptase-PCR amplification. The positions of primer nucleotides were based on the published sequence and contained the 1A rod domain of K9, whose sequence is quite different from the other acidic type I keratins (Langbein *et al*, 1993). Because of the homology among keratin genes, annealing temperature was set $\approx 7^\circ\text{C}$ higher than the calculated T_m for stringent amplification. The cDNA products from cultured palmoplantar keratinocytes showed K9-specific bands in all of the specimens from 12 subjects (ages of the patients ranged from 12 to 47) from early to late passages (Fig 1a, lanes 1–4). Cultured nonpalmoplantar keratinocytes did not express K9 in 16 of 17 specimens (Fig 1a, lanes 5–8), except for one sample from thigh skin (data not shown). This expression of K9 in one sample may be attributed to the PCR amplification of K9 found in the acrosyringia (Moll *et al*, 1987). Sequence analysis was performed to confirm the identity of the cDNA products (Fig 1b). No difference was observed in the 1A region between our sequence and the published sequence of K9 (Langbein *et al*, 1993), whereas 2 bp differences were observed in the variant subdomain V1 of the head region in all of our four samples. One

was an A to T transition at position 468, which did not result in an amino acid change. The other was an A to C transition at position 495, which resulted in a leucine (L) to phenylalanine (F) substitution in codon 133 of K9.

Induction of K9 mRNA expression We next examined the induction of K9 expression in nonpalmoplantar keratinocytes cocultured with palmoplantar fibroblasts by four coculture systems (Fig 2a-e). Obvious K9 mRNA expression occurred when keratinocytes from hair-bearing skin were directly (Fig 2a, e, lane 1) or closely (Fig 2c-e, lanes 3, 4) cocultured with palmoplantar fibroblasts, to mimic a juxtacrine or paracrine system. On the other

hand, only slight K9 expression was observed in the coculture insert system, which separates keratinocytes from fibroblasts (Fig 2b, e, lane 2). The same cell strain of nonpalmoplantar keratinocytes did not express K9 mRNA when cocultured with nonpalmoplantar fibroblasts (Fig 2d, e, lane 5). Moreover, palmoplantar fibroblasts did not express K9 mRNA when cultured alone or with either type of keratinocytes (data not shown). We next determined how long it takes to induce K9 mRNA in nonpalmoplantar keratinocytes by palmoplantar fibroblasts (Fig 2f). For this purpose, we used recombinant cocultures of collagen lattice with embedded fibroblasts and with the insert filled with confluent keratinocytes (Fig 2d). Induction of keratin 9 mRNA expression occurred at 30 min after the recombination, gradually increased in intensity from 2 h to 1 d, and continued for 3 d (Fig 2f).

We next examined the difference in the ability to induce K9 between papillary and reticular dermal fibroblasts from sole skin. Figure 3 shows representative results obtained from nonpalmoplantar keratinocytes cocultured with either type of fibroblasts for 3 d (Fig 2d). Papillary palmoplantar fibroblasts were able to induce K9 mRNA expression in all seven cases (Fig 3, lanes 1, 2), while reticular fibroblasts could moderately induce the K9 expression in five of eight cases (Fig 3, lanes 3, 4). Nonpalmoplantar papillary fibroblasts could not induce K9 mRNA expression in nonpalmoplantar keratinocytes in all four cases (Fig 3, lanes 5, 6). Papillary

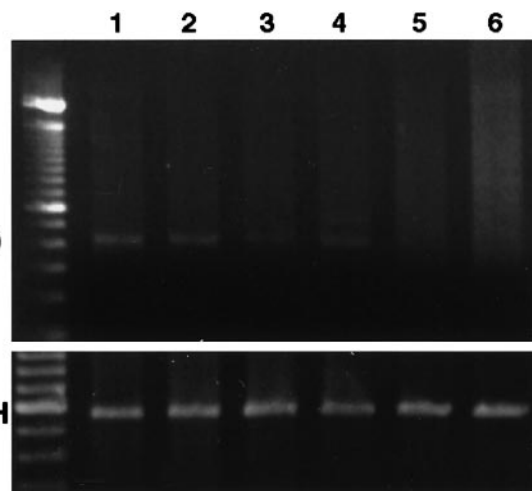
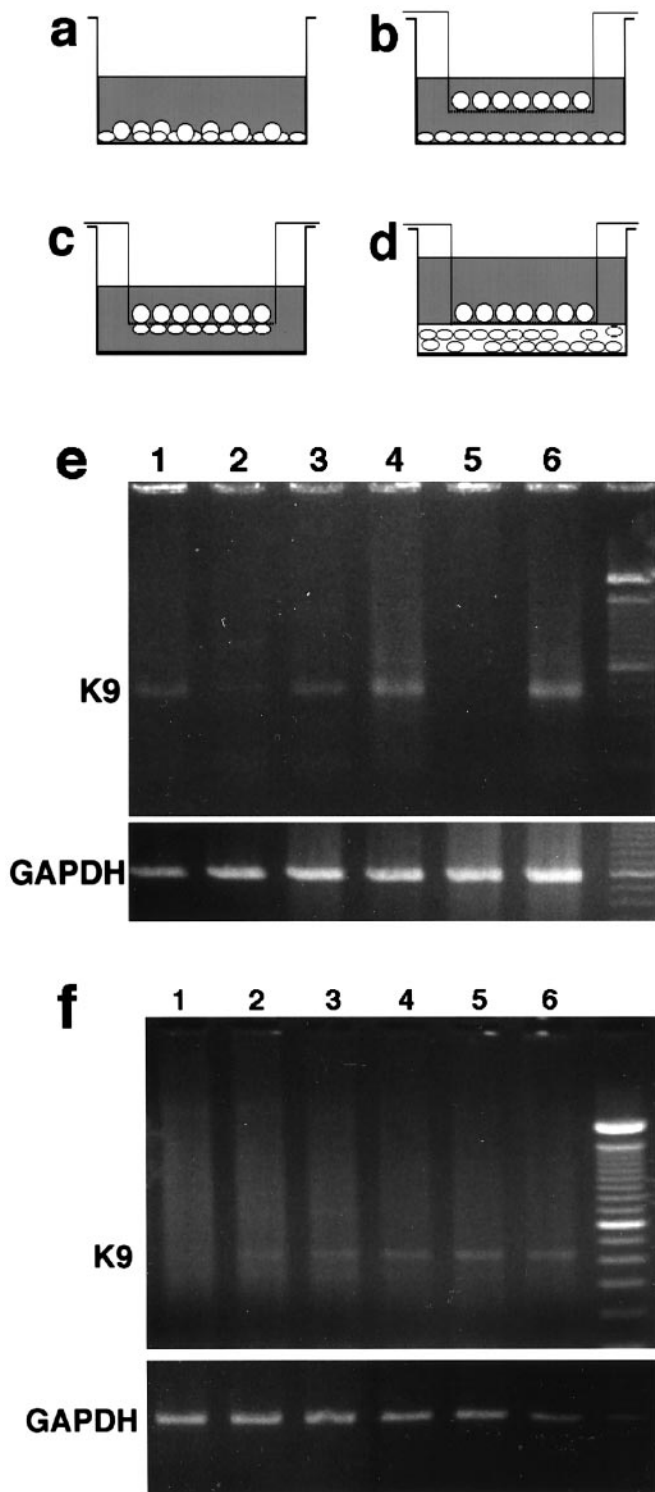


Figure 3. Comparison of papillary and reticular dermal fibroblasts from palmoplantar skin in terms of K9 induction in nonpalmoplantar keratinocytes. The same trunk keratinocytes were cocultured with papillary palmar fibroblasts (lane 1), papillary plantar fibroblasts (lane 2), reticular palmar fibroblasts (lane 3), reticular plantar fibroblasts (lane 4), papillary scalp fibroblasts (lane 5), and papillary trunk fibroblasts (lane 6) as shown in Fig 2(d) for 3 d. Margins indicate size markers. Expression of GAPDH mRNA was shown at the bottom.

Figure 2. Induction of K9 mRNA expressions in nonpalmoplantar keratinocytes. Schematic illustrations of four coculture systems (a-d). (a) Keratinocytes (large circle) were mixed with fibroblasts (small ellipse) in KGM. (b) Keratinocytes were cultured on the inserts in KGM and fibroblasts were seeded on the outer culture plates separately. (c) Fibroblasts were cultured on the bottom of the membrane and keratinocytes were cultured on the top of the membrane in KGM. (d) Keratinocytes were seeded on the inserts in KGM and fibroblasts were embedded in collagen lattices in the outer plates. (e) Trunk keratinocytes were cocultured with plantar fibroblasts as shown in (a)-(d) (lanes 1-4, respectively). The same trunk keratinocytes cocultured with nonpalmoplantar fibroblasts as shown in part (d) (lane 5) and plantar keratinocytes (lane 6) were also examined. (f) Time course of the K9 mRNA induction in nonpalmoplantar keratinocytes. Trunk keratinocytes were cocultured with plantar fibroblasts, as shown in (d) for 0 min (lane 1), 30 min (lane 2), 2 h (lane 3), 6 h (lane 4), 1 d (lane 5), and 3 d (lane 6). Expression of GAPDH mRNA was shown at the bottom.

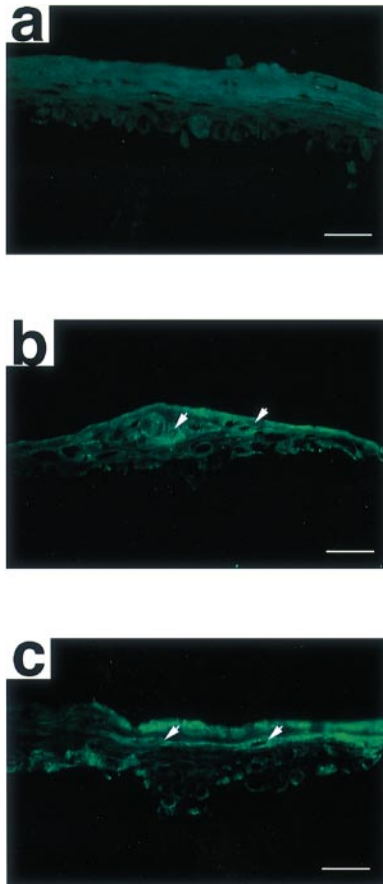


Figure 4. Immunohistochemistry of organotypic cocultures with anti-K9 antibody. The cultures were lifted by the air/liquid interface for 1 wk. No K9-positive cells were found in trunk keratinocytes cocultured with trunk fibroblasts (a). Several K9-positive cells (arrows) were found both in trunk keratinocytes cocultured with palmar fibroblasts (b) and in plantar keratinocytes cocultured with palmar fibroblasts (c). Scale bars: 50 μ m.

palmoplantar fibroblasts had a higher ability to induce K9 in nonpalmoplantar keratinocytes.

Induction of K9 protein K9 protein, a highly differentiated keratin, was not observed in palmoplantar keratinocytes in submerged cultures, as previously reported (Limat *et al*, 1996). To solve this problem, the keratinocyte cultures were lifted to the air/liquid interface with collagen gels containing fibroblasts (Limat *et al*, 1996) using medium consisting of 50% KGM, 2.5% FBS, 7.5% delipidized FBS (Fuchs and Green, 1981), and 40% DMEM with 10^{-7} M vitamin D₃. The organotypic cocultures were studied within 1 wk of preparations. We found that K9 mRNA expression occurred adequately in nonpalmoplantar keratinocytes within 3 d of coculture (Fig 2f), and that long-term cultures resulted in slight degeneration of the suprabasal layers. Immunohistochemical studies with the specific antibodies revealed K9 induction in nonpalmoplantar keratinocytes cocultured with palmoplantar fibroblasts as well as palmoplantar keratinocytes cocultured with palmoplantar fibroblasts (Fig 4). Because long-lasting differentiation could be achieved only by grafting the keratinocytes onto nude mice (Limat *et al*, 1996), we next transplanted 1 wk old organotypic cocultures onto the muscle fascia of the back of SCID mice (Nomura *et al*, 1997) to confirm the K9 induction (Fig 5). Organotypic cocultures of nonpalmoplantar keratinocytes and palmoplantar fibroblasts took well and were excised 3 wk later. The samples, which were shown to be of human origin by testing with anti-human HLA-class 1 antibody (Fig 5a), histologically showed hyperkeratosis and acanthosis-like palmoplantar-type skin (Fig 5b). Furthermore, they showed several K9-positive cells in suprabasal layers of the grafted

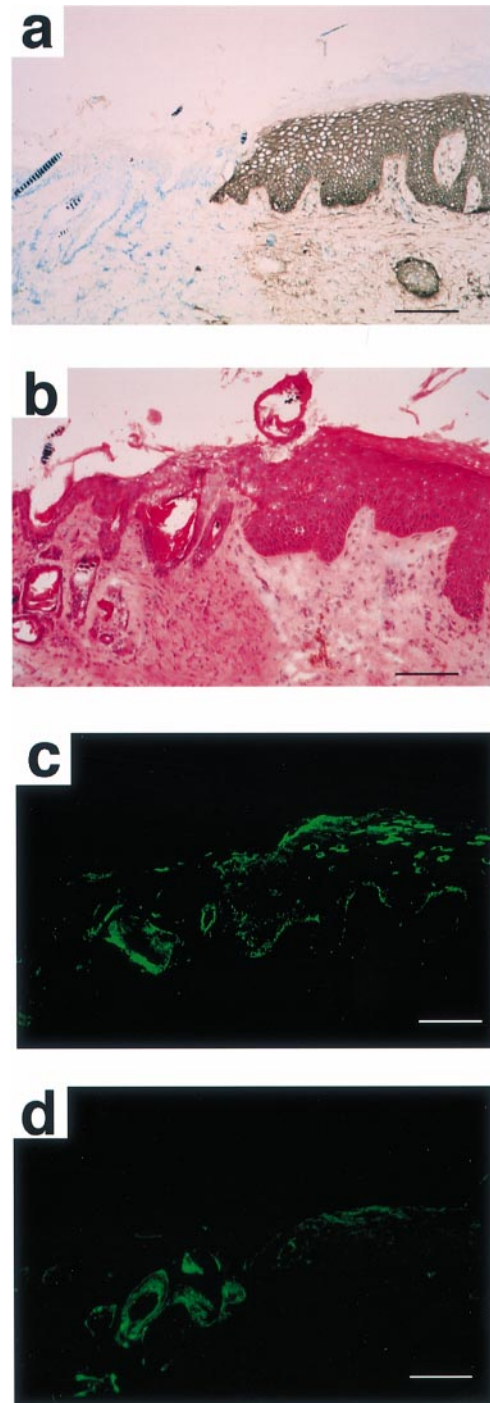


Figure 5. Transplantation of organotypic cocultures on SCID mice. Trunk keratinocytes were organotypically cultured with plantar fibroblasts for 1 wk and grafted onto the dorsal muscle fascia of SCID mice. The transplants were excised at 3 wk post-grafting and examined by anti-human HLA-class 1 antigen antibody (a), hematoxylin and eosin (b), and anti-K9 antibody (c). The left half of each figure shows mouse skin and the right half shows the grafted human culture. Hair follicles of mouse show nonspecific stains both in part (c) and in the negative control that was incubated with preimmune serum (d). Scale bars: 100 μ m.

epidermis (Fig 5c), which never appeared in the cocultures of nonpalmoplantar keratinocytes and fibroblasts (data not shown). The distribution of K9-positive cells in our heterogeneous grafts was comparable with the results reported by Limat *et al* (1996) in their composite grafts of palmoplantar keratinocytes and fibroblasts on nude mice. In this study and in previous work by Limat *et al* (1996), the grafts were harvested shortly after the transplantation;

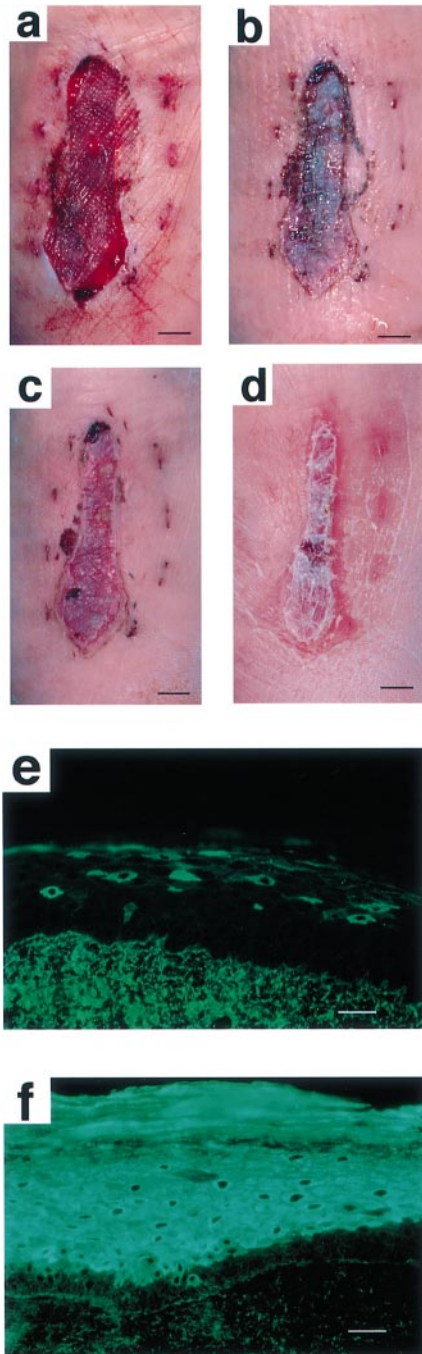


Figure 6. Transplantation of pure epidermal sheets derived from nonpalmoplantar skin on human palmoplantar wounds. The pure epidermis from trunk skin was grafted on the human plantar wound. The graft took well 1 wk later (a) and, gradually, showed typical palmoplantar skin appearance at 2 wk (b), 3 wk (c), and 5 wk (d) post-grafting. Immunohistochemical studies with anti-K9 antibodies were done at 3 wk (e) and 5 wk (f) post-grafting. These results show a representative of five independent studies. Scale bars: (a–d) 1 cm, (e, f) 50 μ m.

long-term interactions may be necessary for the adequate K9 induction and/or contamination with mouse dermal fibroblasts might suppress K9 expression in human keratinocytes. To address these issues and to provide a clinical perspective, we grafted the nonpalmoplantar epidermis on five palmoplantar full-thickness skin wounds due to burn, acute injury, and the resection of acral lentiginous melanoma. We used dispase instead of trypsin in our cultures to minimize contamination with donor fibroblasts. The grafts took well and without contracture (Fig 6a) and, gradually,

showed typical palmoplantar skin appearance (Fig 6b–d). Histologically, the grafted epidermis showed a thick and compact stratum corneum and a well-developed stratum lucidum at 5 wk post-grafting (data not shown). Immunohistochemical examinations also showed a sparse distribution of K9-positive keratinocytes at 3 wk post-grafting (Fig 6e) and expression increased at 5 wk (Fig 6f). These findings are comparable with those observed with normal palmoplantar epidermis (Moll *et al*, 1987). Two years later, the grafted epidermis continued to show expression of K9-positive keratinocytes in the suprabasal layers (data not shown).

DISCUSSION

Once acquired, K9 expression in keratinocytes represents an intrinsic program, as shown by our results that palmoplantar keratinocytes continuously expressed the K9 mRNA during serial subcultivations and continued to express K9 when cocultured with nonpalmoplantar fibroblasts (data not shown). Our results are in agreement with the previous reports, mentioning that differentiation of epithelial cells is generally regulated intrinsically (Doran *et al*, 1980; Boukamp *et al*, 1990; Limat *et al*, 1996; Miller *et al*, 1998).

Epithelial–mesenchymal interactions are considered to play fundamental roles in the growth and differentiation of keratinocytes (Boukamp *et al*, 1990; Jahoda, 1992; Smola *et al*, 1993; Byrne *et al*, 1994). Extrinsic factors, such as mechanical stress, can induce the expression of a keratin probably corresponding to K9 in cultured keratinocytes (Gormar *et al*, 1990). Our report shows exogenous regulation of K9 expression in topologically different keratinocytes by signals from a subset of palmoplantar fibroblasts. Although the culture insert membrane prevented direct contact of the fibroblasts with the overlying keratinocytes, it remains possible that paracrine and/or juxtacrine interactions are involved in K9 induction. The fact that K9 expression could be induced weakly when palmoplantar fibroblasts were distant from keratinocytes is interesting, and suggests a secreted factor may be involved in the induction.

The K9 sequence analysis revealed 2 bp differences between the previously published gene (Langbein *et al*, 1993) and our data obtained from both palmoplantar keratinocytes and nonpalmoplantar keratinocytes cocultured with palmoplantar fibroblasts. Interestingly, our base pair differences were located in the variant end domain, whereas K9 point mutations reported in keratinization disorders occur only in the central rod domain (Bonifas *et al*, 1994; Hennies *et al*, 1994; Reis *et al*, 1994; Torchard *et al*, 1994; Navsaria *et al*, 1995; Rothnagel *et al*, 1995; Kobayashi *et al*, 1996). We regard the L133F substitutions in our experiments as polymorphisms, which have been shown in the other keratin genes (Mischke and Wild, 1987; Zhou *et al*, 1988; Korge *et al*, 1992a, b; Wanner *et al*, 1993). Further investigations along these lines, however, are necessary to answer to this question.

Dermal fibroblasts are heterogeneous populations (Harper and Grove, 1979; Schor and Schor, 1987; Goldring *et al*, 1990; Falanga *et al*, 1995). Our results are in agreement with these previous reports. Fibroblasts derived from palms and soles were different from those from other body sites in terms of K9 induction in nonpalmoplantar keratinocytes. Moreover, papillary palmoplantar fibroblasts had a higher ability to induce K9 mRNA expression than those from reticular palmoplantar dermis.

It is not easy to treat palmoplantar skin defects because palmoplantar skin is difficult to harvest from donor sites and differs from nonpalmoplantar skin in terms of its ability to support mechanical stress. In this report, we grafted epidermal sheets derived from nonpalmoplantar sites on sole wounds. These grafted keratinocytes took and grew well and assumed a palmoplantar phenotype with substantial expression of K9 at 5 wk after the transplantation. There are several possibilities to explain these findings. Because the epidermal sheets were grafted on the fatty layers of sole wounds, the remaining wound fibroblasts might have induced K9 in the grafted epidermis. Possibly, plantar fibroblasts from around the skin defect might have migrated into the wound. Alternatively, K9 expression might have occurred because of keratinocytes around

sweat glands in the wound and which extended into the grafted epidermis. The latter possibility, however, is unlikely because re-epithelialized skin from the sweat apparatus in the deep wound looks different from normal skin and takes longer to be formed than our results indicate (Miller *et al*, 1998). In conclusion, K9 expression in human keratinocytes is controlled not only by intrinsic regulatory mechanism, but also by extrinsic signals from a subset of palmoplantar fibroblasts.

Pure epidermal grafting may be effective in the treatment of palmoplantar skin defects as a result of heterotypic mesenchymal-epithelial interactions.

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