BJD
British Journal of Dermatology

Induction of p38, tumour necrosis factor-α and RANTES by mechanical stretching of keratinocytes expressing mutant keratin 10^{R156H}

M. Obarzanek-Fojt,* B. Favre,* M. Huber,* S. Ryser,* A.M. Moodycliffe,*† P.-J. Wipff,‡ B. Hinz§ and D. Hohl*

*Service of Dermatology and Venereology, CHUV Lausanne, CH-1011 Lausanne, Switzerland
†Department of Bioanalytical Sciences, Nestle Research Center, Lausanne, Switzerland
‡Laboratory of Cell Biophysics, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland
§Laboratory of Tissue Repair and Regeneration, Matrix Dynamics Group, Faculty of Dentistry, University of Toronto, Toronto, ON M5S 3E2, Canada

Correspondence
Daniel Hohl.
E-mail: Daniel.hohl@chuv.ch

Accepted for publication
22 August 2010

Funding sources
This study was funded by grants from the Swiss National Science Foundation to D.H. (3100A0-120343 and 3100A0-107780), M.H. (3100A0-118093), B.H. (3100A0-113733/1) and A.M.M. and S.R. (3100A0-116840).

Conflicts of interest
None declared.

DOI 10.1111/j.1365-2133.2010.10013.x

Summary

Background Epidermolytic hyperkeratosis (bullous congenital ichthyosiform erythroderma), characterized by ichthyotic, rippled hyperkeratosis, erythroderma and skin blistering, is a rare autosomal dominant disease caused by mutations in keratin 1 or keratin 10 (K10) genes. A severe phenotype is caused by a missense mutation in a highly conserved arginine residue at position 156 (R156) in K10.
Objectives To analyse molecular pathomechanisms of hyperproliferation and hyperkeratosis, we investigated the defects in mechanosensation and mechanotransduction in keratinocytes carrying the $K10^{R156H}$ mutation.
Methods Differentiated primary human keratinocytes infected with lentiviral vectors carrying wild-type K10 ($K10^{wt}$) or mutated $K10^{R156H}$ were subjected to 20% isoaxial stretch. Cellular fragility and mechanosensation were studied by analysis of mitogen-activated protein kinase activation and cytokine release.
Results Cultured keratinocytes expressing $K10^{R156H}$ showed keratin aggregate formation at the cell periphery, whereas the filament network in $K10^{wt}$ cells was normal. Under stretching conditions $K10^{R156H}$ keratinocytes exhibited about a twofold higher level of filament collapse compared with steady state. In stretched $K10^{R156H}$ cells, higher p38 activation, higher release of tumour necrosis factor-α and RANTES but reduced interleukin-1β secretion compared with $K10^{wt}$ cells was observed.
Conclusions These results demonstrate that the R156H mutation in K10 destabilizes the keratin intermediate filament network and affects stress signalling and inflammatory responses to mechanical stretch in differentiated cultured keratinocytes.

Keratin intermediate filaments (KIFs) together with the microtubules and microfilaments constitute the cytoskeleton of epithelial cells. Keratins are composed of a central rod domain flanked by nonhelical head and tail domains. The rod domain is subdivided into four α-helical subsegments (1A, 1B, 2A and 2B) separated by non-α-helical linkers (L1, L12, L2). Heterodimerization of basic and acidic keratins (type I and II) takes place via coiled-coil interactions of subsegments 1B and 2B,[1] thus initiating the formation of KIFs, which are crucial for the cell's ability to sustain mechanical and nonmechanical stress.[2]

Mutations in the highly conserved motifs of keratin domain 1A or 2B lead to severe skin disorders,[3–6] which are characterized by the collapse of the intermediate filament network within keratinocytes.[3,4,7,8] Epidermolytic hyperkeratosis (EHK) (bullous congenital ichthyosiform erythroderma) is an autosomal dominant disease characterized by ichthyotic, rippled hyperkeratosis, particularly around joints and folds. Erythroderma and skin blistering due to cellular disruption followed by cytolysis are present at birth, with predominantly hyperkeratotic lesions sustained later in life.[3,9] EHK is caused by mutations in keratin 1 (K1) or keratin 10 (K10) genes.

Mutations in the rod domain of K10 were postulated to affect severely the stability of head to tail overlap between parallel molecules and therefore lead to severe forms of EHK.[4] A mutational hotspot was identified for arginine at position 156 (R156), which is a highly conserved residue in all type I keratins. The corresponding CGC codon within a CpG island is prone to undergo CG to TG or CA transitions on both the coding and noncoding strands.[10] R156 mutations cause severe phenotypes due to misformation and collapse of KIFs as previously shown *in vitro* in keratinocytes from affected patients.[4,7]

© 2010 The Authors
BJD © 2010 British Association of Dermatologists 2010

K10 was shown to be involved in the regulation of the keratinocyte cell cycle.[11] Keratinocytes from K10-null mice showed a higher proliferation rate, activation of extracellular regulated kinase (ERK) 1/2, increased levels of cyclin D1 and c-Myc in the basal layer, and p38 mitogen-activated protein kinase (MAPK) activation in the suprabasal layers.[12,13] Further, ectopic K10 expression in the basal layer of mouse epidermis strongly decreased keratinocyte proliferation due to inhibition of Akt activity and activation of protein kinase C ζ.[14–16]

In this study we postulated that the pathophysiology of EHK might be linked to alterations in stress-signalling responses. As EHK is a very rare skin disorder it is very difficult to collect a sufficient number of human biopsies for detailed studies. Therefore, we established an *in vitro* model to study EHK. By expressing K10 carrying the mutation R156H ($K10^{R156H}$) vs. wild-type K10 ($K10^{wt}$) in primary human keratinocytes, we were able to reproduce the disease phenotype *in vitro*. Furthermore, we analysed changes in the activity of MAPK signalling pathways and the inflammatory response in differentiated transduced keratinocytes subjected to 20% mechanical stretch. Our data demonstrate that our *in vitro* model enables a better understanding of the molecular mechanisms underlying EHK.

Materials and methods

Cell culture

Normal primary human keratinocytes (NHK) were isolated from abdominal tissue as described previously, with minor modifications.[17,18] Primary keratinocytes were cultured in defined serum-free keratinocyte medium (DKSFM; Invitrogen, Carlsbad, CA, U.S.A.) with growth supplement and penicillin/streptomycin. Differentiation of keratinocytes was induced at 80% cell confluence by increasing the calcium concentration in DKSFM to 1·2 mmol L^{-1}.

Cloning of keratin 10 in lentivectors and transduction of normal primary human keratinocytes

Human K10 with Myc-Tag at the N-terminus was amplified by polymerase chain reaction (PCR) amplification (Pfx polymerase) from a vector pCMV-SPORT6.ccdb (Thermo Fisher Scientific, Waltham, MA, U.S.A.) containing the full length K10 cDNA as a template using the following primers: forward F1, 5′-AACTCATCTCAGAAGAGGATCTGTCTGTTCGATACAGCTC-3′ and F2, 5′-CTCGGATCCGAATTCGCCGCCACCATGGAACAAAAACTCATCTCAGAAG-3′, and reverse 5′-CACCTCGAGGAATTCTTAGTATCTTGGTCCCTTAGATG-3′. The PCR products were ligated into pGEM-T Easy vector (Promega, Madison, WI, U.S.A.). The missense mutation replacing arginine 156 by histidine was introduced by directed mutagenesis (G for A) using the following primers: forward 5′-CATGCAGAATCTGAATGACCACCTGGCTTCCTAC-3′ and reverse 5′-CAGTGGACACATTTCGAAGGTCTTTCATTTC-3′. Myc-Tag-$K10^{wt}$ and Myc-Tag-$K10^{R156H}$ were subcloned into pHR'CMV W Sin-18 lentivector.[19] Lentiviral particles were produced according to Wiznerowicz and Trono[20] using psPAX2 instead of pCMV-ΔR8.91. NHK were grown to 50% confluence and infected with lentivirus at a multiplicity of infection of 10 in the presence of 6 μg mL^{-1} of polybrene (Sigma, St Louis, MO, U.S.A.). At a confluence of 80%, keratinocytes were split and used for experimental procedures.

Stretchable culture membranes and stretch assay

Cells were stretched isoaxially in home-made culture devices as described previously.[21,22] Experiments were carried out in six-well cell culture plates. At 80% confluence transduced keratinocytes were cultured for 4 days in a high-calcium medium. A single stretch was applied by screwing down the culture plate on to rings permitting a stretch up to 20%. Stretched keratinocytes were kept in the cell culture incubator for 30 min. After release, the stretch samples were analysed as described below.

Immunofluorescence analysis of cultured keratinocytes

Briefly, cultured primary human keratinocytes were fixed in 4% paraformaldehyde for 20 min and permeabilized with ice-cold acetone for 10 min at −20 °C. Cells were blocked in 12% bovine serum albumin in phosphate-buffered saline for 30 min and incubated with primary antibodies: mouse anti-K10 (Ab-2, clone DE-K10; Thermo Fisher Scientific), rabbit anti-Myc-Tag (sc-789; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and rabbit anti-K1 (PRB 149 P; Covance, Princeton, NJ, U.S.A.), for 2 h at room temperature. Bound antibodies were detected using biotinylated horse antimouse IgG and streptavidin Texas Red, or Alexa Fluor-488 antirabbit secondary antibody. The images were captured by a Zeiss LSM 510 Meta confocal microscope coupled to a CCD camera (Zeiss, Oberkochen, Germany).

Western blotting

Protein extracts were prepared as described previously.[23] Lysates were electrophoresed by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), blotted on to a polyvinylidene difluoride Immobilion®-P transfer membrane (Millipore, Billerica, MA, U.S.A.) and blocked in 1 × Tris-buffered saline–Tween (20 mmol L^{-1} Tris-HCl pH 7·5, 150 mmol L^{-1} NaCl, 0·2% Tween-20) with 5% w/v non-fat dry milk. Expression of exogenous K10 protein was analysed using either mouse anti-K10 or rabbit anti-Myc-Tag antibodies (as indicated above).

For MAPK signalling analysis cells were lysed in RIPA buffer and fractionated by 10% SDS-PAGE. After blocking, membranes were incubated with primary antibodies raised against phosphorylated proteins. After stripping, the membranes were reprobed with antibodies against nonphosphorylated proteins. Antibodies specific for phosphorylated or nonphosphorylated p44/42 MAPK (ERK1/2), stress-activated protein kinase/Jun N-terminal kinase (JNK) (56G8) rabbit monoclonal antibody and p38 MAPK were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Peroxidase-conjugated IgG

© 2010 The Authors
BJD © 2010 British Association of Dermatologists 2010

antimouse or antirabbit was used. Bands were visualized by enhanced chemiluminescence detection kit (Pierce, Rockford, IL, U.S.A.) in LAS 4000 imaging system (GE Healthcare Life Sciences, Little Chalfont, U.K.) and quantified using Multi Gauge densitometry software.

Reverse transcription and quantitative real-time polymerase chain reaction

Total RNA from NHK cultures was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA (1 μg) was reverse transcribed into cDNA using the GeneAmp RNA PCR kit and random hexamers from Applied Biosystems (Foster City, CA, U.S.A.) following the recommendations of the supplier. Real-time PCR analysis using 32 ng cDNA was performed on a StepOne™ PCR machine (Applied Biosystems) using the Power SYBR Green Master Mix (Applied Biosystems) in a reaction volume of 12 μL. Samples were amplified with the following PCR cycling conditions: 95 °C for 10 min, then 40 cycles comprising denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. To evaluate the expression level of RPL13A and K10 mRNAs, commercially available QuantiTect PrimerAssays (Qiagen) were used. Quantification was performed using the comparative $2^{-\Delta\Delta CT}$ method using RPL13A as endogenous control.

Multiplex cytokine measurement

Briefly, 1–3 h before applying the stretch, medium was changed with 1 mL of fresh DKSFM. At the end of the stretch and after membrane release, 500 μL of supernatant was collected, respectively. For kinetic experiments keratinocytes were stretched for 30 min and 500 μL supernatants were collected at 30 min, and every hour up to 8 h after membrane release. An equal amount of the fresh medium was added at every time point. Supernatants were frozen at −80 °C. The concentration of interleukin (IL)-1β, IL-6, IL-8, tumour necrosis factor (TNF)-α and RANTES (regulated on activation, normal T-cell expressed and secreted, CCL5) was measured using the MS2400 cytokine Human 7-plex array (Meso Scale Discovery, Gaithersburg, MD, U.S.A.) in the Sector Imager 2400, model 1250. Calibration curves were prepared with a range of 2·4–10 000 pg mL^{-1} of each cytokine. The results from cytokine measurements are represented as protein concentration in the supernatant relative to nonstretched Myc-$K10^{wt}$ control. Experiments were repeated independently at least twice and gave similar results with slightly shifted time courses, preventing their exact superposition.

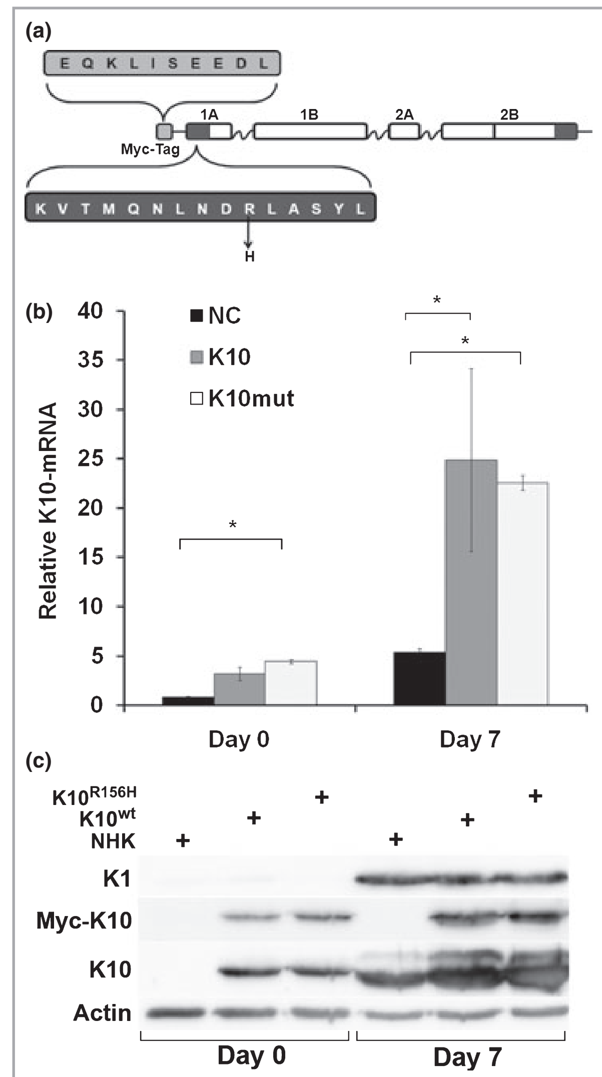

Fig 1. Analysis of $K10^{wt}$ and $K10^{R156H}$ expression in primary human keratinocytes. (a) Schematic representation of the introduced mutation in the human keratin 10 gene. The arginine to histidine mutation at position 156 of the coil 1A consensus domain was introduced by directed mutagenesis. The N-terminus domain of wild-type or mutated K10 was Myc-tagged ($K10^{wt}$ and $K10^{R156H}$). Real-time polymerse chain reaction (b) and immunoblot (c) analysis of K10 expression in infected keratinocytes before (day 0) and after differentiation (day 7). Proteins were detected with anti-Myc (exogenous protein) and anti-K10 (endogenous and exogenous protein) antibodies in the cytoskeletal fraction and compared with noninfected normal primary human keratinocytes (NHK/NC). K1 staining demonstrates proper keratinocyte differentiation while antiactin antibody verifies equal protein loading. Data are presented as mean ± SEM of the fold difference ($n = 2$, $^{*}P < 0{\cdot}05$).

Results

Defective keratin intermediate filament assembly in $K10^{R156H}$ keratinocytes

In order to establish an *in vitro* model to investigate key signalling pathways affected in EHK, primary human keratinocytes were infected by lentiviral vectors carrying Myc-tagged $K10^{wt}$ or mutant $K10^{R156H}$. The mutation in the rod domain was introduced by directed mutagenesis of the $K10^{wt}$ gene (Fig. 1a). Demonstrating that exogenous K10 was efficiently expressed after 7 days of calcium-induced

© 2010 The Authors
BJD © 2010 British Association of Dermatologists 2010

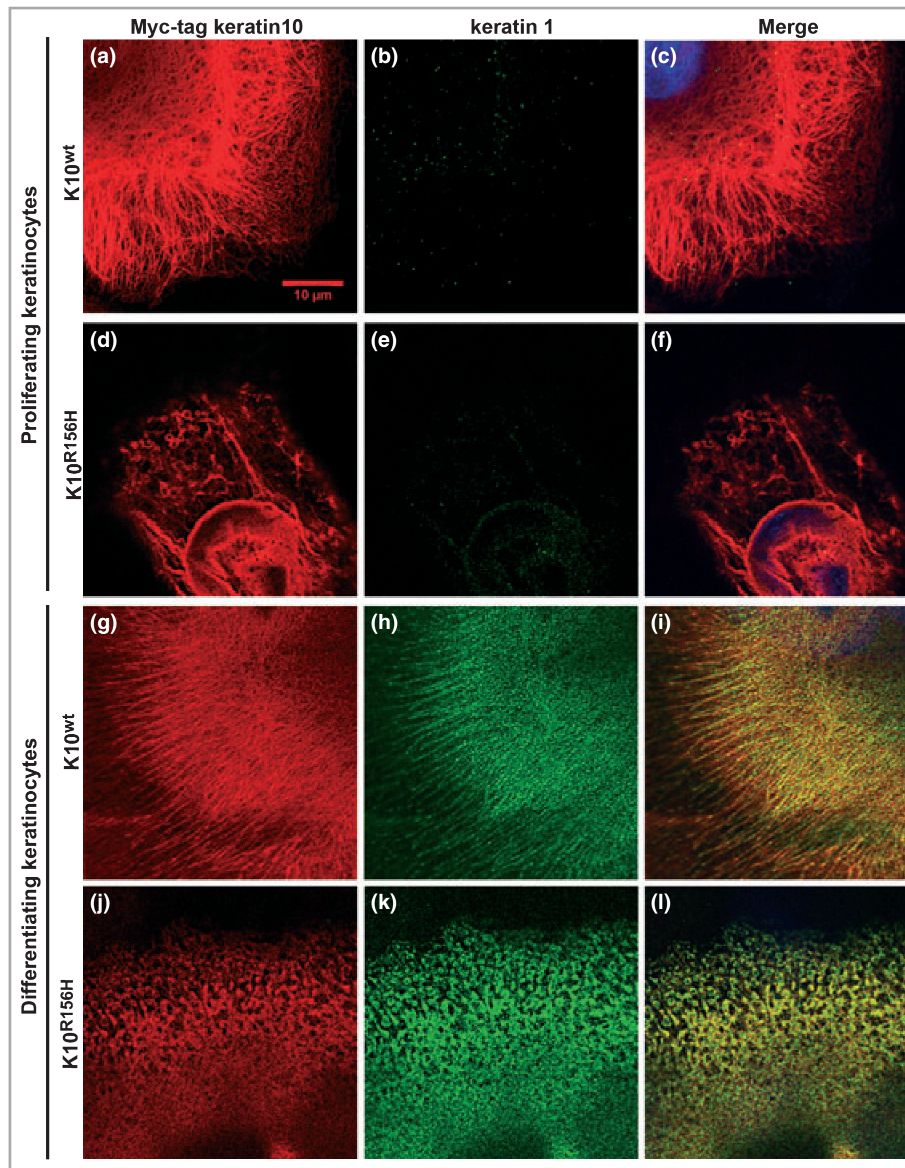

Fig 2. Keratin intermediate filament (KIF) formation in $K10^{wt}$ and $K10^{R156H}$ keratinocytes. Demonstration of KIF arrangement in cell periphery of keratinocytes infected with $K10^{wt}$ (a–c, g–i) or $K10^{R156H}$ (d–f, j–l). Two days post-transduction, subconfluent normal primary human keratinocytes (day 0) (a–f) were differentiated for 6 days (day 6) (g–l). Fixed cells were immunostained for Myc-tagged keratin 10 (a, d, g, j, red) and keratin 1 (b, e, h, k, green). Scale bar = 10 μm.

differentiation in primary human keratinocytes (day 7), K10 mRNA and protein levels were respectively four times and nearly two times higher in infected than in noninfected cells (Fig. 1b, c).

KIF network formation was examined in proliferating (2 days post-transduction, day 0) and in differentiating keratinocytes (6 days in high-calcium medium, day 6). Exogenous $K10^{wt}$ and $K10^{R156H}$ successfully interacted and dimerized with endogenous K1 (day 6) forming KIFs (Fig. 2i, l). No difference in the expression of K1 mRNA was observed between control and transduced cells under proliferating and differentiating conditions (data not shown). As shown in Figure 2 the K1/K10 filament network in differentiated keratinocytes (day 6) was more abundant and compact than in proliferating cells.

Further, we observed that around 30% of cells expressing both endogenous K1 and exogenous $K10^{R156H}$ formed protein aggregates at the cell periphery (Fig. 2j–l), which is a characteristic feature of cultured EHK keratinocytes.[3,4,7] These aggregates are characterized by the formation of round, spot-like or circle-like clumps of proteins, which are well distinguishable from the organized filament network in $K10^{wt}$ (Fig. 2i vs. l). In proliferating $K10^{R156H}$ keratinocytes strong filament collapse was also observed (Fig. 2d–f). No aggregate formation was noted in proliferating or differentiating $K10^{wt}$ cells (Fig. 2a–c, g–i).

© 2010 The Authors
BJD © 2010 British Association of Dermatologists 2010

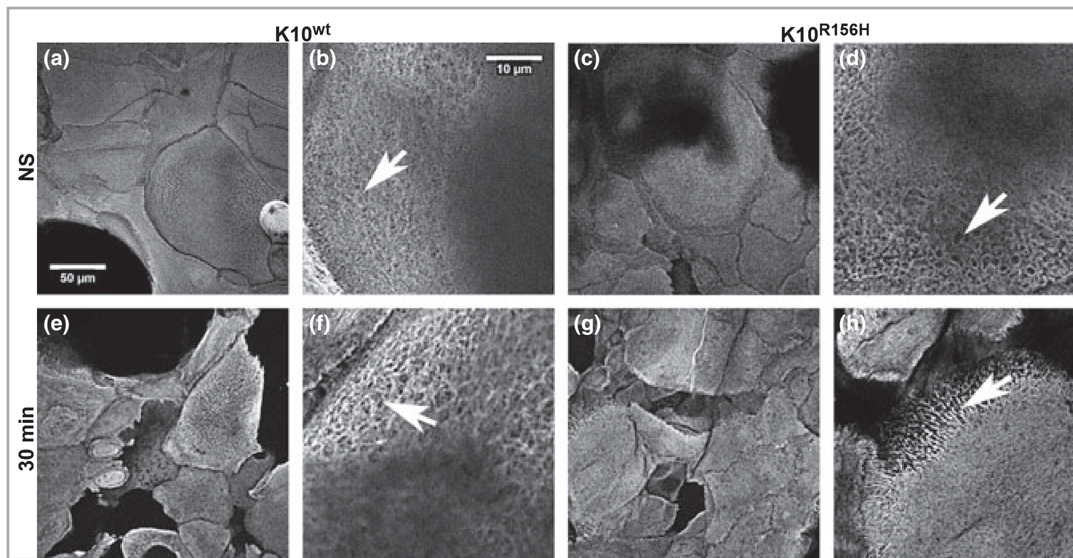

Fig 3. Stretch-induced collapse of keratin intermediate filaments in K10^{R156H} keratinocytes. Normal primary human keratinocytes infected with K10^{wt} (a, b, e, f) or K10^{R156H} (c, d, g, h) were differentiated for 4 days in high-calcium medium. Cells were either untreated (NS) or stretched up to 20% for 30 min. Keratinocytes were stained for Myc to visualize Myc-tagged exogenous K10. The arrows indicate proper (b, f) or altered (d, h) keratin intermediate filaments at cell periphery. Scale bar = 50 µm (a, c, e, g) and 10 µm (b, d, f, h).

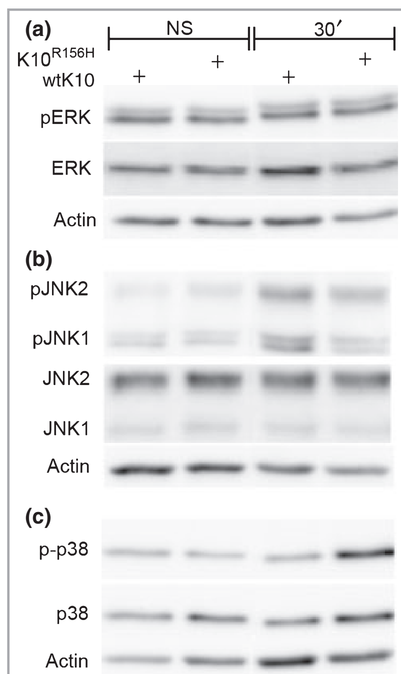

Fig 4. Analysis of the stretching effect on the activation of the mitogen-activated protein kinase signalling pathways in K10^{wt} and K10^{R156H} keratinocytes. Transduced normal primary human keratinocytes were differentiated for 4 days and stretched or not (NS) up to 20% for 30 min. Proteins were then extracted in RIPA buffer, fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and blotted on to a polyvinylidene difluoride membrane. Rabbit antibodies specific for unphosphorylated and phosphorylated forms of (a) extracellular regulated kinase (ERK), (b) stress-activated protein kinase/Jun N-terminal kinase (JNK) and (c) p38 were used. To verify equal loading of protein, the lysates were probed with antiactin antibody.

Strong keratin intermediate filament collapse induced by mechanical stretch of K10^{R156H} keratinocytes

As the KIF network protects cells against mechanical stress and mutations in keratins increase cell fragility, we subjected transduced keratinocytes to mechanical stretch. Keratinocytes differentiated for 4 days in high-calcium medium were isoaxially stretched by 20% for 30 min in a home-made culture device.[22] Immunofluorescence analysis demonstrated an excellent correlation between the amount of KIF aggregates and stretching in K10^{R156H} cells (Fig. 3). In K10^{R156H} keratinocytes, stretched for 30 min, the number of cells with visible KIF aggregates reached 60% of all K1/K10^{R156H}-positive cells, whereas in only 31% of K1/K10^{R156H} nonstretched cells were KIF aggregates observed (Fig. 3c, d vs. g, h). In contrast, 30 min of stretch did not induce any KIF aggregates in K10^{wt} cells (Fig. 3a, b, e, f). In summary, our data demonstrate that KIFs in K10^{R156H} keratinocytes have a tendency to form aggregates under stress conditions. Thus, this *in vitro* model shows features characteristic for EHK. Stretched K10^{R156H} keratinocytes were used to investigate further the molecular mechanisms underlying EHK.

Stronger p38 activation in mechanically stretched K10^{R156H} keratinocytes

ERK1/2, JNK and p38 kinase signalling pathways can be induced under a variety of stress conditions. We investigated the stress responses in stretched K10^{R156H} keratinocytes by using specific antiphosphopeptide antibodies for each kinase.

K10^{wt} and K10^{R156H} keratinocytes growing under static conditions showed equivalent levels of phosphorylated and unphosphorylated ERK, JNK and p38 (Fig. 4). No ERK phosphorylation was detected in cells stretched for 30 min.

© 2010 The Authors

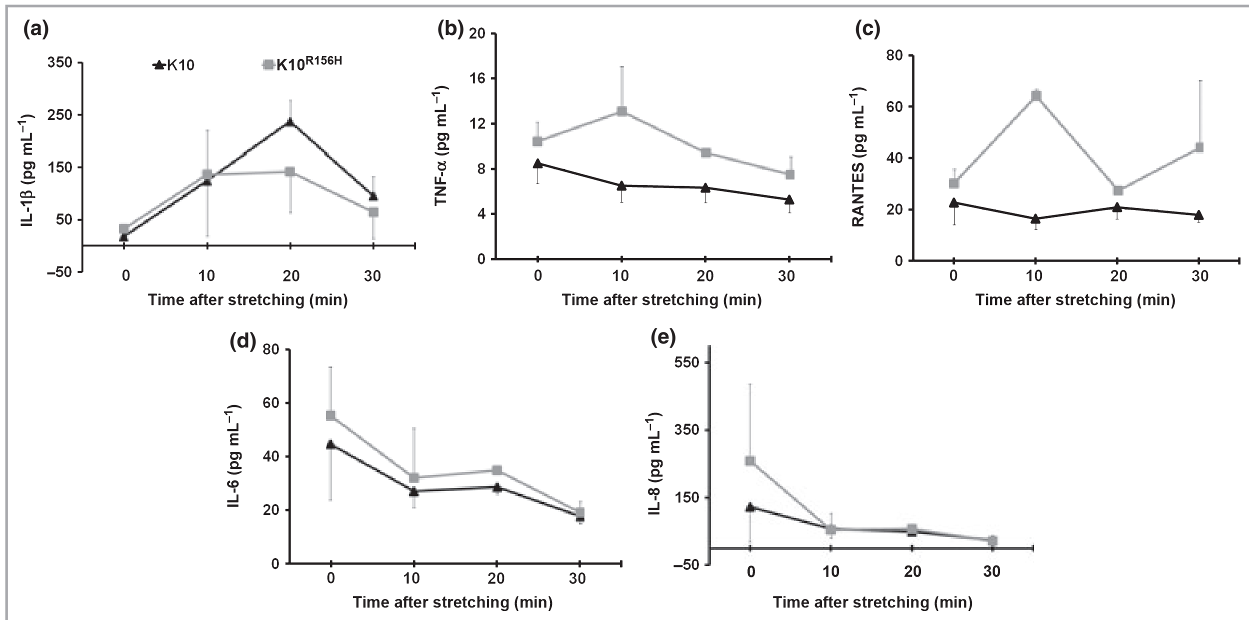

Fig 5. Analysis of early cytokine release in response to stretch in K10^{wt} or K10^{R156H} keratinocytes. Infected normal primary human keratinocytes were differentiated for 4 days and subjected to 20% stretch for the indicated time. Supernatants were collected immediately after membrane release. The time course of the presented cytokine concentrations in the medium was measured using the MS2400 cytokine Human 7-plex array (Meso Scale Discovery, Gaithersburg, MD, U.S.A.). (a) Interleukin (IL)-1β; (b) tumour necrosis factor (TNF)-α; (c) RANTES (regulated on activation, normal T-cell expressed and secreted); (d) IL-6; (e) IL-8. Data are shown as mean ± SEM, $n = 2$.

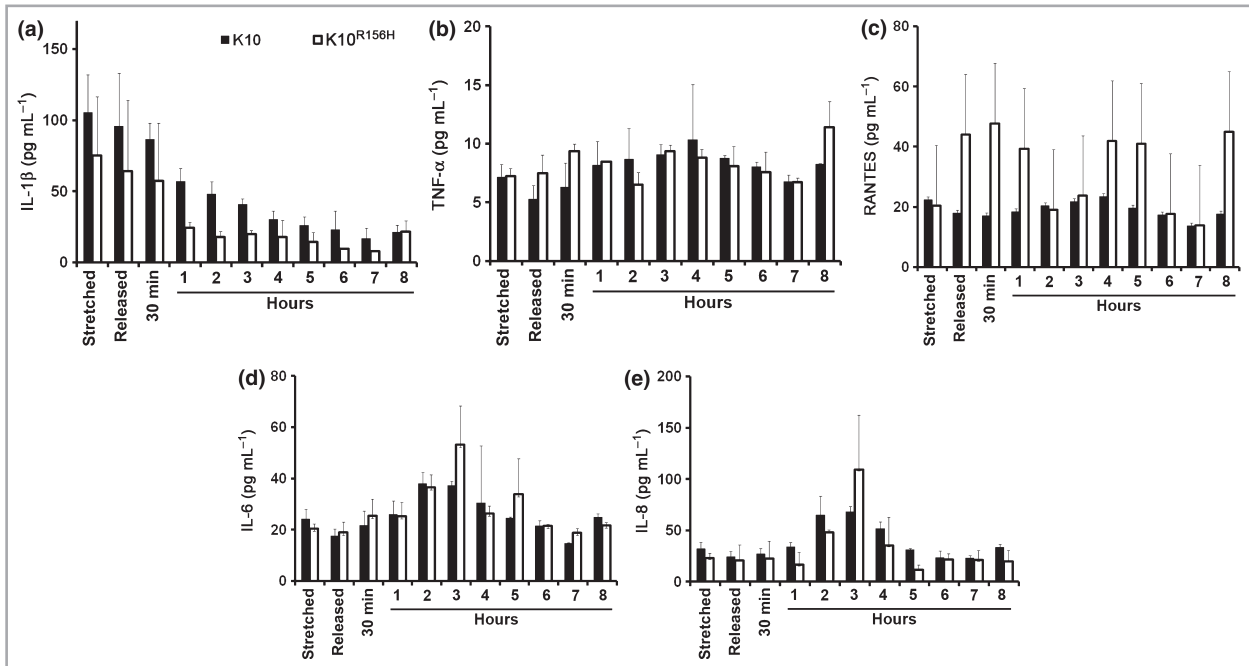

Fig 6. Kinetics of cytokine secretion by K10^{wt} keratinocytes (filled bars) or K10^{R156H} keratinocytes (empty bars) after cell contraction. Normal primary human keratinocytes expressing Myc-K10^{wt} or Myc-K10^{R156H} were subjected to a 20% stretch for 30 min. Culture supernatants were collected at the end of stretch and at the indicated times after membrane release. Cytokines were quantified as in Figure 5. (a) Interleukin (IL)-1β; (b) tumour necrosis factor (TNF)-α; (c) RANTES (regulated on activation, normal T-cell expressed and secreted); (d) IL-6; (e) IL-8. Data are shown as mean ± SEM, $n = 2$.

On the other hand, strong JNK1 and JNK2 phosphorylation was observed after 30 min of stretch. However, JNK1 or JNK2 levels were equivalent in both stretched and nonstretched K10^{wt} and K10^{R156H} cells (Fig. 4b). Surprisingly, we observed that stretching of keratinocytes for 30 min remarkably induced p38 phosphorylation in K10^{R156H} cells compared with K10^{wt}. These data demonstrate that stretch application to primary human keratinocytes induces the MAPK

© 2010 The Authors
BJD © 2010 British Association of Dermatologists 2010

signalling pathways *in vitro*, with stronger activation of p38 in $K10^{R156H}$ when compared with $K10^{wt}$ keratinocytes.

Secretion of interleukin-1β, tumour necrosis factor-α and RANTES is differently modulated in stretched $K10^{wt}$ and $K10^{R156H}$ keratinocytes

Further, we evaluated the proinflammatory response to stretch in these cells. Supernatants collected from cells stretched for 10, 20 and 30 min and after stretch release were analysed using the MS2400 cytokine Human 7-plex array.

Strong IL-1β secretion in $K10^{wt}$ and $K10^{R156H}$ cells was observed upon stretch (Fig. 5a). IL-1β activation was rapid, starting at 20 min of stretch and remaining high for the next 10 min. Under these experimental conditions there was no difference in the levels of IL-1β in the supernatants collected from $K10^{R156H}$ and $K10^{wt}$ cells. Following the 30 min stretch the level of secreted IL-1β decreased slowly. Interestingly, in supernatants collected between 30 min and 3 h following membrane release we found an approximately twofold lower level of IL-1β in $K10^{R156H}$ compared with $K10^{wt}$ keratinocytes (Fig. 6a).

Nonstretched $K10^{wt}$ and $K10^{R156H}$ keratinocytes showed equivalent levels of TNF-α and RANTES (Fig. 5b, c). However, more than twofold or threefold higher levels of TNF-α and RANTES were secreted by $K10^{R156H}$ cells compared with $K10^{wt}$ after 10 min of stretch. After longer stretching periods (20 and 30 min) differences in the TNF-α or RANTES released by $K10^{wt}$ and $K10^{R156H}$ cells were less marked. Interestingly, assessing the kinetics of cytokine release up to 8 h after a single stretch, $K10^{R156H}$ keratinocytes showed repetitive increase and reduction of RANTES levels with a period of 3 h (Fig. 6c). In cells subjected to stretch a diminished secretion of IL-6 and IL-8 was observed in $K10^{wt}$ and $K10^{R156H}$ keratinocytes from 10 to 30 min (Fig. 5d, e). The secretion of these cytokines was not significantly different between $K10^{wt}$ and $K10^{R156H}$ keratinocytes (Fig. 6d, e). Our data indicate a difference in the release of IL-1β, TNF-α and RANTES between $K10^{wt}$ and $K10^{R156H}$ keratinocytes in response to stretch.

Discussion

In this report we analysed the impact of the dominant negative K10 mutation R156H on mechanical stress responses in primary human keratinocytes. To our knowledge, this is the first description of multidirectional/isoaxial stretch application to differentiated keratinocytes expressing the mutated K10 gene. Until now there have been few reports describing the application of mechanical stretch to normal human keratinocytes and all these studies have used techniques applying a unidirectional stretch.[24–28]

It is well established that alterations of cell shape activate tightly regulated and balanced mechanical stress responses such as Ca^{2+} influx, integrin rearrangement and epidermal growth factor receptor activation, which subsequently induce MAPK pathways and prosurvival programmes.[25,29] These mechanisms are partially controlled by quick cytokine release to the body fluid from residual cells.[30–33] Indeed, IL-1 release has already been reported in stretched keratinocytes,[34] supporting our results and validating the keratinocyte mechanosensation in the presented system. Moreover, some cytokines such as IL-1β, IL-6 or TNF-α have already been reported to contribute to the pathogenesis of skin disorders related to mutations in keratin genes, such as epidermolysis bullosa simplex (EBS).[35–37]

We demonstrated that mutation in the K10 gene, which leads to the formation of keratin aggregates, alters the keratinocyte response to physical stress. A strong activation of p38 was demonstrated in $K10^{R156H}$ vs. $K10^{wt}$ keratinocytes following a single stretch. As ultimate regulatory outcome is dependent on the balance between regulatory inputs, it is possible that marked p38 phosphorylation in stretched mutant keratinocytes activates significant responses transmitted to other cells and consequently induces programmes such as hyperproliferation and/or hyperkeratosis. Deregulated p38 activity has already been reported in some pathological cutaneous conditions. Increased activation of p38 was shown in chronic inflammatory disorders such as psoriasis, lichen planus, chronic eczema and prurigo.[38–40] In K10 knockout mice strong activation of p38 leads to increased cell proliferation through upregulation of c-Myc and cyclin D1 expression.[12,13] Moreover, p38 has been reported to suppress the cytoskeleton and microtubule-associated genes in keratinocytes, inducing the expression of proteins involved in cell proliferation.[41] Therefore, we propose that the hyperproliferation characteristic for EHK may be a consequence of strong activation of p38 in the keratinocytes exposed to mechanical stretch.

Interestingly, p38 activity is one of the indispensible factors to induce an inflammatory response in different cells.[39,42,43] Consistent with this, our data demonstrate strong release of inflammatory cytokines such as TNF-α and RANTES in $K10^{R156H}$ keratinocytes subjected to mechanical stretch. As the activity of MAPK and inflammatory response are tightly regulated and interdependent,[31] it is very difficult to demonstrate whether the activation of p38 leads to induction of TNF-α expression and release or vice versa. To clarify which pathway is more possible in the system described here, additional experiments are necessary.

We demonstrated that the TNF-α extracellular level is twice as high in $K10^{R156H}$ as in $K10^{wt}$ keratinocytes following a 10-min stretch. The collapse of KIFs in an *in vitro* EBS model has been shown to lead to autocrine/paracrine TNF-α-mediated receptor activation in keratinocytes.[37] Following TNF-α activation, TRADD protein (TNF receptor type 1-associated death domain) interacts with procaspase 8 and induces cell death. TRADD was recently shown to interact with keratins (K14, K18) and to inhibit TNF-α-induced apoptosis.[37,44] As keratin/TRADD interaction takes place through the 1A rod domain of keratins, 1A domain mutants may affect TRADD binding resulting in increased TNF-α/caspase 8-dependent

© 2010 The Authors
BJD © 2010 British Association of Dermatologists 2010

apoptosis.[45] Whether the $K10^{R156H}$ mutation induces TNF-α-dependent apoptosis remains to be seen. To our knowledge the K10/TRADD interaction has not been studied; however, it might be possible that decreased TRADD sequestration takes place in differentiated $K10^{R156H}$ keratinocytes.

Finally, we observed a strong activation of RANTES in $K10^{R156H}$ keratinocytes, suggesting an important function of RANTES in the pathogenesis of EHK. RANTES belongs to the C-C class of cytokines and is a chemoattractant for blood monocytes, memory T-helper cells and eosinophils.[46–48] It is involved in pathogenesis of psoriasis where it is thought to contribute to the migration of activated T cells and, therefore, has been proposed to be a good therapeutic target.[49,50] RANTES is stimulated upon TNF-α[50,51] and p38 activation. A strong induction of RANTES synthesis was reported in epithelial cells in response to constitutive p38 activation.[52] The repetitive increase and decrease of secreted RANTES in $K10^{R156H}$ keratinocytes after cell stretching and contraction suggest that RANTES is tightly regulated both at the level of cytokine secretion and at the level of gene transcription by the mechanical stress signalling pathways. Notably, it has recently been demonstrated that strong RANTES activation and *de novo* mRNA synthesis are inhibited by acitretin, a second-generation retinoid.[53] This drug is commonly used for therapy of EHK. As the mechanisms of acitretin action are not clear, our *in vitro* data may help to understand better the basis for its successful clinical use in patients with EHK.

In summary, we report the impact of K10 aggregates on the mechanotransduction, signalling and inflammatory responses in primary human keratinocytes. We propose a model that KIF collapse caused by mutation in the K10 gene leads to release of TNF-α, and turns on the p38 pathway. Strong p38 activity leads to the synthesis and release of cytokines such as TNF-α or RANTES. We suggest that the presented *in vitro* model may represent a good technique to monitor the effects of K10 mutations on cell behaviour and to assess the effect of gene therapy for EHK. To our knowledge this is the first study demonstrating an impact of multidirectional stretch on keratinocytes carrying the mutation in the K10 gene. A recent study by Russell *et al.*[54] presented an application of the oscillating stretch on proliferating keratinocytes carrying a K14 mutation. Possibly the combination of both isoaxial and oscillating stretch would be the most suitable to study the skin disorders caused by mutations in structural proteins, first, by improving data reproducibility through automation of the assay and second, through mimicking the *in vivo* multidirectional stress conditions.

What's already known about this topic?

- Mutations in keratin 10 (K10) cause the most severe forms of epidermolytic hyperkeratosis.
- Keratins have been shown to be implicated in the regulation of the cell cycle.
- K10 knockout mice show high activity of p38 and extracellular regulated kinase signalling, leading to induction of cell proliferation.

What does this study add?

- Our results show that K10 mutation affects mechanosensation and mechanotransduction leading to deregulation of p38 signalling and release of cytokines.

Acknowledgments

We are grateful to Gaëlle Bussard for her contribution to plasmid cloning and Josiane Smith for preparation of silicone plates. We thank Dr Magdalini Kypriotou for her generous support.

References

1 Wu KC, Bryan JT, Morasso MI *et al.* Coiled-coil trigger motifs in the 1B and 2B rod domain segments are required for the stability of keratin intermediate filaments. *Mol Biol Cell* 2000; **11**:3539–58.
2 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.
3 Syder AJ, Yu QC, Paller AS *et al.* Genetic mutations in the K1 and K10 genes of patients with epidermolytic hyperkeratosis. Correlation between location and disease severity. *J Clin Invest* 1994; **93**:1533–42.
4 Rothnagel JA, Dominey AM, Dempsey LD *et al.* Mutations in the rod domains of keratins 1 and 10 in epidermolytic hyperkeratosis. *Science* 1992; **257**:1128–30.
5 Terron-Kwiatkowski A, van Steensel MA, van Geel M *et al.* Mutation S233L in the 1B domain of keratin 1 causes epidermolytic palmoplantar keratoderma with 'tonotubular' keratin. *J Invest Dermatol* 2006; **126**:607–13.
6 Haruna K, Suga Y, Mizuno Y *et al.* R156C mutation of keratin 10 causes mild form of epidermolytic hyperkeratosis. *J Dermatol* 2007; **34**:545–8.
7 Huber M, Scaletta C, Benathan M *et al.* Abnormal keratin 1 and 10 cytoskeleton in cultured keratinocytes from epidermolytic hyperkeratosis caused by keratin 10 mutations. *J Invest Dermatol* 1994; **102**:691–4.
8 D'Alessandro M, Russell D, Morley SM *et al.* Keratin mutations of epidermolysis bullosa simplex alter the kinetics of stress response to osmotic shock. *J Cell Sci* 2002; **115**:4341–51.
9 Irvine AD, McLean WH. Human keratin diseases: the increasing spectrum of disease and subtlety of the phenotype–genotype correlation. *Br J Dermatol* 1999; **140**:815–28.
10 Cooper DN, Youssoufian H. The CpG dinucleotide and human genetic disease. *Hum Genet* 1988; **78**:151–5.
11 Frost P, Weinstein GD, Van Scott EJ. The ichthyosiform dermatoses. II. Autoradiographic studies of epidermal proliferation. *J Invest Dermatol* 1966; **47**:561–7.
12 Reichelt J, Magin TM. Hyperproliferation, induction of c-Myc and 14-3-3sigma, but no cell fragility in keratin-10-null mice. *J Cell Sci* 2002; **115**:2639–50.

© 2010 The Authors
BJD © 2010 British Association of Dermatologists 2010

13 Reichelt J, Furstenberger G, Magin TM. Loss of keratin 10 leads to mitogen-activated protein kinase (MAPK) activation, increased keratinocyte turnover, and decreased tumor formation in mice. *J Invest Dermatol* 2004; **123**:973–81.
14 Paramio JM, Casanova ML, Segrelles C *et al.* Modulation of cell proliferation by cytokeratins K10 and K16. *Mol Cell Biol* 1999; **19**:3086–94.
15 Paramio JM, Segrelles C, Ruiz S *et al.* Inhibition of protein kinase B (PKB) and PKCzeta mediates keratin K10-induced cell cycle arrest. *Mol Cell Biol* 2001; **21**:7449–59.
16 Santos M, Paramio JM, Bravo A *et al.* The expression of keratin k10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis. *J Biol Chem* 2002; **277**:19122–30.
17 Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975; **6**:331–43.
18 Green H, Kehinde O, Thomas J. Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. *Proc Natl Acad Sci USA* 1979; **76**:5665–8.
19 Zufferey R, Dull T, Mandel RJ *et al.* Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J Virol* 1998; **72**:9873–80.
20 Wiznerowicz M, Trono D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J Virol* 2003; **77**:8957–61.
21 Goffin JM, Pittet P, Csucs G *et al.* Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. *J Cell Biol* 2006; **172**:259–68.
22 Wipff PJ, Rifkin DB, Meister JJ *et al.* Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J Cell Biol* 2007; **179**:1311–23.
23 Hohl D, Mehrel T, Lichti U *et al.* Characterization of human loricrin. Structure and function of a new class of epidermal cell envelope proteins. *J Biol Chem* 1991; **266**:6626–36.
24 Reichelt J. Mechanotransduction of keratinocytes in culture and in the epidermis. *Eur J Cell Biol* 2007; **86**:807–16.
25 Yano S, Komine M, Fujimoto M *et al.* Mechanical stretching *in vitro* regulates signal transduction pathways and cellular proliferation in human epidermal keratinocytes. *J Invest Dermatol* 2004; **122**:783–90.
26 Yano S, Komine M, Fujimoto M *et al.* Activation of Akt by mechanical stretching in human epidermal keratinocytes. *Exp Dermatol* 2006; **15**:356–61.
27 Kippenberger S, Bernd A, Loitsch S *et al.* Signaling of mechanical stretch in human keratinocytes via MAP kinases. *J Invest Dermatol* 2000; **114**:408–12.
28 Knies Y, Bernd A, Kaufmann R *et al.* Mechanical stretch induces clustering of beta1-integrins and facilitates adhesion. *Exp Dermatol* 2006; **15**:347–55.
29 Eckert RL, Efimova T, Dashti SR *et al.* Keratinocyte survival, differentiation, and death: many roads lead to mitogen-activated protein kinase. *J Investig Dermatol Symp Proc* 2002; **7**:36–40.
30 Chen Y, Pat B, Zheng J *et al.* Tumor necrosis factor-alpha produced in cardiomyocytes mediates a predominant myocardial inflammatory response to stretch in early volume overload. *J Mol Cell Cardiol* 2010; **49**:70–8.
31 Zhan M, Jin B, Chen SE *et al.* TACE release of TNF-alpha mediates mechanotransduction-induced activation of p38 MAPK and myogenesis. *J Cell Sci* 2007; **120**:692–701.
32 Sasamoto A, Nagino M, Kobayashi S *et al.* Mechanotransduction by integrin is essential for IL-6 secretion from endothelial cells in response to uniaxial continuous stretch. *Am J Physiol Cell Physiol* 2005; **288**:C1012–22.
33 Thomas RA, Norman JC, Huynh TT *et al.* Mechanical stretch has contrasting effects on mediator release from bronchial epithelial cells, with a rho-kinase-dependent component to the mechanotransduction pathway. *Respir Med* 2006; **100**:1588–97.
34 Takei T, Kito H, Du W *et al.* Induction of interleukin (IL)-1 alpha and beta gene expression in human keratinocytes exposed to repetitive strain: their role in strain-induced keratinocyte proliferation and morphological change. *J Cell Biochem* 1998; **69**:95–103.
35 Lu H, Chen J, Planko L *et al.* Induction of inflammatory cytokines by a keratin mutation and their repression by a small molecule in a mouse model for EBS. *J Invest Dermatol* 2007; **127**:2781–9.
36 Roth W, Reuter U, Wohlenberg C *et al.* Cytokines as genetic modifiers in K5−/− mice and in human epidermolysis bullosa simplex. *Hum Mutat* 2009; **30**:832–41.
37 Yoneda K, Furukawa T, Zheng YJ *et al.* An autocrine/paracrine loop linking keratin 14 aggregates to tumor necrosis factor alpha-mediated cytotoxicity in a keratinocyte model of epidermolysis bullosa simplex. *J Biol Chem* 2004; **279**:7296–303.
38 Johansen C, Kragballe K, Westergaard M *et al.* The mitogen-activated protein kinases p38 and ERK1/2 are increased in lesional psoriatic skin. *Br J Dermatol* 2005; **152**:37–42.
39 Funding AT, Johansen C, Kragballe K *et al.* Mitogen- and stress-activated protein kinase 1 is activated in lesional psoriatic epidermis and regulates the expression of pro-inflammatory cytokines. *J Invest Dermatol* 2006; **126**:1784–91.
40 Wang S, Uchi H, Hayashida S *et al.* Differential expression of phosphorylated extracellular signal-regulated kinase 1/2, phosphorylated p38 mitogen-activated protein kinase and nuclear factor-kappaB p105/p50 in chronic inflammatory skin diseases. *J Dermatol* 2009; **36**:534–40.
41 Gazel A, Nijhawan RI, Walsh R *et al.* Transcriptional profiling defines the roles of ERK and p38 kinases in epidermal keratinocytes. *J Cell Physiol* 2008; **215**:292–308.
42 Jackson JR, Bolognese B, Hillegass L *et al.* Pharmacological effects of SB 220025, a selective inhibitor of p38 mitogen-activated protein kinase, in angiogenesis and chronic inflammatory disease models. *J Pharmacol Exp Ther* 1998; **284**:687–92.
43 Kjellerup RB, Kragballe K, Iversen L *et al.* Pro-inflammatory cytokine release in keratinocytes is mediated through the MAPK signal-integrating kinases. *Exp Dermatol* 2008; **17**:498–504.
44 Inada H, Izawa I, Nishizawa M *et al.* Keratin attenuates tumor necrosis factor-induced cytotoxicity through association with TRADD. *J Cell Biol* 2001; **155**:415–26.
45 Lugassy J, McGrath JA, Itin P *et al.* *KRT14* haploinsufficiency results in increased susceptibility of keratinocytes to TNF-alpha-induced apoptosis and causes Naegeli–Franceschetti–Jadassohn syndrome. *J Invest Dermatol* 2008; **128**:1517–24.
46 Schall TJ, Bacon K, Toy KJ *et al.* Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* 1990; **347**:669–71.
47 Alam R, Stafford S, Forsythe P *et al.* RANTES is a chemotactic and activating factor for human eosinophils. *J Immunol* 1993; **150**:3442–8.
48 Schroder JM, Noso N, Sticherling M *et al.* Role of eosinophil-chemotactic C-C chemokines in cutaneous inflammation. *J Leukoc Biol* 1996; **59**:1–5.
49 Raychaudhuri SP, Jiang WY, Farber EM *et al.* Upregulation of RANTES in psoriatic keratinocytes: a possible pathogenic mechanism for psoriasis. *Acta Derm Venereol (Stockh)* 1999; **79**:9–11.
50 Kanda N, Watanabe S. 17beta-estradiol inhibits the production of RANTES in human keratinocytes. *J Invest Dermatol* 2003; **120**:420–7.

© 2010 The Authors
BJD © 2010 British Association of Dermatologists 2010

51 Wakugawa M, Nakamura K, Akatsuka M *et al*. Interferon-gamma-induced RANTES production by human keratinocytes is enhanced by IL-1beta, TNF-alpha, IL-4 and IL-13 and is inhibited by dexamethasone and tacrolimus. *Dermatology* 2001; **202**: 239–45.

52 Colombara M, Antonini V, Riviera AP *et al*. Constitutive activation of p38 and ERK1/2 MAPKs in epithelial cells of myasthenic thymus leads to IL-6 and RANTES overexpression: effects on survival and migration of peripheral T and B cells. *J Immunol* 2005; **175**:7021–8.

53 Zhang M, Zhu L, Feng Y *et al*. Effects of acitretin on proliferative inhibition and RANTES production of HaCaT cells. *Arch Dermatol Res* 2008; **300**:575–81.

54 Russell D, Ross H, Lane EB. ERK involvement in resistance to apoptosis in keratinocytes with mutant keratin. *J Invest Dermatol* 2010; **130**:671–81.

© 2010 The Authors
BJD © 2010 British Association of Dermatologists 2010