Expression of Keratin K2e in Cutaneous and Oral Lesions

Association with Keratinocyte Activation, Proliferation, and Keratinization

Balvinder K. Bloor,* Nicholas Tidman,[†]
Irene M. Leigh,[†] Edward Odell,* Bilal Dogan,[‡]
Uwe Wollina,[§] Lucy Ghali,[†] and Ahmad Waseem*

From the Head and Neck Cancer Research Program,* Guy's, King's, and St. Thomas's Dental Institute, King's College London, London, United Kingdom; the Cancer Research United Kingdom Skin Tumour Biology Unit,[†] Centre for Cutaneous Research, Queen Mary, University of London, London, United Kingdom; the Department of Dermatology,[‡] Gulbane Askeri Tip Akademisi (GATA) Haydarpasa Teaching Hospital, Istanbul, Turkey; and the Department of Dermatology,[§] General Hospital Dresden-Friedrichstadt, Dresden, Germany

The cytoskeleton in keratinocytes is a complex of highly homologous structural proteins derived from two families of type I and type II polypeptides. Keratin K2e is a type II polypeptide that is expressed in epidermis late in differentiation. Here we report the influence of keratinocyte activation, proliferation, and keratinization on K2e expression in samples of cutaneous and oral lesions. The normal expression of K2e in the upper spinous and granular layers of interfollicular epidermis is increased in keloid scars but showed distinct down-regulation in psoriasis and hypertrophic scars where keratinocytes are known to undergo activation. Unlike normal and psoriatic skin, K2e expression in hypertrophic and keloid scars began in the deepest suprabasal layer. In cutaneous basal and squamous cell carcinomas, K2e was absent in most tumor islands but the overlying epidermis showed strong expression. No significant K2e expression in nonkeratinized or keratinized oral epithelia, including buccal mucosa, lateral border of tongue and gingiva was detected. In oral lichen planus K2e expression was undetectable, but in benign keratoses of lingual mucosa induction of K2e along with K1 and K10 was observed. In mild-to-moderate oral dysplasia with orthokeratinization, K2e was highly expressed compared with parakeratinized areas but in severe dysplasia as well as in oral squamous cell carcinoma, K2e expression was undetectable. Taken together, the data suggest that K2e expression in skin is sensitive to

keratinocyte activation but its up-regulation in oral lesions is a reflection of the degree of orthokeratinization. (Am J Pathol 2003; 162:963–975)

The normal adult epidermis is a self-renewing tissue consisting of 10 to 20 layers in which cell proliferation is primarily restricted to the basal layer. When keratinocytes commit to differentiate, they down-regulate cell surface integrins to lose adhesiveness, leave the basal layer, exit the cell cycle,^{1,2} and undergo a program of terminal differentiation as they move through the suprabasal layers to the tissue surface.³ During this journey the keratinocytes undergo a series of physiological and morphological changes that culminate in the production of dead, flattened enucleated squames that are shed and replenished by differentiating keratinocytes.^{4,5} In the oral cavity, however, huge regional variations are found in the degree and type of keratinization. Orthokeratinized epithelium similar to that in skin is seen in the hard palate, whereas other regions are either parakeratinized (gingiva) or nonkeratinized (buccal mucosa).⁶

Injury to the epidermis activates a homeostatic response resulting in inflammation, re-epithelialization, followed by tissue remodeling.^{7,8} Several studies have suggested release of interleukin-1 from keratinocytes at the wound site as the initial trigger for the inflammatory reaction. This serves as an autocrine signal to surrounding keratinocytes and paracrine signal to other cells, such as fibroblasts, endothelial cells, and lymphocytes resulting in a pleiotropic effect on them.^{9,10} The changes in gene expression that accompany re-epithelialization are similar to those seen in other disorders associated with hyperproliferation such as psoriasis, contact dermatitis, and squamous cell carcinoma (SCC) suggesting considerable overlap in the signaling cascades. The development of a normal scar is dependent on the reversal of expres-

Supported by Cancer Research UK and the Queen Mary and Westfield Special Trustees.

Accepted for publication December 10, 2002.

Address reprint requests to Dr. A. Waseem, King's College London, Department of Craniofacial Development, Floor 28, Guy's Tower, London Bridge, London SE1 9RT UK. E-mail: ahmed.waseem@kcl.ac.uk.

sion of these genes at the wound site. However, in some cases the inflammatory and proliferative signals persist even after wound closure resulting in pathological scars, such as hypertrophic (HTS) and keloid scars. Although most previous studies have considered these scars as dermal phenomena,^{11,12} we and others have identified abnormalities associated with epidermal keratinocytes in HTS perhaps as a result of aberrant epidermal-mesenchymal interactions.^{13,14}

One of the most sensitive biochemical markers of terminal differentiation in keratinocytes is the keratin protein family that constitutes the major cytoskeletal architecture of all epithelia. In humans, the family consists of ~30 polypeptides (including trichocytic keratins of hair and nail) that are divided into two types; type I is acidic and includes K9 to K20; type II is basic/neutral and includes K1 to K8.^{15,16} Keratins are always expressed in pairs of type I and type II polypeptides in epithelia and undergo heterotypic association to form filaments. In stratified epithelia the basal keratinocytes express K5, K14, K19 (mucosal epithelia), and K15 as major keratins.^{17–19} In the suprabasal compartment the differentiating keratinocytes express different keratin pairs depending on the specific pathway of differentiation, for example, in skin the suprabasal keratinocytes express K1/K10, in buccal epithelia they express K4/K13, and in cornea they express K3/K12.¹⁷ Keratins K6, K16, and K17 are associated with hyperproliferation, such as during wound healing, psoriasis, HTS, and in various cancers.^{13,20–22} In the oral cavity the keratinized epithelia covering gingiva and hard palate express K1/K10 as the major keratins²³ and small amounts of these proteins are present in nonkeratinized epithelia as well.²⁴

Besides K1 and K10 there are two other keratins, K2 and K9, that are expressed in epidermis. K9 is a type I keratin that is expressed almost exclusively in palmo-plantar epidermis,^{25,26} whereas K2 is a type II keratin that was first identified in skin and oral masticatory epithelia as a polypeptide of molecular mass 65.5 kd.¹⁷ The identity of keratin K2 as an independent gene product remained elusive until 1992 when Collin and co-workers^{27,28} cloned the cDNA for this protein. They showed two different K2 genes; K2e, expressed predominantly in epidermis, and K2p, expressed in masticatory epithelia such as hard palate and gingiva. Unlike K1 and K10 that are expressed in the deepest suprabasal layer in epidermis, K2e is mostly localized in the upper spinous and granular layers suggesting that K2e is a marker of late epidermal differentiation.²⁸ The mouse homologue of K2e is a 70-kd protein that is expressed much more in murine epidermis than the human counterpart.^{29,30} The human K2e protein is expressed in prenatal fetal epidermis³¹ whereas synthesis of the murine homologue is induced only postnatally.³⁰ Mutations in this polypeptide have been associated with ichthyosis bullosa of Siemens that is characterized by disruption of keratin filaments and cell lysis in the spinous and granular layers.^{32,33}

An attractive approach to explore the molecular perturbation leading to an epidermal or an oral lesion is to investigate the expression of keratin genes in those lesions. A large body of data in the literature suggests that in cutane-

ous and mucosal lesions, keratinocytes take an alternative pathway of differentiation that is reflected in aberrant expression of keratin genes.^{34–37} How this altered pathway of differentiation at the onset and subsequent progression of a disease affects K2e expression is not known. This study describes the first study in which K2e expression has been systematically investigated along with the expression of other keratins in cutaneous and oral lesions. Our data suggest that expression of K2e is not exclusive to epidermis and given the appropriate signaling will be expressed where it is not normally expressed.

Materials and Methods

Antibodies and Tissue Samples

Mouse monoclonal anti-Ki67, MIB-1, and anti-keratin K10, RKSE 60 were obtained commercially from DAKO, UK and ICN, UK respectively. The rest of the anti-keratin antibodies used in this study were as follows: LHK1 for K1,³⁸ LL001 for K14,³⁹ LHK6 for K6, and LL025 for K16.¹³ These in-house monoclonal antibodies were obtained by culturing respective hybridomas in 10% fetal calf serum in Dulbecco's modified Eagle's medium and the supernatants from confluent cultures were stored in 0.2% (w/v) sodium azide until used in immunoassays. Biotinylated rabbit anti-mouse antibody and streptavidin-biotin horseradish peroxidase were purchased from DAKO, UK Ltd.

All cutaneous and oral tissue samples were snap-frozen in iso-pentane immediately after their surgical removal from patients and stored at -70°C until used. For long term storage (more than a year) samples were stored in liquid nitrogen. The protocols for the use of human tissues were approved by the local ethical committee. Pathological scars were distinguished clinically by their appearance and medical history, whereas HTS were confined to the boundary of original wound, keloids grew much larger, invaded healthy dermal tissues, and had a tendency to recur.

Frozen tissues were serially sectioned at 5 μm in a cryostat (Shandon, UK) and mounted onto slides. Sections for *in situ* hybridization were mounted onto slides coated with a 2% (v/v) solution of aminopropyl triethoxysilane (Sigma, UK), fixed for 30 minutes in 4% (w/v) paraformaldehyde (Sigma, UK), and stored at -70°C . Tissue sections for immunohistochemistry were left unfixed at the same temperature.

Production of LHK2e Monoclonal Antibody

The monoclonal antibody LHK2e was raised in mice against a synthetic peptide (NH₂-GEAFGSSVTF₃SFR-COOH) identical to the last 13 amino acids at the C-terminus of the K2e polypeptide. The same peptide has been used previously to generate a polyclonal antiserum against this polypeptide.³¹ For monoclonal antibody production BALB/c mice were immunized subcutaneously against thyroglobulin conjugate of the peptide as described previously.¹⁹ Tail bleeds were tested against a bovine serum albumin conjugate of the peptide using

enzyme-linked immunosorbent assays. Mice with the strongest response (minimum titer = 1/10,000 by enzyme-linked immunosorbent assay) were boosted intravenously 5 day before fusion. Spleen cells were fused with Sp2/O-Ag14 nonproducer myeloma cells using polyethylene glycol 6000 (Merck, UK) as described previously.⁴⁰ Hybridomas were grown in a selection medium containing azaserine and hypoxanthine and hybridoma specific for K2e were screened against the bovine serum albumin conjugate. The positive wells were further characterized by immunofluorescence on sections of normal human epidermis. The wells with the highest reactivity were cloned twice by limiting dilution and the supernatant was collected and stored in 0.2% (w/v) sodium azide at 4°C until required. The antibody was isotyped using a commercially available kit (Amersham Pharmacia Biotech, UK) and was found to be of IgM isotype. Because IgMs are known to be less stable and may give nonspecific reactivity after prolonged storage, we routinely stored LHK2e supernatant in small aliquots at -70°C until just before use.

Preparation of Keratin Proteins from Epidermis and Primary Keratinocytes

Keratin polypeptides were isolated from normal breast skin obtained after cosmetic surgery within 6 hours of tissue removal. Excess dermis and connective tissue were removed using a dermatome and the skin was cut into small pieces. The skin fragments were washed in Dulbecco's modified Eagle's medium and used to separate epidermis either by dispase or by trypsin. For dispase treatment the skin fragments were incubated in 20 ml of 2 U/ml dispase at 37°C for 2 to 3 hours or at 4°C for 16 hours and the epidermis was peeled off with a pair of tweezers and used for keratin extraction. For trypsin treatment, the skin fragments were incubated in 20 ml of 0.25 mg/ml trypsin in Dulbecco's modified Eagle's medium at 37°C for 2 hours with occasional gentle shaking. The suspension was filtered with a plastic net and the filtrate was split into two halves, A and B. Fraction A was spun and the pellet (keratinocytes) was used for isolation of keratins. Fraction B was cultured into two 75-cm² flasks in complete keratinocyte culture medium⁴¹ and the cells were used to extract keratins after they had achieved 90% confluence (after ~2 weeks).

Keratin polypeptides were extracted from epidermis and keratinocytes using the procedure published earlier.⁴² Briefly, the epidermis separated by dispase, keratinocyte pellet isolated by trypsin, or keratinocytes in monolayer were washed with phosphate-buffered saline (PBS) containing complete protease inhibitor cocktail (Roche, UK) in PBS. The cells were treated with 50 ml of low-salt extraction buffer (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 5 mmol/L ethylene glycol-bis (β -amino ethyl)-N,N,N',N'-tetraacetic acid (EGTA), 0.5% Triton X-100 supplemented with protease inhibitor) for 10 minutes on ice with occasional mixing. The pellet was recovered by centrifugation and extracted twice in 50 ml of high-salt

extraction buffer (1.5 mol/L NaCl with the low-salt buffer) for 20 minutes on ice with occasional sonication. The pellet was washed twice with PBS plus protease inhibitors and suspended in 3× sample buffer [60 mmol/L Tris-HCl, pH 8.0, 300 mmol/L dithiothreitol, 4.5% sodium dodecyl sulfate (SDS), 30% glycerol], incubated in a boiling water bath for 10 minutes and stored in aliquots at -20°C. Aliquots were diluted threefold with water before loading onto a 12% (w/v) SDS-polyacrylamide gel.

Polymerase Chain Reaction and cDNA Cloning

For preparation of the cRNA probe, a small fragment of the K2e cDNA (kindly provided by Dr. Langbein, Heidelberg, Germany) was amplified by polymerase chain reaction using a forward (GGAGGAGAATTCTCTGGAGGTGGAAAA-CACAGCTCT) and a reverse primer (GGGGGCGGATCCT-TATCTAAAAGAGAAGGTCACGCT). Either an *Eco*RI or a *Bam*HI site, shown in italics, was introduced in the forward and reverse primers, respectively, to facilitate cloning. The polymerase chain reaction was performed using thermostable PWO DNA polymerase (Roche Diagnostics, UK) as described before¹³ and the product, identified on a 2.2% (w/v) agarose gel, was cut with *Eco*RI and *Bam*HI and ligated to the corresponding sites of pGEM-4 (Promega, Madison, WI). The ligation mixture was transformed into *E. coli* Sure cells (Stratagene, La Jolla, CA), identified by restriction analysis and the insert confirmed by nucleotide sequencing. Of the six clones sequenced, five produced correct sequence whereas the sixth one had a six-base deletion. The confirmed pGEM-K2e construct contained a 150-bp insert from nucleotide 1822 to 1971 of the K2e cDNA.²⁸

Synthesis of Digoxigenin-Labeled Riboprobes

Anti-sense and sense riboprobes for K2e were prepared from a cDNA clone containing the keratin insert. Riboprobes for K2e were synthesized by *in vitro* transcription for 2 hours at 37°C with digoxigenin-11-dUTP (Roche Diagnostics, UK) and an RNA polymerase (T7 or SP6, Promega) as described previously.¹⁹

Nonradioactive in Situ Hybridization

The procedure for *in situ* hybridization is explained in detail elsewhere,²⁴ and only summarized here. Tissue sections prefixed with paraformaldehyde were washed for 5 minutes in PBS and 100 mmol/L of glycine, and then pretreated for 10 minutes at 37°C with 1 μ g/ml of proteinase K. After postfixing in 4% paraformaldehyde, sections were incubated for 10 minutes at 45°C in 50% formamide and 4× standard saline citrate. Next, 2 ng/ μ l of digoxigenin-labeled riboprobe was applied onto each section in buffer containing 50% formamide, 4× standard saline citrate, 10 mmol/L dithiothreitol, 10% dextran sulfate, 5× Denhardt's solution, 500 μ g/ml yeast t-RNA, and 500 μ g/ml salmon sperm DNA. Target and probe RNA were denatured for 7 minutes at 65°C and the sections hybridized for 18 hours at 45°C in a controlled chamber (Hy-

bailed, UK). Unbound probe was removed with a 30-minute wash at 52°C in 50% formamide and 2× standard saline citrate, followed by a 30-minute incubation at 37°C with 50 µg/ml ribonuclease A and 30 minutes at 52°C in 50% formamide and 0.1× standard saline citrate. Sections were washed for 5 minutes in buffer I (100 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 8.0), blocked for 30 minutes in 10% sheep serum, and incubated for 2 hours with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche Diagnostics, UK). Excess antibody was removed with two 10-minute washes, first in buffer I followed by buffer II (100 mmol/L Tris-HCl, 100 mmol/L NaCl, and 50 mmol/L MgCl₂, pH 9.5). Alkaline phosphatase activity was developed for 2 hours with 350 µg/µl of nitro blue tetrazolium chloride and 175 µg/µl of 5-bromo-4-chloro-indoyl-phosphate. Finally, sections were washed for 30 minutes in buffer III (10 mmol/L Tris-HCl and 1 mmol/L ethylenediaminetetraacetic acid, pH 8.0) and mounted in aqueous mounting fluid (Raymond Lamb, UK). All expression data by *in situ* hybridization produced intense specific cytoplasmic staining.

Immunocytochemistry

The indirect immunohistochemical staining method was used to detect keratin polypeptides with mouse monoclonal antibodies. For K1 and K10, antibodies included LHK1³⁸ and RKSE60,⁴³ respectively, and for K2e, LHK2e was used. Tissue sections were fixed either in acetone or in a mixture of acetone and methanol (1:1) for 10 minutes at 4°C, incubated for 60 minutes with monoclonal antibody (1:10 for K1 and K10; neat supernatant for K2e) followed by 30 minutes in biotinylated rabbit anti-mouse antibody (1:300) and another 30 minutes in streptavidin-biotin horseradish peroxidase (1:50). Sections were developed for 2 minutes with diaminobenzidine as chromogen substrate (DAKO Ltd.), counterstained with hematoxylin, and mounted in a xylene-based mountant (BDH-Merck, UK).

Formalin-fixed and paraffin-embedded tissue blocks were cut into 5-µm sections, dewaxed by incubating twice in xylene for 5 minutes each. The sections were incubated three times in absolute ethanol followed by a 5-minute incubation in 90% ethanol followed by distilled water and PBS. Before immunohistochemical staining, antigen retrieval was performed by microwave oven in citrate buffer, pH 6.0, for 10 minutes at 800 W. The endogenous tissue peroxidase activity was blocked with 3% H₂O₂ (v/v) in 50% methanol. The nonspecific protein binding was blocked with a 1:5 dilution of normal serum from the animal species of the secondary antibody for 30 minutes and the sections were incubated with primary and secondary antibodies and color developed as described above for the cryosections.

Control Experiments

Positive tissue controls for K2e, K1, and K10 included normal skin and attached gingiva, whereas negative con-

Figure 1. Immunoreactivity of keratin polypeptides with keratin monoclonal antibodies. Human epidermis from breast skin was removed either by treatment with dispase (**lane 1**) or keratinocytes isolated by treatment with trypsin (**lane 2**) and cultured until they became confluent (**lane 3**). Keratin polypeptides were isolated from these samples as described in the Materials and Methods section and analyzed on SDS polyacrylamide gels. The protein profile as determined by Coomassie brilliant blue staining is shown in the CBB panel. The polypeptides were transferred onto nitrocellulose membranes and probed for keratins K14 (LLOO1), K6 (LLO25), K2e (LHK2e), K10 (RKSE60), and K1 (LHK1).

trols included buccal mucosa. The specificity of *in situ* hybridization for K2e was determined with a digoxigenin-labeled sense riboprobe. The specificity of immunohistochemistry staining was determined by substituting the primary antibody with PBS or Sp2/O-Ag14 supernatant or an irrelevant antibody of the same subclass.

Other Methods

SDS-polyacrylamide gel electrophoresis of keratin polypeptides and their immunodetection by Western blotting was performed as described elsewhere.^{44,45} Restriction digestion of plasmid DNA, ligation, and bacterial transformation was performed using standard procedures.⁴⁶ Nucleotide sequencing was conducted on an ABI automatic sequencer using cycle sequencing protocol provided by the manufacturer. All pictures were scanned and assembled using Adobe PhotoShop version 6.01.

Results

The Monoclonal Antibody LHK2e (IL39) Reacts Specifically with the Human Keratin K2e Polypeptide

The monoclonal antibody against keratin K2e was originally named IL39 but to distinguish it from other anti-keratin antibodies and to be consistent with the terminology used in this laboratory we have changed its name to LHK2e. Although the antibody has been used previously,⁴⁷ its specificity and reaction characteristics have not been established. In this study we report its production and reactivity with the K2e polypeptide on blots and tissue sections.

Using cell sorting we established that most hybridoma cells in the positive clone were producing IgM and the number of IgG producers were either none or undetectable (not shown). The specificity of LHK2e was tested by Western blotting using keratin polypeptides extracted from epidermis and primary cultured keratinocytes. The SDS-polyacrylamide gel electrophoresis profile of keratin polypeptides extracted from the epidermis, stripped either with dispase or with trypsin, was identical (Figure 1,

compare lanes 1 and 2 in the CBB panel). However, as expected the high-molecular weight protein bands containing primarily keratin K1 and K2e polypeptides were absent in the keratins extracted from the primary keratinocytes (Figure 1, compare lanes 1 and 2 with lane 3 in the CBB panel). The reactivity of these keratin polypeptides with antibodies directed against keratins K14, K6, K1, K10, and K2e is shown in Figure 1. Every keratin antibody reacted only with a single polypeptide with no sign of proteolysis and the molecular size corresponded exactly to the size of its antigen reported in the literature.⁴⁸ As expected, keratin K14 was detected in the cytoskeletal proteins isolated from epidermis as well as from primary keratinocytes. The keratin K6 polypeptide was detected in keratinocytes but not in epidermis and keratins K1 and K10 were detected only in epidermis but not in primary keratinocytes. LHK2e reacted with a single polypeptide of ~66 kd extracted from epidermis either by trypsin or dispase but not in primary keratinocytes.

To test whether LHK2e was species-specific, we reacted the antibody with cryosections derived from rat, mouse, pig, and bovine skin using immunoperoxidase staining. As predicted, we observed no reactivity of LHK2e with any other species that we tested (not shown). Although we have described only the use of cryosections in this study we observed LHK2e reactivity with formalin-fixed paraffin-embedded sections after antigen microwave retrieval (not shown).

Variable Expression of Keratin K2e in Normal and Hyperproliferating Epidermis of Psoriasis, HTS, and Keloid Scars

In almost all normal cases K1 and K10 was seen in the deepest suprabasal layer whereas K2e distribution was variable. In most body sites, including abdomen, arm, leg, and breast, we observed K2e expression in the spinous and granular layers, which is consistent with a marker of late keratinocyte differentiation (Figure 2E). However, in facial skin we observed K2e in all layers above the basal layer (Figure 2F). Furthermore, contrary to the reported absence of K2e in penile epidermis,²⁸ we observed specific suprabasal expression in foreskin that was patchy and discontinuous (Figure 2G). In foot and sole epidermis K2e expression was weak or absent in the cornified layer but expression was detectable below the cornified layer (Figure 2H).

We investigated the expression of keratins K2e, K1, and K10 in five different cases each of hyperproliferating epidermis including psoriasis, HTS, and keloid scars (Table 1). Keratinocyte activation and proliferation in these samples was investigated by the expression of keratin K6 and K16 pair and Ki67, respectively. Expression of Ki67 was clearly stronger than normal and spread to the suprabasal layers of most lesional epidermis (compare Figure 2B with Figure 3, F and J) indicating hyperproliferation of keratinocytes. In all hyperproliferating epidermis, we observed strong expression of K1 throughout suprabasal layers (Figure 3; A, G, and K) and a similar pattern was seen for keratin K10 (Table 1). The hyperproliferation

Figure 2. Expression of keratin polypeptides and Ki67 in normal epidermis from different body sites. Histological appearance of normal abdominal epidermis (A) and a few Ki67-positive cells restricted to the normal basal layer in an adjacent section (B). In the same specimen, protein for K1 (C) and K10 (D) are homogeneously expressed throughout the suprabasal compartment whereas expression of K2e protein is restricted to the upper suprabasal layers (E). However, in a sample of facial skin K2e expression appears to begin in the deepest suprabasal layer (F). In penile epidermis K2e shows sporadic and patchy expression (G) whereas in foot arch epidermis K2e is restricted to layers immediately beneath the stratum corneum (H). Original magnifications: $\times 115$ (A–F); $\times 57$ (G, H).

marker K16 was also strongly expressed homogeneously throughout all psoriatic samples except in the basal layer (Figure 3B), whereas in HTS it was strong but heterogeneous (Figure 3I). Keratin K6 expression was similar to K16 in psoriatic and HTS samples, except in some cases it was weaker than K16, and was present in most upper suprabasal layers (Figure 3C). In the epidermis of keloid scars, however, we observed little or no expression of K6 and modest expression of K16 (Table 1), suggesting that although the epidermis was hyperproliferating there was little or no activation of keratinocytes. Staining with LHK2e showed expression in suprabasal layers but there was a clear down-regulation in psoriasis and HTS (Figure 3D and Table 1). In some psoriatic samples where K2e was strongly expressed we found little or no expression in keratinocytes above the dermal papillae (Figure 3E). However, in the epidermis of HTS and keloid scars, K2e was expressed homogeneously throughout all suprabasal layers right from the deepest layer (Figure 3, H and L). In some HTS samples, K2e expression was much stronger than in others, whereas in keloids it was mostly strong (Table 1). In addition we analyzed five different formalin-fixed, paraffin-embedded samples of psoriatic

Table 1. Expression of Epidermal Keratins in Cutaneous Lesions*

Case no.	Disease [‡]	Site [†]	Keratin protein expression in suprabasal compartment					
			K1	K10	K6	K16	K2e	Ki67
C-1	NS	Abdomen	+++	+++	—	—	+++	+/-, B & EB
C-2	NS	Arm	+++	+++	—	—	++	+/-, B & EB
C-3	NS	Arm	+++	+++	—	—	++	+/-, B & EB
C-4	NS	Leg	+++	+++	+/-	+/-	++	+/-, B & EB
C-5	NS	Leg	+++	+++	—	+/-	++	+/-, B & EB
C-6	Psoriasis	Abdomen	++	++	+++	+++	++	++, B & SB
C-7	Psoriasis	Arm	++	+++	++	+++	+/-	++, B & SB
C-8	Psoriasis	Arm	+++	+++	++	+++	++	++, B & SB
C-9	Psoriasis	Leg	+++	+++	+++	+++	++	++, B & SB
C-10	Psoriasis	Leg	+	++	+++	+++	+	++, B & SB
C-11	HTS	Abdomen	+++	+++	+++	+++	+	+, mostly B
C-12	HTS	Abdomen	+++	+++	+++	+++	+/-	+, mostly B
C-13	HTS	Arm	+++	+++	++	++	+++	+, mostly B
C-14	HTS	Arm	+++	+++	++	++	+++	+, B & EB
C-15	HTS	Leg	+++	+++	+++	+++	+	+, mostly B
C-16	Keloid	Abdomen	+++	+++	+/-	++	+++	+, B & EB
C-17	Keloid	Abdomen	+++	+++	+/-	+/-	++	+, B & EB
C-18	Keloid	Neck	+++	+++	+/-	+++	+++	+, B & EB
C-19	Keloid	Arm	+++	+++	—	—	++	+, mostly B
C-20	Keloid	Leg	+++	+++	—	—	+	+, mostly B
C-21	BCC	NK	—	+/-	ND	ND	+/-	ND
C-22	BCC	NK	+/-	+/-	ND	ND	—	ND
C-23	SCC	NK	+/-	++	ND	ND	+/-	ND
C-24	SCC	NK	—	+/-	ND	ND	—	ND
C-25	SCC	NK	—	+/-	ND	ND	—	ND

*All frozen samples used in this study are listed here to highlight individual variations.

[†]Normal skin samples were site matched as far as possible.

[‡]Expression in normal skin of other body sites is given in Figure 2.

NS, normal skin; B, basal; EB, epibasal; SB, suprabasal. Immunoreactivity was absent (—), <5% cells positive (+/-), 10 to 30% cells positive (+), 40 to 70% cells positive (++), 80 to 100% cells positive (+++). NK, not known; ND, not determined.

epidermis for the expression of K1, K10, and K2e and the overall pattern was very similar to that observed with frozen sections (not shown).

Keratin K2e Expression Is Down-Regulated in Cutaneous SCCs and Basal Cell Carcinomas (BCCs)

Two BCC and three SCC samples were used to study expression of keratins K1, K10, and K2e by immunocytochemistry. In both BCCs the keratinocytes at the center of tumor islands were positive for K1 and showed weak sporadic staining for K10 and no reactivity for K2e (not shown). In cutaneous SCCs the tumor islands showed stronger reactivity for K10 compared with K1 and K2e (Table 1). The overlying epidermis both in BCCs and SCCs was reactive with antibodies specific for these keratins. In five cases each of formalin-fixed, paraffin-embedded BCCs and SCCs, we observed a pattern very similar to that described for frozen tissues (not shown).

Low-Level Expression of Keratin K2e in Normal Oral Epithelia

We determined expression of keratin K2e along with K1 and K10 in cryosections of keratinizing and nonkeratinizing oral tissues including gingiva, tongue, and floor of the mouth (Table 2). Strong expression of K1 and K10 in

keratinizing layers of essentially normal gingiva (clinical samples with mild degree of inflammatory hyperplasia) was observed (Figure 4, B and C). However, the K2e expression in gingiva was very weak and spread throughout the suprabasal layers (Figure 4D). In lateral border of the tongue the expression of K1 was more widespread than K10 (Figure 4, G and H). The K1 expression was stronger than K10 and extended into the rete-ridges and occasionally even to the basal keratinocytes. The K10 expression on the other hand was mainly restricted at interpapillary junctions (Figure 4H). The K2e expression in the tongue epithelia was very weak and restricted to the keratinizing suprabasal epithelia (Figure 4F). In epithelium from buccal mucosa and floor of mouth, we observed weak expression of K1 and K10 but none for K2e (not shown). Although we observed little or no protein expression in oral epithelia, the K2e mRNA expression was widespread in the basal and suprabasal layers (Figure 4E).

K2e Expression Is Up-Regulated in Oral Hyperkeratotic Lesions but Not in Oral Lichen Planus

Hyperkeratinized lesions in oral epithelia are associated with smoking and exposure to other genotoxic agents. Lichen planus is a chronic inflammatory condition typified by a change in keratin expression induced by basal cell destruction and that carries a small risk of developing into

Figure 3. Expression of keratin polypeptides in cutaneous hyperproliferative lesions. Sections from frozen psoriatic, HTS, and keloid scar epidermis were stained with different keratin and Ki67 antibodies. In psoriatic epidermis, K1 (**A**) and K16 (**B**) show homogeneous distribution in the suprabasal compartment whereas K6 is heterogeneously expressed (**C**) and K2e is either down-regulated (**D**) or heterogeneously expressed with little or no expression above dermal papillae (**E**). Increased proliferation compared to normal epidermis was demonstrated by Ki67 staining. **F:** In HTS epidermis the expression of K1 (**G**) and K2e (**H**) was uniformly distributed throughout the suprabasal compartment, K16 is heterogeneously expressed (**I**), and Ki67 was localized in basal and epibasal layers (**J**). In keloid scar epidermis keratins K1 (**K**) and K2e (**L**) were primarily homogeneously expressed in the suprabasal compartment. Original magnifications: $\times 78$ (**A–E**); $\times 155$ (**F–L**).

SCC.⁴⁹ We investigated keratins K1, K10, and K2e expression in six hyperkeratotic lesions and five lichen planus samples taken from oral mucosa, tongue, and gingiva (Table 2). In most hyperkeratotic lesions K1 and K10 were strongly expressed, with intense reactivity in columns of keratinocytes located over the connective tissue papillae (Figure 5, A and B). The K2e expression in these lesions was observed only in keratinocytes situated underneath the orthokeratinized layer (Figure 5D). As shown for normal oral epithelia, K2e mRNA was strongly expressed in basal and suprabasal layers of the hyperkeratotic lesions (Figure 5C).

In oral lichen planus the keratin expression was very different in areas of active basal cell destruction from inactive areas. In active areas, low levels of K1 but no

K2e was detectable (Figure 5, E and F). However, inactive areas of lichen planus expressed very high levels of K1 and K10 (Figure 5, G and H) without significant expression of K2e (not shown).

Expression of Keratin K2e Is Up-Regulated in Oral Dysplasia

Oral dysplasia is a premalignant condition that is often accompanied by keratinization. Histologically the condition can be classified into mild, moderate, and severe depending on the level and extent of morphological changes. In mild, moderate, and moderate-to-severe dysplasia, expression of K1 and K10 was induced

Table 2. Expression of K1, K2e, and K10 in Normal and Diseased Oral Epithelia*

Case no.	Disease	Site	Expression of keratin proteins in suprabasal compartment or tumour islands		
			K2e	K1	K10
O-1	Normal	Buccal mucosa	—	+/-	—
O-2	Normal	Buccal mucosa	—	+	+
O-3	Normal	Ventral tongue	+/-	+	+
O-4	Normal	Ventral tongue	+/-	+/-	+
O-5	Normal	Gingiva	+/-	+	++
O-6	Normal	Gingiva	+	+	+
O-7	Keratosis	Buccal mucosa	—	++	+
O-8	Keratosis	Buccal mucosa	+/-	++	++
O-9	Keratosis	Buccal mucosa	+/-	++	+
O-10	Keratosis	Lingual mucosa	+	+++	+++
O-11	Keratosis	Dorsal tongue	+	+	+
O-12	Keratosis	Lateral tongue	—	+/-	—
O-13	Lichen planus	Buccal mucosa	—	+	++
O-14	Lichen planus	Buccal mucosa	—	++	++
O-15	Lichen planus	Lingual mucosa	—	+/-	+/-
O-16	Lichen planus	Gingiva	—	+	+
O-17	Lichen planus	Gingiva	—	++	++
O-18	Mild dysplasia	Verical tongue	+/-	++	++
O-19	Mild dysplasia	Verical tongue	+++	+++	+++
O-20	Moderate dysplasia	Buccal mucosa	+/-	+/-	++
O-21	Moderate dysplasia	Floor of mouth	—	+	+
O-22	Moderate dysplasia	Labial mucosa	+/-	+	+
O-23	Severe dysplasia	Lateral tongue	+/-	+++	+++
O-24	Severe dysplasia	Soft palate	+/-	++	++
O-25	WDSCC	Gingiva	—	++	—
O-26	WDSCC	Alveolar ridge	+/-	+++	++
O-27	MDSCC	Alveolar ridge	—	+/-	+/-
O-28	MDSCC	Floor of mouth	—	—	+/-
O-29	PDSCC	Ventral tongue	—	—	—
O-30	PDSCC	Lateral tongue	—	—	—
O-31	PDSCC	Floor of mouth	—	—	+/-

*To show individual variations in keratin gene expression for a clinical situation all cases used in this study are listed here.

SCC, Squamous cell carcinoma; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; B, basal and LS lower 1/3 of the suprabasal compartment (2 to 5 layers). No immunostaining for protein (—), <5% cells positive (+/-), 10 to 30% cells positive (+), 40 to 70% cells positive (++), 80 to 100% cells positive (+++).

strongly in the upper suprabasal layers (Figure 6; A, B, E, F, and J). The K2e protein was barely detectable in mild dysplasia but the mRNA was strongly expressed (Figure 6, C and G). In samples histologically classified as moderate dysplasia, K2e expression was very strong in areas underneath the orthokeratinized layer (Figure 6I). However, in adjacent areas where the tissue was thin and parakeratinized there was little or no expression of K2e (Figure 6H). The expression of K1 (Figure 6, E and F) and K10 (not shown) in ortho- and parakeratinized oral dysplasia mirrored the expression seen for K2e, raising the possibility that a signaling mechanism conducive for the expression of these keratins might be active in keratinocytes located underneath the orthokeratinized layers. None of the antibodies used for K1, K10, or K2e reacted with the orthokeratinized layer of the lesional epithelia, perhaps because of masking of reactive epitopes caused by extensive cornification. In transitional regions, histologically described as moderate-to-severe dysplasia, K2e expression was induced in suprabasal keratinocytes but not to the extent observed in orthokeratinized areas (compare Figure 6, L and I).

Absence of K2e Expression in Oral SCCs Positive for K1 and K10

In poorly differentiated SCCs we did not observe detectable levels of K1, K10, or K2e expression (Table 2).⁵⁰ However, in well-differentiated oral SCCs these proteins were detectable by immunocytochemistry albeit at different levels. In some oral SCCs, K1 and K10 were completely absent but in others these proteins were expressed at high levels in keratinized areas at the center of tumor islands (Figure 7; A, B, and C). Most oral SCCs were negative for LHK2e and only very weak and sporadic expression was observed rarely (Figure 7, D and E). However, consistent with our previous data on K1 and K10,⁵⁰ the mRNA for K2e was still expressed in oral SCCs (Figure 7F).

Discussion

In this report we have investigated the expression of K2e and other differentiation-specific keratin genes, including K1, K10, K6, and K16 in cutaneous and oral lesions

Figure 4. Expression of keratins K1, K10, and K2e in normal oral epithelia. Histological appearance of essentially normal gingiva showing parakeratinization (A). In adjacent sections, expression of K1 (B) and K10 (C) is restricted to the upper suprabasal compartment whereas K2e is absent (D). In the ventral surface of tongue, transcript for K2e (E) shows a much wider suprabasal distribution than its protein, which is localized to isolated cells (F). Proteins for K1 (G) and K10 (H) are present in columns of keratinocytes over the connective tissue papillae, although K1 is more widely distributed. Original magnifications, $\times 50$.

Figure 5. Expression of keratins K1, K10, and K2e in hyperkeratotic lesions and oral lichen planus. In hyperkeratotic lesions, K1 is homogeneously distributed throughout the suprabasal layers (A) whereas expression of K10 is restricted to cells over connective tissue papillae (B) and mRNA for K2e is uniformly expressed in the basal and lower suprabasal layers (C). K2e protein was expressed mostly in cells underneath the orthokeratinized layer (D). In an active area (lymphocytes in epithelium, apoptosis, atrophy; visible only at higher magnification) of oral lichen planus, K1 protein is sporadically expressed in the suprabasal compartment (E) whereas K2e was absent (F). In inactive areas (minimal lymphocytic infiltration and apoptosis, epithelium normal or acanthotic), K1 shows strong but heterogeneous suprabasal distribution (G) whereas K10 is uniformly expressed in the same compartment (H). Original magnifications: $\times 25$ (A, B); $\times 50$ (C-H).

where keratinocytes are activated, become hyperproliferative or undergo keratinization. The influence of keratinocyte activation and hyperproliferation on keratin gene expression was investigated in epidermis derived from patients with psoriasis, HTS, and keloid scars and the effect of keratinization was studied in oral epithelia, which are prone to keratinization in response to disease-specific signaling. Keratinocyte activation involves autocrine and paracrine signaling, allowing them to become hyperproliferative, migratory, alter their cytoskeleton, augment synthesis of extracellular matrix and cell surface receptors. These changes are necessary to enable keratinocytes to accelerate wound closure.

The specificity of the monoclonal antibody LHK2e for keratin K2e was evaluated by Western blotting using keratin polypeptides isolated from epidermis and cultured keratinocytes. The absence of K1 and K10 in keratin extract isolated from cultured keratinocytes is in contrast with previous reports in which these proteins are reportedly expressed in differentiating keratinocytes.^{38,51} However, it is possible that low levels of K1 and K10 in cultured keratinocytes were washed out during high-salt extraction as reported for SCC cell lines.⁵² The reactivity of LHK2e on blots suggested that K2e is expressed in epidermal keratinocytes but down-regulated as they begin to grow in culture, consistent with previous reports.^{28,29} This antibody also did not react with K2e of any other species tested suggesting that it is highly specific for human K2e and could be used in transgenic experiments.

Keratin Expression in Activated Epidermal Keratinocytes

Our data suggest that although psoriatic and HTS epidermis strongly express keratins K6 and K16, the specific markers of keratinocyte activation, the keratinocytes in the two lesions differed in keratin K2e expression. Although keratins K6 and K16 were expressed homogeneously throughout the psoriatic epidermis, K2e and K1 expression was heterogeneous in most cases (four of five), in some cases with little or no expression in keratinocytes located above dermal papillae (Figure 3E), indicating a different pathological state. Furthermore, in some cases K2e expression was barely detectable in the involved region (Figure 3D and case C-7 in Table 1). Based on these observations, we propose that psoriatic keratinocytes exist in two levels of activation: those located above the dermal papillae are most activated and the remainder is mild to moderately activated. This is consistent with a previous report in which the existence of different types of keratinocyte activation has been proposed.¹⁰ These types cannot be distinguished by the expression of K6 and K16 but can be distinguished by K2e expression because the most activated keratinocytes do not express K2e whereas mild to moderately activated cells do. The exact role of K2e in epidermis is not known but mutations in this gene cause ichthyosis bullosa of Siemens, a dominant-negative disease characterized by skin blistering and mild epidermolytic hyper-

Figure 6. Expression of keratins K1, K10, and K2e in oral dysplasia. In mild oral dysplasia, K1 is homogeneously expressed in suprabasal layers (A) whereas K10 shows patchy expression mostly as columns of keratinocytes located over connective tissue papillae (B), K2e mRNA is uniformly expressed in the basal and lower suprabasal layers (C) in the presence of little or none of its protein (D). In moderate dysplasia, protein expression of K1 was weak and heterogeneous in parakeratinized region (E) but in the adjacent orthokeratinized region K1 expression was strong and homogeneous (F). K2e mRNA expression was detected in the para- and orthokeratinized regions (G) but protein expression was weaker in the parakeratinized region (H) compared with the orthokeratinized (I). In moderate/severe dysplasia, K1 was mostly localized in the upper suprabasal layers (J) and K2e mRNA was detectable in basal and suprabasal layers (K) but the protein was only expressed focally in the upper suprabasal keratinocytes (L). Original magnifications: $\times 160$ (A–I); $\times 80$ (J–L).

keratosis.³³ This suggests that K2e may be important for the stabilization of keratin cytoskeleton in epidermis and therefore down-regulation of this gene in epidermal lesions, associated with strong keratinocyte activation, is likely to cause local cutaneous fragility.

In three HTS cases, 80 to 100% suprabasal keratinocytes expressed K6/K16 and only 10 to 30% were positive for K2e. However, in two HTS and two keloid cases, we observed a high level of K2e expression along with K16 and/or K6 suggesting involvement of factors other than keratinocyte activation in the regulation. One striking difference was the expression of K2e in the deepest suprabasal layer in HTS and keloid compared with the late expression observed in normal and psoriatic epidermis. In a previous study we also reported precocious

expression of filaggrin in HTS samples.¹³ This taken together with our present observations suggest changes in signaling leading to induction of early differentiation in HTS and keloid scars. In three keloid cases, virtual absence of K6 and K16 indicated that keratinocytes, although hyperproliferating as shown by Ki67 expression, were not activated and K2e in these samples continued to be expressed at high levels (Table 1). Although the basis for this up-regulation in some HTS and keloid scars is not known, it may be linked to increased expression of transforming growth factor- β in HTS and keloid scars, which also express higher levels of K5 and K14 compared with normal epidermis.⁵³

The pattern of keratin K1 and K10 expression in BCCs and SCCs was consistent with that reported previous-

Figure 7. Expression of keratin polypeptides in oral SCCs. In a well-differentiated oral SCC protein expression for K1 (A) and K10 (B, C) was detected within tumor islands. The K2e expression in these samples was weak or undetectable (D, E) although mRNA was still transcribed in most cells of the tumor islands (F). Original magnifications, $\times 50$.

ly.^{34,35} In BCCs, K2e was absent in tumor islands, which is not surprising as K2e is not expressed in basal keratinocytes. The down-regulation of K2e in SCCs could occur as a result of keratinocyte activation as well as because of the acquisition of embryonic phenotype, characterized by the expression of K8 and K18.^{34,44} In most cutaneous tumors we observed suppression of K1 and K10 expression to be less marked than K2e, suggesting differential regulation of these genes. Recent studies have shown that expression of K10 inhibits cell proliferation and suppresses tumor development,^{54,55} and could explain a requirement for its down-regulation in keratinocyte transformation. However, why keratinocytes might down-regulate K2e in carcinomas is not clear. It is conceivable that the molecular changes brought about to suppress K10 expression are not compatible with K2e expression.

Among the factors influencing epithelial differentiation, retinoids are the most extensively investigated. The effects of retinoids on epidermis differ widely from those on cultured keratinocytes. *In vitro* retinoic acid is known to suppress expression of differentiation markers including filaggrin,⁵⁶ transglutaminase,⁵⁷ loricrin,⁵⁸ and keratins K1 and K10.⁵⁹ However, exposure of epidermis to retinoic acid does not change expression of keratin K1 and K10 in human⁶⁰ and murine⁶¹ epidermis. No data on the influence of retinoic acid on *in vitro* expression of K2e are yet available. However, recent studies on human volunteers showed that retinoic acid can suppress K2e transcription by 100- to 1000-fold,⁴⁷ although down-regulation was not evident at the protein level perhaps because of slow turnover of the polypeptide. Exposure to retinoids also induces expression of K6 and K16, markers of the activated phenotype.^{60,61} Given these observations, it is tempting to speculate that an endogenous retinoid imbalance may partially contribute to keratinocyte activation

and down-regulation of K2e. A gradient of retinoic acid with the highest level in the basal layer and declining to much lower levels in suprabasal layers has been proposed in epidermis.⁶² This would potentially suppress K2e gene expression in lower suprabasal layers.

Expression of Keratin K2e in Keratinizing Oral Epithelia

This is the first study in which K2e expression has been shown to be induced in oral lesions. In 27 cases investigated we found up-regulation of K2e in most hyperkeratotic lesions and dysplasia, albeit to varying degrees. The K2e mRNA was also induced in all cases in the basal and suprabasal layers. This observation is consistent with our previous data in which transcription of epidermal keratins has been shown in basal keratinocytes of oral epithelia.²⁴ As reported previously, keratins K1 and K10 are also induced in these lesions⁶³ but the extent of K2e induction was invariably higher in dysplasia than in simple hyperkeratotic lesions. The level of K1, K10, and K2e expression showed strong dependence on the type and degree of keratinization. In parakeratinized dysplasia these keratins were induced but to a very low extent. On the other hand, in orthokeratinized dysplasia these keratins were strongly induced in upper spinous to granular layers. The dependence of K1, K10, and K2e expression on ortho- versus parakeratinization seems to rely on the degree of keratinization; parakeratinized oral epithelia are thin and lightly keratinized and show weak expression compared with the orthokeratinized epithelia. Whether the lesional changes in oral epithelia lead to induction of epidermal keratin genes or vice versa is not clear. However, a recent study has found that overexpression of K10, a minor component of normal buccal mucosa,²⁴ causes widespread abnormality in oral mucosa indicating alterations in biological behavior of oral keratinocytes expressing high levels of epidermal keratins.⁶⁴ Although the gene for human K2e has been cloned and characterized,⁶⁵ the *cis*-regulatory elements and transcription factors required for its regulation have not been identified. However, the data presented in this study suggest that K2e expression, unlike K9, requires a specific signaling cascade that seems to be independent of the keratinocyte origin. For example, expression of K2e in oral dysplasia and hyperkeratotic lesions shows that induction of K2e is not an intrinsic feature of the epidermal keratinocyte but, given the right signals, this gene can be induced in epithelia in which it is not normally expressed. Nevertheless, the molecular interactions that suppress K2e in normal oral epithelia and the perturbations that lead to its induction in oral lesions remained to be determined.

Oral dysplasia is a premalignant lesion but some cases respond to treatment or revert spontaneously and therefore it is desirable to define molecular changes that would allow pathologists to identify the highest risk lesions. Although the significance of K2e induction in oral dysplasia remains to be elucidated, the observation offers an opportunity to decipher the molecular changes underlying the onset of cellular transformation. Retinoids are known to be important for the normal function of

mucosal epithelia, and dietary deficiency can lead to widespread metaplasia in rats and humans.⁶⁶ It is therefore conceivable that localized retinoid deficiency in certain clinical situations, such as oral dysplasias and hyperkeratotic lesions, might provide the molecular environment permissive for the induction of K2e gene.

In conclusion, we have studied expression of several keratins including K1, K10, K6, K16, and K2e in cutaneous and oral lesions. Using a monospecific monoclonal antibody, LHK2e, we have shown that K2e, which is normally expressed late during epidermal differentiation, begins to be expressed in the deepest suprabasal layer of HTS and keloid scar epidermis. This gene is sensitive to keratinocyte activation and in markedly activated keratinocytes of psoriatic samples K2e appears to be down-regulated. However, a mild-to-moderate level of activation does not influence K2e expression and appears to up-regulate K2e in some keloid and HTS cases. In oral epithelia no significant expression of K2e was detected in buccal mucosa, gingiva, and tongue whereas other differentiation-specific keratins (K1 and K10) were highly expressed. In lesions of inactive oral lichen planus, we observed induction of K1 and K10 but not K2e. However, we observed large increases in the expression of all of the three keratins in oral dysplasia and hyperkeratotic lesions. The up-regulation was strongly dependent on the degree of keratinization and was highest in orthokeratinized oral epithelia. These observations suggest that K2e expression is not exclusive to epidermis and given an appropriate signaling cascade, this gene can be induced in tissues in which it is not normally expressed.

Acknowledgments

We thank Mrs. Patricia Purkis for helping us with initial immunostaining experiments on paraffin and frozen tissue sections; Dr. L. Langbein for the full-length K2e cDNA clone; Dr. Andrew Yeudall for helpful suggestions on the manuscript; and Ian Goldsmith at Cancer Research UK for supplying synthetic oligonucleotides used in this study.

References

- Hotchin NA, Gandarillas A, Watt FM: Regulation of cell surface beta 1 integrin levels during keratinocyte terminal differentiation. *J Cell Biol* 1995, 128:1209–1219
- Adams JC, Watt FM: Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes alpha 5 beta 1 integrin loss from the cell surface. *Cell* 1990, 63:425–435
- Watt FM, Kubler MD, Hotchin NA, Nicholson LJ, Adams JC: Regulation of keratinocyte terminal differentiation by integrin-extracellular matrix interactions. *J Cell Sci* 1993, 106:175–182
- Fuchs E: Keratins and the skin. *Annu Rev Cell Dev Biol* 1995, 11:123–153
- Eckert RL, Crish JF, Robinson NA: The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiol Rev* 1997, 77:397–424
- Squier CA, Johnson NW, Hopps RM: Human Oral Mucosa: Development, Structure and Function. Oxford, Blackwell Scientific Publications, 1976, pp 7–44
- Martin P: Wound healing—aiming for perfect skin regeneration. *Science* 1997, 276:75–81
- Clark RAF: Mechanisms of Cutaneous Wound Healing. New York, McGraw-Hill, 1993
- Coulombe PA: Towards a molecular definition of keratinocyte activation after acute injury to stratified epithelia. *Biochem Biophys Res Commun* 1997, 236:231–238
- Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M: Keratins and the keratinocyte activation cycle. *J Invest Dermatol* 2001, 116: 633–640
- Akagi A, Tajima S, Ishibashi A, Yamaguchi N, Nagai Y: Expression of type XVI collagen in human skin fibroblasts: enhanced expression in fibrotic skin diseases. *J Invest Dermatol* 1999, 113:246–250
- Niessen FB, Spauwen PH, Schalkwijk J, Kon M: On the nature of hypertrophic scars and keloids: a review. *Plast Reconstr Surg* 1999, 104:1435–1458
- Machesney M, Tidman N, Waseem A, Kirby L, Leigh I: Activated keratinocytes in the epidermis of hypertrophic scars. *Am J Pathol* 1998, 152:1133–1141
- Niessen FB, Andriessen MP, Schalkwijk J, Visser L, Timens W: Keratinocyte-derived growth factors play a role in the formation of hypertrophic scars. *J Pathol* 2001, 194:207–216
- Sun TT, Tseng SC, Huang AJ, Cooper D, Schermer A, Lynch MH, Weiss R, Eichner R: Monoclonal antibody studies of mammalian epithelial keratins: a review. *Ann NY Acad Sci* 1985, 455:307–329
- Fuchs E, Weber K: Intermediate filaments: structure, dynamics, function, and disease. *Annu Rev Biochem* 1994, 63:345–382
- Moll R, Franke WW, Schiller DL, Geiger B, Krepler R: The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 1982, 31:11–24
- Lloyd C, Yu QC, Cheng J, Turksen K, Degenstein L, Hutton E, Fuchs E: The basal keratin network of stratified squamous epithelia: defining K15 function in the absence of K14. *J Cell Biol* 1995, 129:1329–1344
- Waseem A, Dogan B, Tidman N, Alam Y, Purkis P, Jackson S, Lalli A, Machesney M, Leigh IM: Keratin 15 expression in stratified epithelia: downregulation in activated keratinocytes. *J Invest Dermatol* 1999, 112:362–369
- Weiss RA, Eichner R, Sun TT: Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. *J Cell Biol* 1984, 98:1397–1406
- Leigh IM, Navsaria H, Purkis PE, McKay IA, Bowden PE, Riddle PN: Keratins (K16 and K17) as markers of keratinocyte hyperproliferation in psoriasis in vivo and in vitro. *Br J Dermatol* 1995, 133:501–511
- Kallioinen M, Koivukangas V, Jarvinen M, Oikarinen A: Expression of cytokeratins in regenerating human epidermis. *Br J Dermatol* 1995, 133:830–835
- Sawaf MH, Ouhayoun JP, Forest N: Cytokeratin profiles in oral epithelial: a review and a new classification. *J Biol Buccale* 1991, 19:187–198
- Bloor BK, Su L, Shirlaw PJ, Morgan PR: Gene expression of differentiation-specific keratins (4/13 and 1/10) in normal human buccal mucosa. *Lab Invest* 1998, 78:787–795
- Langbein L, Heid HW, Moll I, Franke WW: Molecular characterization of the body site-specific human epidermal cytokeratin 9: cDNA cloning, amino acid sequence, and tissue specificity of gene expression. *Differentiation* 1993, 55:57–71
- Moll I, Heid H, Franke WW, Moll R: Distribution of a special subset of keratinocytes characterized by the expression of cytokeratin 9 in adult and fetal human epidermis of various body sites. *Differentiation* 1987, 33:254–265
- Collin C, Ouhayoun JP, Grund C, Franke WW: Suprabasal marker proteins distinguishing keratinizing squamous epithelia: cytokeratin 2 polypeptides of oral masticatory epithelium and epidermis are different. *Differentiation* 1992, 51:137–148
- Collin C, Moll R, Kubicka S, Ouhayoun JP, Franke WW: Characterization of human cytokeratin 2, an epidermal cytoskeletal protein synthesized late during differentiation. *Exp Cell Res* 1992, 202:132–141
- Herzog F, Winter H, Schweizer J: The large type II 70-kDa keratin of mouse epidermis is the ortholog of human keratin K2e. *J Invest Dermatol* 1994, 102:165–170
- Rentrop M, Nischt R, Knapp B, Schweizer J, Winter H: An unusual type-II 70-kilodalton keratin protein of mouse epidermis exhibiting

- postnatal body-site specificity and sensitivity to hyperproliferation. *Differentiation* 1987, 34:189–200
31. Smith LT, Underwood RA, McLean WH: Ontogeny and regional variability of keratin 2e (K2e) in developing human fetal skin: a unique spatial and temporal pattern of keratin expression in development. *Br J Dermatol* 1999, 140:582–591
32. Rothnagel JA, Traupe H, Wojcik S, Huber M, Hohl D, Pittelkow MR, Saeki H, Ishibashi Y, Roop DR: Mutations in the rod domain of keratin 2e in patients with ichthyosis bullosa of Siemens. *Nat Genet* 1994, 7:485–490
33. McLean WH, Morley SM, Lane EB, Eady RA, Griffiths WA, Paige DG, Harper JI, Higgins C, Leigh IM: Ichthyosis bullosa of Siemens—a disease involving keratin 2e. *J Invest Dermatol* 1994, 103:277–281
34. Markey AC, Lane EB, Churchill LJ, MacDonald DM, Leigh IM: Expression of simple epithelial keratins 8 and 18 in epidermal neoplasia. *J Invest Dermatol* 1991, 97:763–770
35. Markey AC, Lane EB, MacDonald DM, Leigh IM: Keratin expression in basal cell carcinomas. *Br J Dermatol* 1992, 126:154–160
36. Morgan PR, Su L: Intermediate filaments in oral neoplasia. I. Oral cancer and epithelial dysplasia. *Eur J Cancer B Oral Oncol* 1994, 30B:160–166
37. Wetzels RH, Kuijpers HJ, Lane EB, Leigh IM, Troyanovsky SM, Holland R, van Haelst UJ, Ramaekers FC: Basal cell-specific and hyperproliferation-related keratins in human breast cancer. *Am J Pathol* 1991, 138:751–763
38. Leigh IM, Purkis PE, Whitehead P, Lane EB: Monospecific monoclonal antibodies to keratin 1 carboxy terminal (synthetic peptide) and to keratin 10 as markers of epidermal differentiation. *Br J Dermatol* 1993, 129:110–119
39. Purkis PE, Steel JB, Mackenzie IC, Nathrath WB, Leigh IM, Lane EB: Antibody markers of basal cells in complex epithelia. *J Cell Sci* 1990, 97:39–50
40. Waseem NH, Lane DP: Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. *J Cell Sci* 1990, 96:121–129
41. Navasaria HA, Sexton C, Bouvard V, Leigh IM: Human epidermal keratinocytes—growth of keratinocytes with a 3T3 feeder layer: basic techniques. *Keratinocytes Methods*. Edited by IM Leigh, FM Watt. Cambridge, Cambridge University Press, 1994, pp 5–12
42. Waseem A, Lane EB, Harrison D, Waseem N: A keratin antibody recognizing a heterotypic complex: epitope mapping to complementary locations on both components of the complex. *Exp Cell Res* 1996, 223:203–214
43. Ramaekers FC, Moesker O, Huysmans A, Schaart G, Westerhof G, Wagenaar SS, Herman CJ, Vooijs GP: Intermediate filament proteins in the study of tumor heterogeneity: an in-depth study of tumors of the urinary and respiratory tracts. *Ann NY Acad Sci* 1985, 455:614–634
44. Waseem A, Alexander CM, Steel JB, Lane EB: Embryonic simple epithelial keratins 8 and 18: chromosomal location emphasizes difference from other keratin pairs. *New Biol* 1990, 2:464–478
45. Waseem A, White K, Waseem NH: Identification of a novel keratin epitope: evidence for association between non-helical sub-domains L12 during filament assembly. *Int J Biochem Cell Biol* 1997, 29:971–983
46. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning—A Laboratory Manual*. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1989
47. Virtanen M, Torma H, Vahlquist A: Keratin 4 upregulation by retinoic acid in vivo: a sensitive marker for retinoid bioactivity in human epidermis. *J Invest Dermatol* 2000, 114:487–493
48. Quinlan RA, Schiller DL, Hatzfeld M, Achtstatter T, Moll R, Jorcano JL, Magin TM, Franke WW: Patterns of expression and organization of cytokeratin intermediate filaments. *Ann NY Acad Sci* 1985, 455:282–306
49. Eisen D: The clinical features, malignant potential, and systemic associations of oral lichen planus: a study of 723 patients. *J Am Acad Dermatol* 2002, 46:207–214
50. Bloor BK, Seddon SV, Morgan PR: Gene expression of differentiation-specific keratins in oral epithelial dysplasia and squamous cell carcinoma. *Oral Oncol* 2001, 37:251–261
51. Lee YS, Yuspa SH, Dlugosz AA: Differentiation of cultured human epidermal keratinocytes at high cell densities is mediated by endogenous activation of the protein kinase C signaling pathway. *J Invest Dermatol* 1998, 111:762–766
52. Katagata Y: Evidence of differentiated keratin peptide (K1) in cultured human squamous cell carcinomas: demonstration of generality by three different approaches. *J Dermatol* 1992, 19:781–785
53. Prathiba V, Rao KS, Gupta PD: Altered expression of keratins during abnormal wound healing in human skin. *Cytobios* 2001, 104:43–51
54. Santos M, Paramio JM, Bravo A, Ramirez A, Jorcano JL: The expression of keratin k10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis. *J Biol Chem* 2002, 277:19122–19130
55. Santos M, Ballestin C, Garcia-Martin R, Jorcano JL: Delays in malignant tumor development in transgenic mice by forced epidermal keratin 10 expression in mouse skin carcinomas. *Mol Carcinog* 1997, 20:3–9
56. Asselineau D, Dale BA, Bernard BA: Filaggrin production by cultured human epidermal keratinocytes and its regulation by retinoic acid. *Differentiation* 1990, 45:221–229
57. Griffiths CE, Rosenthal DS, Reddy AP, Elder JT, Astrom A, Leach K, Wang TS, Finkel LJ, Yuspa SH, Voorhees JJ: Short-term retinoic acid treatment increases in vivo, but decreases in vitro, epidermal transglutaminase-K enzyme activity and immunoreactivity. *J Invest Dermatol* 1992, 99:283–288
58. Hohl D, Licht U, Breitkreutz D, Steinert PM, Roop DR: Transcription of the human loricrin gene in vitro is induced by calcium and cell density and suppressed by retinoic acid. *J Invest Dermatol* 1991, 96:414–418
59. Kopan R, Fuchs E: The use of retinoic acid to probe the relation between hyperproliferation-associated keratins and cell proliferation in normal and malignant epidermal cells. *J Cell Biol* 1989, 109:295–307
60. Rosenthal DS, Griffiths CE, Yuspa SH, Roop DR, Voorhees JJ: Acute or chronic topical retinoic acid treatment of human skin in vivo alters the expression of epidermal transglutaminase, loricrin, involucrin, filaggrin, and keratins 6 and 13 but not keratins 1, 10, and 14. *J Invest Dermatol* 1992, 98:343–350
61. Eichner R, Kahn M, Capetola RJ, Gendimenico GJ, Mezick JA: Effects of topical retinoids on cytoskeletal proteins: implications for retinoid effects on epidermal differentiation. *J Invest Dermatol* 1992, 98:154–161
62. Vahlquist A, Stenstrom E, Torma H: Vitamin A and beta-carotene concentrations at different depths of the epidermis: a preliminary study in the cow snout. *Ups J Med Sci* 1987, 92:253–257
63. Bloor BK, Seddon SV, Morgan PR: Gene expression of differentiation-specific keratin (K4, K13, K1 and K10) in oral non-dysplastic keratoses and lichen planus. *J Oral Pathol Med* 1999, 29:376–384
64. Santos M, Bravo A, Lopez C, Paramio JM, Jorcano JL: Severe abnormalities in the oral mucosa induced by suprabasal expression of epidermal keratin K10 in transgenic mice. *J Biol Chem* 2002, 277:35371–35377
65. Smith FJ, Maingi C, Covello SP, Higgins C, Schmidt M, Lane EB, Uitto J, Leigh IM, McLean WH: Genomic organization and fine mapping of the keratin 2e gene (KRT2E): K2e V1 domain polymorphism and novel mutations in ichthyosis bullosa of Siemens. *J Invest Dermatol* 1998, 111:817–821
66. Lotan R: Squamous cell differentiation markers in normal, premalignant, and malignant epithelium: effects of retinoids. *J Cell Biochem* 1993:S167–S174