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Keratins (K16 and K17) as markers of keratinocyte hyperproliferation in psoriasis *in vivo* and *in vitro*

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Summary Keratinocyte differentiation in psoriasis was examined using a panel of monospecific monoclonal antibodies to keratins (K), including two recently developed monoclonal antibodies raised to carboxy terminal peptides of K6 (LL020) and K16 (LL025). Keratinocytes from normal skin, untreated psoriatic plaques and non-lesional psoriatic skin, were cultured using multiple *in vitro* systems. Time-lapse cinephotography was used to measure the intermitotic time of normal and psoriatic keratinocytes in both low calcium-defined and serum-containing media. The intermitotic time did not differ significantly between psoriatic and normal keratinocytes. Keratin expression of psoriatic and normal keratinocytes *in vitro* was examined by both gel electrophoresis and immunocytochemistry. K6, K16 and K17 were detected suprabasally in all culture systems *in vitro*, but only in interfollicular psoriatic epidermis *in vivo*, and not in normal skin. Small subpopulations of keratinocytes expressed simple epithelial keratins K7, K8, K18 and K19 in cultures on plastic substrates, but these keratins were absent in skin equivalents of normal or psoriatic skin. No psoriasis-specific pattern of differentiation was found *in vitro*. As the K6 peptide antibody reacted with basal cells of normal skin, probably due to K5 cross-reactivity, K16 expression determined by LL025 was found to be the most sensitive indicator of the psoriatic state of differentiation, and this antibody is recommended for future work on psoriasis. K17 had a distinct pattern of tissue distribution in normal skin: K17, but not K16, was present in basal myoepithelial cells in sweat glands, and the deep outer root sheath, but K17 distribution paralleled that of K16 in suprabasal psoriatic epidermis. As keratins K6, K16 and K17 are expressed in keratinocyte hyperproliferation, when high levels of certain cytokines are also expressed, the role of growth factors and regulatory nuclear transcription factors in the control of K6, K16 and K17 expression in psoriasis requires further study, in order to provide insight into the relationship between proliferation and differentiation.

Epidermal changes in psoriasis primarily involve hyperproliferation and a greatly abbreviated epidermal turnover time, a shortened keratinocyte cell cycle time, and an increased growth fraction.[1] The resulting hyperplastic epidermis is poorly differentiated, with immature cornification.[2] A sequential study of the advancing edge of psoriatic plaques showed that the initial event was a change in keratin (K) expression.[3] This characteristically comprised reduced suprabasal keratins (K1 and K10) and novel suprabasal expression of K6 (48 kDa) and K16 (56 kDa), as occurs in hyperproliferative epidermis in other diseases.[4] A study of cultured epidermal keratinocytes has produced similar findings.[5] Psoriatic keratinocytes have been cultured *in vitro*,[6–12] but there have been few studies comparing the differentiation of psoriatic and normal keratinocytes *in vitro*. As we have recently developed monospecific monoclonal antibodies to keratins K6 and K16, and novel reagents to detect keratin 17 are available,[13,14] we have examined, by both immunocytochemical and biochemical techniques, the expression of keratins in both normal and psoriatic individuals, using different culture systems which produce different stages of morphological differentiation. We have also compared the proliferation of psoriatic and normal keratinocytes *in vitro* by time-lapse cinephotography (TLP).

Methods

Tissue samples

Normal skin was obtained from operative samples taken from several body sites (breast, abdomen, scalp and foreskin), and an extensive bank of frozen tissues was available through collaborative projects. Punch biopsies of paired non-lesional and lesional psoriatic epidermis (from untreated stable plaque psoriasis) [12 samples each] were taken and snap-frozen for immunohistochemistry. Further punch and shave biopsies were collected in culture medium and immediately transported to the laboratory for keratinocyte and fibroblast culture.

Keratinocyte culture

Primary keratinocytes were cultured from infant foreskin, adult breast and abdominal skin, psoriatic plaques and non-lesional psoriatic skin, using irradiated 3T3 feeder cells.[15] Passaged keratinocytes from all strains were plated on glass coverslips for immunocytochemistry. Samples of passaged keratinocytes were also subcultured into keratinocyte growth medium (KGM™; Clonetics, Denver, CO, U.S.A.) without feeders.[10] Parallel cultures of keratinocytes were also passaged into organotypical cultures in silicone chambers, with gels of rat tail collagen separating the epithelial and mesenchymal components.[16] Both normal human fibroblasts and psoriatic fibroblasts were seeded on the undersurface of the collagen gel as feeder cells in the organotypical cultures. After 21 days, the collagen-based cultures were removed from the silicone chamber and snap-frozen. Transverse cross-sections across the cultures were cut and sectioned for immunohistochemistry.

Immunocytochemistry

Fixed coverslips, frozen cross-sections of keratinocyte sheets and complex cultures, and frozen sections of psoriatic and normal skin were stored at −70°C. Monoclonal antibodies to K6 (LL020) and K16 (LL025) had been raised to carboxy terminal peptides, as previously described.[17] Coverslips and cryostat sections were examined using the monoclonal antibodies listed in Table 1, employing routine immunoperoxidase and immunofluorescence techniques. All the other antibodies have been characterized previously, as shown in the table. In addition, anti-peptide antibodies had been raised to keratins K1 and K14. LL001 reacted with keratin K14 throughout the epidermis, as expected from biochemical studies. Basally reactive antibodies to

Table 1. Keratin expression *in vivo* (interfollicular epidermis)

Keratin	Antibody/reference	Normal	Non-lesional psoriasis	Lesional psoriasis
K8	LE41 (50)	–	–	–
K18	LE61 (50)	–	–	–
K19	LP2K (51)	–	–	–
K7	LPIK (52)	–	–	–
K7/8	Cam 5.2 (53)	–	–	–
Basal	pAb421 (27)	B	B	B
Basal	pAb601 (27)	B	B	B
Basal	LH6 (17)	B	B	B+
Basal	LH8 (17)	B	B	B
K14	LL001 (17)	++	++	++
K5	PAb (17)	++	++	++
K10	LHP 1,2,3 (54)	SB	SB	SB
K1	LL017 (54)	SB	SB	SB
K4	6B10 (55)	–	–	–
K13	IC7 (55)	–	–	–
K6	LL020 (17)	SB(B)	SB(B)	SB(B)
K6	KA12	–	–	SB
K16	LL025 (17)	–	–	SB
K17	E3 (13)	–	–	SB(h)
K7/17	C46 q (14)	–	–	SB(h)
Vimentin		–	–	–

B, basal; SB, suprabasal; ++, pan-epithelial; pAb, polyclonal antibody; h, high in epidermis; B+, more than one layer.

keratins K5 and K14 do not detect keratins K5 and K14 in all cells, as the epitopes are conformational and masked by suprabasal expression of further differentiation-linked keratins.

Gel electrophoresis of keratin extracts

Cultured keratinocytes, biopsy specimens, or scale scrapings from normal, non-lesional and lesional psoriatic skin, were examined by one- and two-dimensional electrophoresis.[18–20] Keratins were isolated from cells, epidermal tissue, or stratum corneum, by a standard cytoskeletal method.[20,21] Briefly, cells or tissue were extracted serially with 1% Triton-X100 and 2 mM DTT in a Tris–NaCl–EGTA buffer (low salt), 0·5% Triton-X100 and 2 mM DTT in a Tris-1·5 M KCl–EGTA buffer (high salt) and Tris-buffered saline. Keratins were then extracted from the final pellet with 2% SDS and 2% β-mercaptoethanol. The keratins were analysed by SDS–PAGE on 7·5–17·5% gradient slab gels,[20] and by a combination of isoelectric focusing (IEF: pH 5–8) and SDS–PAGE on gradient slab gels. Keratin proteins were visualized by staining with Coomassie blue R250.

Time-lapse cinemicroscopy

Normal and lesional psoriatic keratinocytes were cultured on 60-mm Petri dishes in the presence of gamma-irradiated 3T3 feeders for 48 h.[15] The dishes were then washed with EDTA to remove 3T3s, leaving behind very small colonies of keratinocytes (the presence of 3T3s makes it difficult to follow the mitosis of keratinocytes on time-lapse photography). Each sample was cultured in duplicate and time-lapse cinemicroscopy repeated three times. The cinemicroscopy unit was enclosed within a controlled environment at a temperature of 37°C, and was constantly perfused with 5% CO_2. The equipment comprised four Olympus time-lapse units, 16-mm Bolex cameras and inverted Olympus IMT microscopes, fitted with × 10 phase objectives. The cultures were filmed at a rate of one frame every 4 min, using Kodak Infocaptur film, as previously described.[22,23] The culture medium was changed every 2 days over a period of 5–6 days and the cultures were maintained as long as possible. The films, which comprised 360 frames per 24 h, were then viewed using an analytical projector. At the start of measurement, a mitosis was clearly identified within the very small clump of cells, and then both daughter cells were tracked on the film until they divided. Using the frame timings, the interval between the mother and daughter mitoses was calculated as the intermitotic time. By following the ensuing progeny, keratinocyte family trees could be constructed and the intermitotic time thus calculated for each generation. Numerous mitoses (up to 86) were counted for each film, and counting was continued until the films were completed; the cells became confluent (when mitoses are difficult to see) or senesced and ceased to divide.[22] Psoriatic and normal keratinocytes were also cultured in low-calcium-defined medium (KGM Clonetics), in order to compare the intermitotic times of psoriatic and normal keratinocytes in low- and high-calcium media. A large number of biopsies (> 300) were performed to obtain approximately 30 strains of psoriatic keratinocytes (see Results). Many films were unsuccessful because of a number of problems. High cell motility meant that in some films keratinocytes moved out of the field of view during filming, and in others cells failed to divide, or were overconfluent and could not be analysed. Statistical analysis of generational change and changes with ageing of culture and cell cycle dispersion were performed by Dr Y.Rochev, Institute of Theoretical and Experimental Biophysics, Puschino, Moscow, and as this showed no significant differences, further filming was thought to be unnecessary. The selection of keratinocytes by such a demanding mode of measurement may make comparisons with other methods difficult, as it selects cells of high proliferative potential.

Results

Immunohistochemistry (Table 1)

Keratins 6 and 16 in psoriatic and normal skin. On screening a bank of normal tissues, mAb LL025 reacted with suprabasal keratinocytes in oral mucosa at all sites (buccal, palate, gingiva and tongue) and in the genital mucosae of the vagina, vulva and ectocervix. In normal skin, there was no reaction with interfollicular epidermis, but there was a cone of reaction with the suprabasal upper outer root sheath. In all samples of lesional psoriatic epidermis, suprabasal staining with LL025 was seen in interfollicular epidermis from the epibasal cells to the surface (Fig. 1). Hair follicles were seldom seen in psoriatic plaques, but a reaction could also be seen in psoriatic outer root sheath. Occasional areas of K16 (LL025) reactivity were seen in non-lesional epidermis, perhaps reflecting incipient disease.

K6 (detected by LL020) was also found predominantly suprabasally in lesional psoriatic epidermis, and the distribution mirrored K16. However, LL020

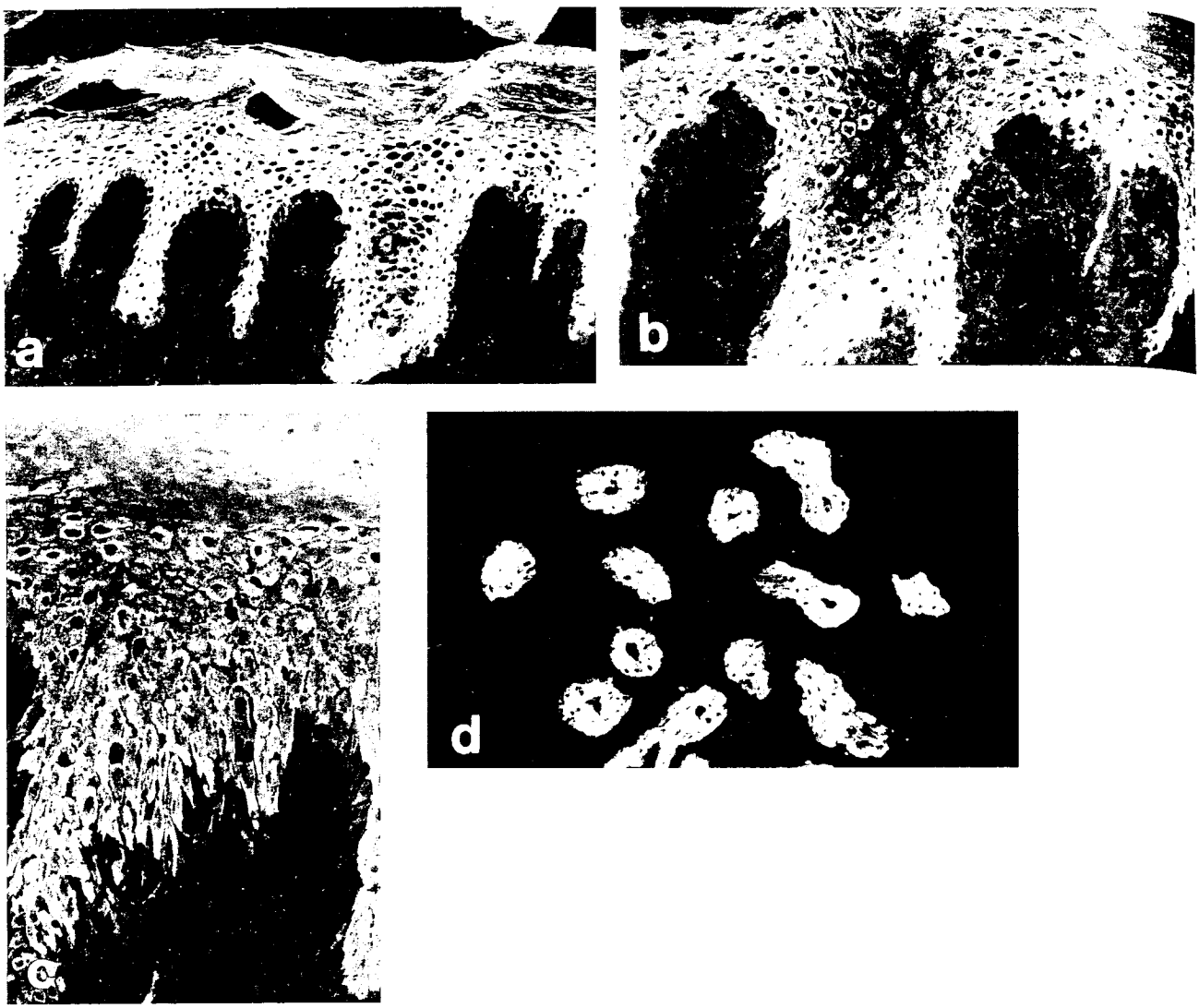

Figure 1. Immunohistochemistry of psoriasis skin to demonstrate reaction with (a) KA12, (b) LL025 and (c) C46, all of which show suprabasal reactivity. (d) Normal skin shows basal reactivity of a sweat gland with E3.

reactivity was also found in a high proportion of basal cells (80%), as well as throughout the suprabasal cell layer. A distinct difference from LL025 staining was seen in interfollicular normal skin, where basal and suprabasal LL020 staining could be seen. Recent immunoblotting has shown weak reactivity of LL020 with K5 and K6 (E.B.Lane *et al.*, unpubl. obs.). However, staining with KA12 (a keratin antibody reacting only with K6, kindly donated by R.Nagle) mirrored LL025 staining in that it was localized to suprabasal psoriatic epidermis only (Fig. 1).

Keratin 17 in normal and psoriatic skin. K17 was shown, by antibody E3,[13] (and checked by cross-reaction with C46 [K7 and K17])[14] to be present in the deep outer root sheath and the basal cells of sweat glands in normal skin (Fig. 1). Intense basal reactivity was seen adjacent to entry points of the acrosyringium, and more extensively in scalp skin (up to 50% of basal cells), but no suprabasal reactivity could be seen in normal skin. In lesional psoriatic epidermis, the suprabasal keratinocytes reacted with both E3 and C46 at a higher position in the epidermis than LL025, being more than 2–3 cells away from the basal lamina and not in an epibasal position (Fig. 1). C46 staining was invariably more intense.

Keratins K5 and K14 in normal and psoriatic skin. As shown in Table 1, the polyclonal/monoclonal antibodies to K5 and K14 peptides showed reactivity throughout

the epidermis in normal skin, and in non-lesional and lesional psoriatic skin. This is a well established feature of these antibodies, as keratins 5 and 14 continue to be expressed in suprabasal cells, whereas basal-restricted epitopes detected by conformation-dependent antibodies react with basal cells only. Thus, basal cell markers to basal keratin conformational epitopes reacted with a single layer of basal cells only in normal skin, and a similar distribution was found in psoriatic plaques. The extent of reaction of the basal cell markers varied from antibody to antibody in psoriatic lesions. The antibody LH8 gave the most restricted reaction, with the majority of psoriatic samples showing a basal cell monolayer, whereas other basal antibodies reacted with an expanded population of basal cells forming 2–3 layers.

Keratins K1 and K10 in normal and psoriatic skin. Monoclonal antibodies to K1 and K10 reacted with suprabasal keratinocytes in psoriatic plaques, as in normal skin. With the weaker antibodies, reduced intensity and restricted distribution of staining could be seen. The non-lesional psoriasis biopsies were indistinguishable from normal skin. K10 detection varied according to the antibodies, as LHP1 gave high suprabasal staining whereas polyclonal antisera to K10 reacted epibasally.

Simple epithelial keratins. No expression of simple epithelial keratins K7, K8, K18, K19 was found in interfollicular epidermis in lesional and non-lesional psoriatic skin, or in normal skin, apart from Merkel cells.

Cultured keratinocytes from normal and psoriatic skin (Table 2)

Normal epidermal keratinocytes on irradiated 3T3 feeders (Fig. 2). Normal keratinocytes initially formed small colonies, which subsequently enlarged and became confluent. Stratification could be seen in the centre of colonies, with squames forming and covering several basal cells. All keratinocytes expressed antigens detected by the anti-K14 peptide antibodies LL001 and LL002 (Table 1). All other keratins were expressed in a heterogeneous distribution. Patchy expression of keratins 1 and 10 was found suprabasally on dispase-separated sheets, and this also applied to K4 and K13, K6 and K16. Islands of expression of K19 were found, and here the cells appeared to be stratifying less. Keratin 7 expression was also seen in normal foreskin and adult keratinocytes. Small numbers of isolated cells expressing K8 and K18 were also found in cultures of foreskin keratinocytes.

Table 2. Keratin expression in psoriatic and normal keratinocytes

	Plastic			Organotypical	
	FsK p1/2/3	NK p1/2/3	PsK p1	Nk	PsK
K8	+/−	−	−	−	−
K18	+/−	−	−	−	−
K19	+/−	+/−	+	(+)	(+) sup
K7	+/++	+/−	−	−	−
K7/8	+	+/−	−	−	−
pAb421	+	−	+	B/SB	B/SB
pAb601	+	+	+	B/SB	B/SB
LH6	++	+	−	B/SB	B/SB
LH8	+	+	−	B/EB	B/EB
LL001	+−	++	++	++	++
pAbK5	+	+	+	+	+
K10	−	−	+	SB	SB
K1	−	+/−	+	SB	SB
K4	+/−	−	+	+/− sup	+/− sup
K13	−	+/−	+	−	−
K6	(+)	+	+	++	++
K16	+/−	+/−	++	SB	SB
Vimentin	+h	+h	+h	−	−

FsK, foreskin keratinocytes; NK, normal adult keratinocytes; PsK, psoriatic keratinocytes; ND, not done; h, heterogeneous; p1, passage 1; +, positive; ++, strongly positive; +/− variable positive; B, basal; SB, suprabasal; EB, epibasal; B/SB, basal and suprabasal; sup, superficial.

Normal epidermal keratinocytes in low-calcium medium. Confluent keratinocyte cultures, 24 h after they were exposed to low-calcium medium, lost desmosomal interconnections and the suprabasal cell layers detached. When examined with the monoclonal antibodies, the cells were uniformly reactive with basal cell markers and LL001, and there was minimal reactivity of K1/K10, K6/K16 and K4/K13. The simple epithelial keratins were all expressed heterogeneously. When cells were cultured in low-calcium medium for longer periods, larger cells expressing differentiation-specific keratins were seen in increasing numbers.

Epidermal keratinocytes: complex cultures. Keratinocytes were subcultured on to collagenous substrates and cross-sections were examined. The cultures were stratified and a distinct layering could be seen, with cuboidal basal cells and attenuated larger spinous cells. Several layers of cornifying cells were seen superficially, and they had retained nuclei (resembling parakeratosis). There was orderly basal–suprabasal compartmentalization of keratin expression, with basal cell markers reacting with

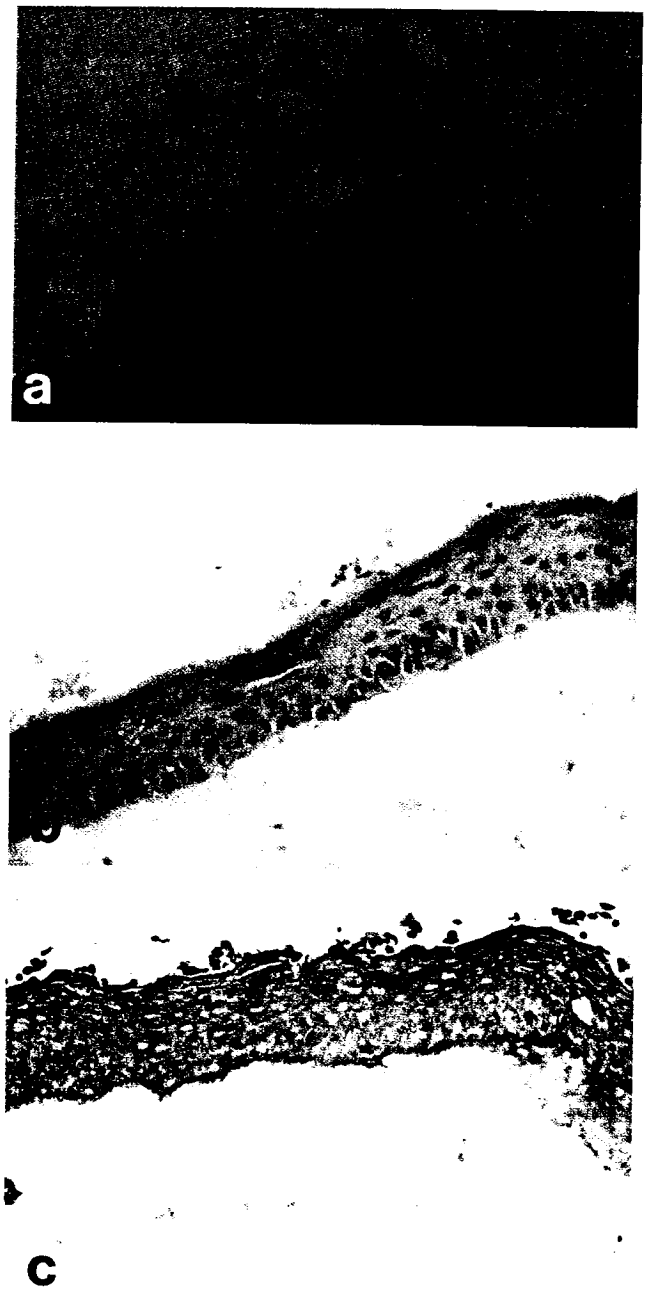

Figure 2. Immunocytochemistry of keratinocytes to show: (a) plastic substrate with psoriatic keratinocytes staining with polyclonal anti-keratin antibody; (b) organotypical culture of psoriatic keratinocytes—gross morphology; (c) organotypical culture of psoriatic keratinocytes and K14 (LL001) reactivity.

basal cells, whereas K1/K10 and K6/K16 were found suprabasally. Expression of K1 was less than that of K10 (probably because the antibody is always weaker), and both were in high suprabasal layers only. More extensive staining of basal cells was found than in normal skin *in vivo*, with a complementary reduction in suprabasal keratins in higher layers. K4 was barely visible in complex cultures (minute traces in the most superficial layers) and K13 was absent. No simple epithelial keratins (K8 and K18) were found, and only superficial traces of K19 were observed. Thus, the pattern of keratin expression in complex cultures was closer to that in hyperproliferative skin, and the simple epithelial keratins were reduced compared with cultures on plastic.

Psoriatic keratinocytes compared with normal epidermal keratinocytes (Fig. 2). Although there was great difficulty in establishing primary keratinocyte cultures from lesional psoriatic skin (10% of biopsies produced cultures, compared with 65% of normal adult skin

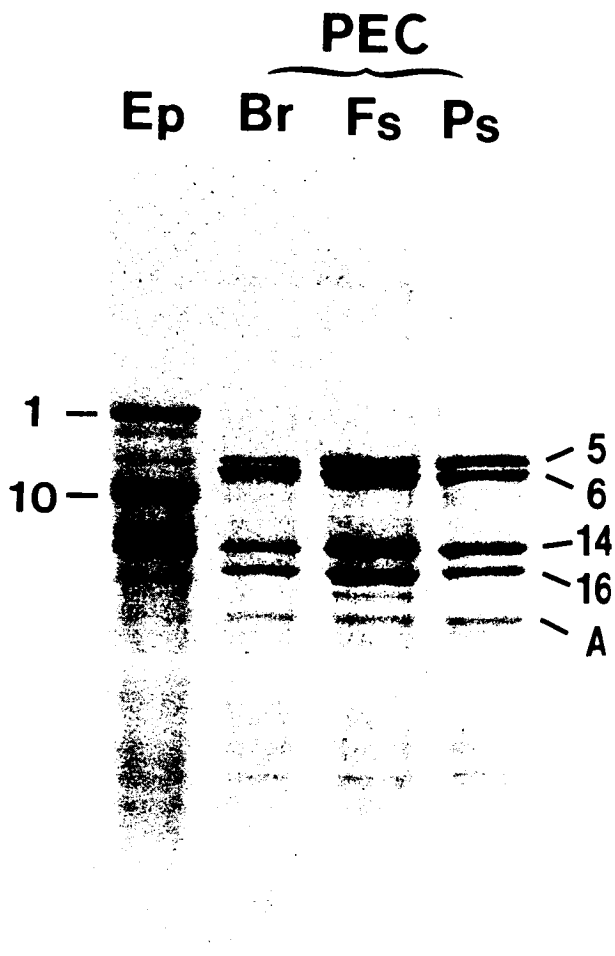

Figure 3. One-dimensional electrophoresis of keratin proteins purified from normal interfollicular epidermis (Ep) and different primary epidermal cultures [PEC; keratinocytes isolated from breast skin (Br) foreskin (Fs) and psoriatic skin (Ps)]. The keratins were purified by a standard cytoskeletal method, and analysed by SDS–PAGE on 7·5–17·5% gradient slab gels. The proteins were visualized by staining with Coomassie blue-R250. Keratins are labelled numerically (1, 10, 5, 6, 14, 16) according to Moll *et al.* (1982), and β-actin is marked A.

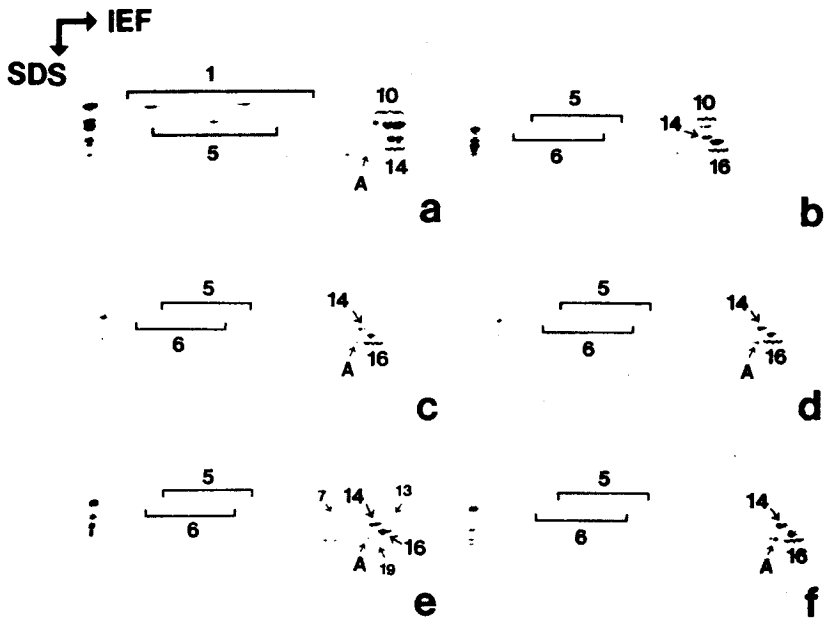

Figure 4. Two-dimensional electrophoresis of keratin proteins purified from normal epidermis (a), psoriatic epidermis (b), and primary epidermal cultures (c, normal breast keratinocytes; d, non-lesional psoriatic keratinocytes; e, normal foreskin keratinocytes; f, lesional psoriatic keratinocytes). Keratins were extracted by a standard cytoskeletal method, and separated initially by isoelectric focusing (IEF; pH4–8), and then in the second dimension by SDS–PAGE on 7·5–17·5% gradient slab gels. The sample was also applied to the left of each gel in the second dimension, to show a direct relationship to the one-dimensional separation. The keratins are labelled according to the standard nomenclature (1 = K1, 5 = K5, etc.), and A is β-actin.

biopsies), there was no consistent discernible difference between psoriatic and normal keratinocytes in keratin immunocytochemistry or gross cell morphology. However, psoriatic keratinocyte cultures appeared very sensitive to trypsinization, and could not be readily passaged from primary culture. The gross morphology of complex cultures of psoriatic keratinocytes occasionally gave thicker epithelia than normal controls, but this was not reproducible in different experiments using the same cells of origin, or different biopsy sources, and did not relate to the fibroblast type used, whether psoriatic or normal.

Keratin expression in normal and psoriatic keratinocytes (gel electrophoresis). The protein profile of interfollicular epidermis shows that four major keratins (K1, K5, K10 and K14) are expressed in normal skin (Fig. 3, Ep). Psoriatic epidermis has an altered profile, displaying a reduction in K1 and K10 expression, and a concomitant increase in K6 and K16 expression (data not shown). However, when cells are removed from normal epidermis and cultured on a plastic substrate (PEC), a total loss of K1 and K10 expression and a rapid increase in K6 and K16 expression are observed (Fig. 3). Keratinocytes from lesional psoriatic skin have a similar response to culture conditions (Fig. 3). Hence, a similar keratin profile is obtained for all cultured cells.

More detailed analysis by two-dimensional (2-D) electrophoresis (Fig. 4a–f) showed a similar picture, and confirmed the absence of K1 and K10 in cultured keratinocytes from both normal and psoriatic epidermis. Normal epidermis expressed K1, K5, K10 and K14, together with small amounts of K15 and β-actin (Fig. 4a). Psoriatic epidermis, on the other hand, showed reduced amounts of K1 and K10 and an induction of K6 and K16 expression (Fig. 4b). However, two-dimensional profiles from the cultured keratinocytes were all similar. Cultured keratinocytes from breast skin (Fig. 4c) expressed K5, K6, K14 and K16, together with small amounts of β-actin and vimentin. The keratin profile of foreskin keratinocytes was similar, but low levels of K7, K13 and K19 were observed, in addition to a low level of β-actin. Keratinocytes from both non-lesional (Fig. 4d) and lesional (Fig. 4f) psoriatic skin also expressed the major 'culture-type' keratins (K5, K6, K14 and K16) but, unlike normal cells, had a much higher level of β-actin expression. Isoelectric focusing was used for the 2-D gels, which provides better resolution of acidic keratins, but the 1-D gels showed good quantitative similarity between K5 and K14, and K6 and K16. The increased number of isoelectric variants for K5 and K6 also gives a lower intensity on the 2-D gel. Thus, in order to study the defects in keratin expression in psoriatic keratinocytes, culture systems which induce differentiation defined by expression of K1 and K10 are required.

Keratinocyte intermitotic time by time-lapse photography (Table 3)

There were interpretable data from multiple keratinocyte samples, but equivalent sets tended to grow well in

Table 3. Time-lapse cinemicroscopy

Sample	Medium	Mean intermitotic time	SD	SE	Mitoses observed
Psoriasis 1	RM	22·5	6·6	1·55	19
Psoriasis 2	RM	24·0	7·95	1·82	19
Psoriasis 3	RM	20·6	8·65	0·93	86*
Psoriasis 1	KGM	23·5	5·93	0·92	42
Psoriasis 2	KGM	27·9	7·43	1·92	15
Normal 1	RM	17·2	4·82	0·74	42
Normal 1	KGM	30·1	7·57	1·41	29
Normal 2	KGM	33·0	9·49	2·63	13

Time in hours. Intermitotic time = the observed time on the film frame markings from mitosis of the mother cell to mitosis of the daughter cell. Mitoses observed = the total number of mitoses observed for a single culture. Mean intermitotic time = the mean of the intermitotic times for the number of mitoses. RM, serum-containing medium.[15] KGM, defined low-calcium medium.[11] SD, standard deviation.
*Correlation of mother/daughter cell cycle ($R = 0{\cdot}46$, $a = 0{\cdot}001$).

the test conditions regardless of the medium used. Although only a small number of successful time-lapse photography films was obtained following the use of large numbers of samples, laborious frame by frame analysis detected a large number of mitoses in both psoriatic and normal cells, and the data obtained were sufficient to enable a satisfactory statistical analysis. Pooling data from all samples and numerous individuals gave the following results (the original data are shown in Table 3). When psoriatic keratinocytes were cultured in serum-containing medium, the mean intermitotic time per sample varied from 20·6 to 24 h, whereas with normal adult keratinocytes under identical conditions the mean intermitotic time (one sample, 42 mitoses) was 17·2 h. In KGM, psoriatic keratinocytes had a mean intermitotic time of between 23·5 and 27·9 h, and equivalent cultures of normal adult keratinocytes gave a mean intermitotic time of 30·1–33 h. There was no statistically significant difference in the intermitotic times between the various groups.

Analysis of the cell cycle, or effects of ageing, showed no increase with cultivation across three–four generations. Some clones showed homogeneity amongst cells of an individual clone, with dispersion of the cell cycle in the clone being less than in the entire population. Some correlation between mother and daughter cell cycle times was observed, particularly in larger clones, in which mother cells with a faster replication cycle produced daughter cells with a faster replication cycle, but the numbers were not sufficient to yield statistically valid data.

Discussion

Kinetics of keratinocytes in vitro

Studies of psoriatic keratinocyte kinetics *in vitro* have produced differing results with different systems, but overall they suggest that there is no consistent difference in kinetics between psoriatic keratinocytes from lesional or non-lesional skin and appropriate adult normal controls.[6–12] Our data would support this conclusion, as we did not observe any significant difference in intermitotic time between normal and psoriatic keratinocytes when cultured in low-calcium or serum-containing media. Direct observation of the intermitotic time by TLP avoids the problem of labelling interfering with the cell cycle. The keratinocyte *in vitro* has a much shorter cell cycle time than those reported for normal epidermis (311 h) or psoriatic epidermis (36 h) *in vivo*.[1] This suggests that *in vitro* models are so hyperproliferative that they may obscure differences between psoriatic and normal keratinocytes. Skin equivalents and animal grafts promote epidermal differentiation, and may be better able to discriminate between different cell kinetics.

Hyperproliferative keratins as markers of altered differentiation

Our study confirms that K6 and K16 are constitutively expressed in certain stratified squamous mucosal epithelia, and are not found in normal epidermis external to the outer hair root sheath and junctional region, but are found in suprabasal psoriatic epidermis.[4,24,25] The distribution of K17 in normal tissue appears to be distinct from that of K16. In normal skin, K16 is found suprabasally in the upper outer root sheath, and K17 is found basally in myoepithelial basal glandular cells and in the deep outer root sheath.[26] However, we have found that both K16 and K17 are expressed suprabasally in psoriatic hyperproliferation. Our earlier study of psoriasis and keratin compartmentalization used only immunofluorescence techniques and a limited number of antibodies.[27] Clearly, basal cell antibodies show considerable variation in the extent of their reactivity with basal epidermis, partly due to differing antibody affinity, which is in accord with recent reports showing quantitative alteration in K5 and K15 in psoriasis.[25] Our suggestion that psoriatic hyperproliferation occurs predominantly in the differentiating compartment was subsequently supported by lectin binding studies,[28] and by involucrin staining of thymidine-labelled suprabasal psoriatic

keratinocytes.[29] In transgenic mice, keratinocytes expressing human keratin 1 can divide *in vivo* and *in vitro*.[30] Studies of psoriatic hair follicles have shown changes in the upper outer root sheath and sebaceous gland,[31,32] but not in the putative stem cell region in the bulge. These observations suggest that psoriatic hyperproliferation largely results from recruitment of cell division in the transit amplifying population and not amongst stem cells. This would explain the particular difficulty of both establishing and maintaining psoriatic keratinocytes *in vitro* as, by definition, amplifying cells would have limited proliferation potential.

The expression of K6, K16 and K17 in psoriasis supports the widespread view that psoriatic keratinocytes respond with an alternative pathway of differentiation.[24,33,34] However, this response is rather stereotyped. There are no disease-specific changes, as identical changes are induced in normal keratinocytes whenever migration and mitosis are necessary. Oral mucosa, palmoplantar epidermis and wound healing are good examples of this. The alternative pathway of differentiation in psoriasis and wound healing includes changes in integrin expression[35,36] and in other hyperproliferation antigens,[31] and the activation of growth factors[37] [interleukin 1(IL-1), interleukin 6 (IL-6),[37,38] transforming growth factor α (TGF-α)[39] and transforming growth factor $\beta 1$ (TGF-$\beta 1$)[40]] and growth factor receptors.[41] Recent 2-D gel cataloguing of proteins present in the psoriatic plaque has shown upregulation of low-molecular-weight proteins in psoriasis, particularly a protein called psoriasin.[42] All these proteins are also found in normal keratinocytes *in vitro*.

Recent research has shown the importance of cytokines in the regulation of keratins K6, K16 and K17. In transient expression assays, keratins K6 and K16 are the only keratins upregulated by epidermal growth factor (EGF) and transforming growth factor α (TGF-α), whereas keratin K17 is upregulated by gamma interferon (IFN-γ).[43] As psoriatic hyperplasia is thought to result from dysregulation of growth factors in psoriatic epidermis, transgenic mice overexpressing TGF-α,[44] and IL-6 transgenic mice, have been found to exhibit marked phenotypic abnormalities, but without epidermal hyperproliferation or the induction of keratins K6/K16 in affected skin.[45] Several factors are probably required to reproduce the psoriatic phenotype.

Cultured keratinocytes and the hyperproliferative phenotype

Comparison of keratins from cultured non-lesional psoriatic and normal keratinocytes on 1-D and 2-D gel electrophoresis showed no major differences; both predominantly express K5, K6, K14 and K16. Immunocytochemistry revealed that all keratinocytes continued to express K6, K16 and K17 suprabasally, whether from psoriatic or normal skin, on plastic or collagenous substrate, in the presence of feeder 3T3 cells, or in normal or low-calcium medium. This suggests that normal keratinocytes *in vitro* adopt a pattern of psoriatic differentiation, rather than that psoriatic keratinocytes lose phenotype. As keratinocytes are so hyperproliferative in culture (as shown by TLP), this may obscure subtle differences in differentiation between diseased and normal keratinocytes.

Organotypical cultures as models of psoriasis

On adding a dermal component or extracellular matrix to both psoriatic and normal keratinocyte cultures, the resulting epidermis more closely resembled hyperproliferative skin *in vivo*, with K5/K14 in basal cells and K6/K16, K1/K10 suprabasally, and minor amounts of K17 and K19. There was loss of the simple epithelial keratins found in the same cells on a plastic substrate. Mesenchymal influences regulate keratinocyte differentiation by both directive and permissive signals.[16,46] Recent studies have also suggested that provision of a mesenchyme is important in maintaining stem cell function *in vitro*. Saiaig *et al.*[47] found that normal keratinocytes were more proliferative in explant outgrowth on the living skin equivalent when psoriatic fibroblasts were included in the matrix, whereas psoriatic keratinocytes remained hyperproliferative throughout. Animal studies have confirmed the necessity for appropriate mesenchymal components to reproduce the psoriatic phenotype.[48] As organotypical cultures have both mesenchymal and epithelial components, they provide optimal models for epidermal hyperproliferation, as normal keratinocytes adopt the hyperproliferative phenotype, i.e. psoriatic-type differentiation. Keratinocytes cultured on plastic substrates cannot be considered a model for normal or psoriatic epidermis, because of the widespread activation of genes not found in normal skin (such as simple epithelial keratins). When grown on collagenous or dermal equivalent substrates, the keratinocytes approximate to psoriatic or wounded skin, and may be a model for these disorders, but not for normal skin.

Response to growth factors

We found no significant differences in intermitotic time between psoriatic and normal keratinocytes in

both serum-free, low-calcium medium and serum-containing medium. The current theory is that psoriatic hyperplasia is triggered by T-cell activation and results from altered keratinocyte susceptibility to cytokines such as IFN-γ.[11,49] Activated keratinocytes synthesize and respond to a large number of growth factors, but overactivity of single growth factors in transgenic mice does not reproduce the hyperproliferative phenotype. As nuclear transcription factors such as NFIL6 can act as master switches for a large number of genes, and many of the genes expressed in psoriatic epidermis contain NFIL6 binding sequences in their URR, further understanding of the role of nuclear transcription factors and growth factors in the regulation of expression of K6/K16 and K17 genes must advance our knowledge of the fundamental basis of psoriasis, and also provide a novel target for therapeutic intervention.

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