

INVESTIGATIVE REPORT

Comparison of Growth-inhibitory Agents by Fluorescence Imaging of Human Skin Re-epithelialization *in vitro*

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Drug screening procedures should preferably utilize experimental settings mimicking the *in vivo* situation. The aim of this study was to evaluate a skin explant model as a tool to identify topical agents with anti-proliferative properties in human epidermis. Re-epithelialization was initiated from a skin punch biopsy explanted onto de-epidermized dermis and cultured at the air–liquid interface in the presence of the epidermal growth factor receptor kinase inhibitor PKI166, tacrolimus or established topical anti-psoriatic drugs: betamethasone, calcipotriol, dithranol and tazarotene. Neo-epidermal extension was traced by fluorescence microscopy prior to histomorphometric analysis. PKI166 at 1 μ M decreased the mean radial outgrowth rate (–19%), frequency of BrdU-positive (–37%) and laminin 5-positive (–45%) cells, indicating reduced proliferation and migration of neo-epidermal keratinocytes. However, the papillomatosis index and epithelial thickness were not significantly affected. Calcipotriol at 1 μ M had a similar effect on the outgrowth rate (–15%) and fraction of laminin 5-stained keratinocytes (–40%). Furthermore, calcipotriol significantly reduced mean neo-epidermal thickness. Equimolar concentrations of the other test compounds had no apparent effect on histology or outgrowth parameters. This study exemplifies the versatility of combined dynamic and morphological analysis and emphasizes the potential of epidermal growth factor receptor-directed inhibition in hyperproliferative disorders of the epidermis. **Key words:** epidermal growth factor; tyrosine kinase inhibitors; proliferation; epidermis; fluorescent tracer.

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In vitro assessment of dermatological drugs generally uses cell assays. However, since multilayered and differentiated epithelium is easy to establish and mimics native tissue better than monolayer cultures do, human epidermis equivalents are generally advocated in skin biology and toxicology research (reviewed in ref. 1). The dermis-based skin explant model represents one such *in*

vitro technique, supporting radial outgrowth of a solely keratinocyte-containing neo-epidermis on a matrix of cell-free human dermis (2, 3). To visualize the process of re-surfacing without harming the tissue in culture, the model was recently complemented with successive fluoroprobing steps (4). Here, this imaging technique was utilized to co-investigate dynamic and morphological effects of topical anti-psoriatic drugs and other growth-inhibitory agents on neo-epidermis in culture.

MATERIALS AND METHODS

Test compounds

Stock solutions (0.1, 1, 10 mM) of betamethasone (Sigma-Aldrich, St Louis, MO, USA), calcipotriol (LeoPharma, Ballerup, Denmark), dithranol (Sigma-Aldrich), PKI166 (Novartis, Basel, Switzerland), tacrolimus (Fujisawa Pharmaceuticals, Osaka, Japan) and tazarotene (Allergan, Irvine, CA, USA) were prepared in 99.5% ethanol.

Skin explant culture

Normal human skin explants (superficial 2-mm punch biopsies) were fibrin-glued onto cell-free de-epidermized dermis (DED; 10-mm diameter) (3, 5) and cultured as described previously in detail (4). Explants were prepared from reduction mamoplasty skin stored at 4°C for less than 48 h. Two explanted DEDs were placed on a modified cell strainer (70 μ m, Falcon, BD Biosciences, Bedford, MA, USA) in each well of a 6-well culture plate and grown at the air–liquid interface (Fig. 1a–b) in an atmosphere of 5% CO₂/air at 37°C. The culture medium comprised Dulbecco's Modified Eagle's Medium:Ham's F12 medium (3:1), 10% foetal bovine serum, 100 μ g/ml streptomycin, 100 U/ml penicillin, non-essential amino acids (all Gibco, Paisley, Scotland, UK), 5 μ g/ml insulin (Lilly, Solna, Sweden), 0.18 mM adenine, 0.5 μ g/ml hydrocortisone, 10^{–10} M cholera enterotoxin, 10 ng/ml epidermal growth factor (all Sigma-Aldrich) and a test compound or vehicle (ethanol, final concentration 0.01%). Medium was renewed after 72 h and then at 24-h intervals throughout the culture period. Three experimental series were performed with quadruplicates of explanted DEDs exposed to each test compound at specified concentrations. Each series included explants and DEDs, respectively, from a common donor. Proliferative cells (entering S-phase) were labelled by incubation with 30 μ M bromodeoxyuridine (BrdU, Roche Diagnostics, Mannheim, Germany) 4 h prior to harvest (4). As positive control for apoptosis, cultures were exposed to 1 μ M staurosporine (Sigma-Aldrich) during the final 24 h of culture (6). The study was approved by the local ethics committee at Uppsala University.

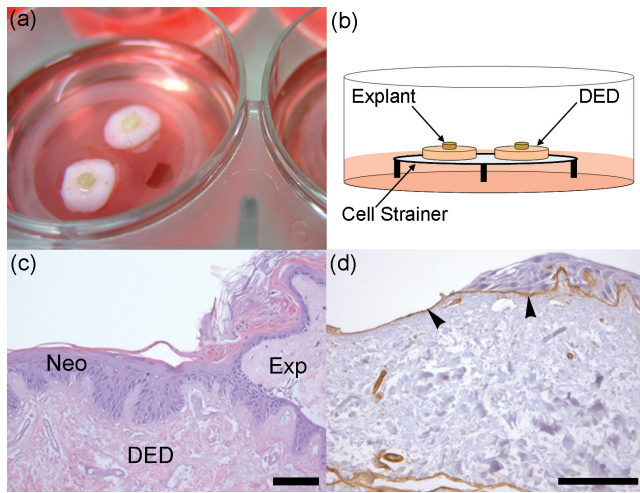


Fig. 1. Skin explant culture on acellular dermis at the air-liquid interface. (a) A modified cell strainer placed in a culture well and used as "raft" for a pair of explant cultures. (b) Schematic representation of the model used in this study. (c) Haematoxylin-eosin staining of neo-epidermis (Neo) generated from a skin explant (Exp) cultured on top of de-epidermized dermis (DED) for 9 days. Bar = 100 μ m. (d) Type IV collagen immunostaining (arrowheads) of vessels and basement membrane zone at the neo-epidermal front. Bar = 100 μ m.

Visualization and outgrowth measurement

Neo-epidermal surface area was traced before medium renewals by fluorescence imaging of re-epithelialization (FIRE) technique (4) using fluorescein diacetate (FDA, Sigma-Aldrich) as indicator of live cells (7). Epidermal outgrowth (total fluorescent area minus explant area, Fig. 2, insert) was determined daily by image analysis while maintaining the tissue in culture and quantified using DP-soft image analysis programme (Olympus, Hamburg, Germany).

Histology and immunostaining

Cultures were harvested on day 9 and fixed in 4% neutral-buffered formaldehyde before embedding in paraffin. De-paraffinized cross-sections (5 μ m) were stained with haematoxylin-eosin and

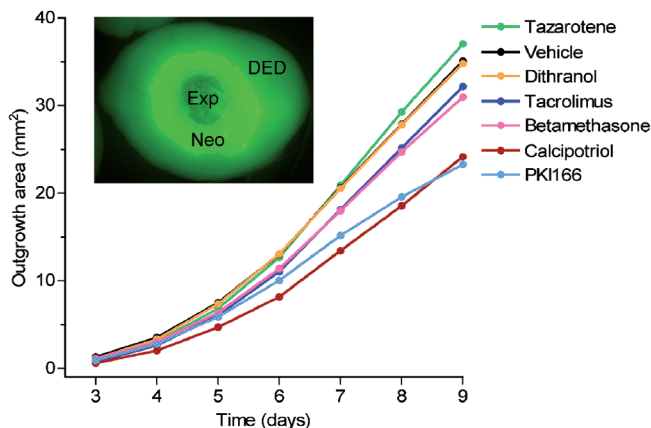


Fig. 2. Mean neo-epidermal outgrowth areas (mm²) generated over time from explants cultured in presence of different test compounds (1 μ M). Values represent means from three experimental series. Insert: DED with a central skin explant (Exp); visualization of neo-epidermis (Neo) during culture by fluorescein diacetate probing and fluorescence microscopy (day 7).

evaluated for gross morphology, neo-epidermal thickness (total area of viable neo-epidermis/horizontal length of neo-epidermis) and papillomatosis index (length of neo-epidermis basement membrane zone (BMZ)/horizontal length of neo-epidermis) (4). BrdU-immunostaining was conducted as described and proliferative cell density was defined as the number of BrdU-reactive cells per mm length of the BMZ (4). Histometry and BrdU-density were analysed in duplicate sections from two (series 1–2) or four (series 3) samples of each treatment group using Leica Q500 image analysis software. For type IV collagen detection, sections were pre-treated for 20 min in 95°C Target Retrieval Solution (S1699, Dako-Cytomation, Glostrup, Denmark) to improve staining results, and incubated for 30 min at room temperature with primary antibody (1:25 dilution, clone CIV 22, DakoCytomation) (8). Laminin 5-producing cells were identified with antibodies (1:1700) against the γ -2 chain (9, 10). Sections were pre-treated with 1% trypsin for 30 min at 37°C. StreptABComplex/HRP Duet mouse/rabbit (DakoCytomation) kit was used for visualization of collagen IV and laminin 5. The number of laminin 5-positive cells was determined in a blinded manner within the outermost (1.2 mm) neo-epidermal front (day 9) in duplicate sections from two (series 1–2) or four (series 3) samples per group. Apoptotic cells were immunostained for cleaved caspase-3 (1:200, Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. For antigen unmasking, slides were brought to boiling in 1 mM EDTA pH 8.0 and maintained at sub-boiling temperature for 15 min in a microwave. PBS/0.1% Tween-20 was used as wash buffer. Biotinylated anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA) was detected with ABC technique. Diaminobenzidine (DAB, Vector Laboratories) was used as chromogenic substrate. Slides were counterstained with haematoxylin and mounted in glycerol jelly. The density of caspase-3-positive cells in neo-epidermis (day 9) was assessed in a blinded manner in duplicate sections from four samples per group in series 3; (–): 0–3 positive (pos) cells/mm neo-epidermis, (+): 4–50 pos cells/mm, (++) : > 50 pos cells/mm. All immunohistochemical staining procedures were performed for the 1- μ M groups only and included negative controls omitting the primary antibody.

Statistical analysis

To test statistical differences in radial growth rates between drug- and vehicle-exposed samples, individual growth rates were estimated from serial area measurements by a mixed effects model (11). In this model, the factors: treatment, experimental series and interaction between treatment and series were included as fixed effects. The intra-sample correlation was accounted for by allowing individual intercepts for the samples as random effects. The model ignores deviation in linearity and estimates radial progression over day 3–9, regardless of when curve linearity occurs. Since both intra-experimental and intra-sample correlations were considered and the deviant samples were few, the model was regarded as appropriate for our calculations. Analyses were performed with SAS version 8.02 (SAS Institute Inc., Cary, NC, USA). Absolute differences of means in BrdU-density, laminin 5-positive cells and histometric values between drug- and vehicle-exposed groups were calculated for each experimental series and analysed statistically by paired *t*-test using SPSS version 12.01 (SPSS Inc., Chicago, IL, USA).

RESULTS

Morphology

The neo-epidermis formed in the presence of either test compound was multilayered, containing the microscopically

layers typical of native human epidermis attached to a BMZ expressing type IV collagen (Fig. 1c–d). The epithelium was somewhat thicker (8–10 viable cell layers) with a prominent orthokeratotic stratum corneum nearby the explant, compared with the advancing edge. In general, the keratinocytes had more frequent mitotic figures and were less polarized than in natural skin. A few keratinocytes displayed dyskeratosis or intracellular vacuoles and the horny layer contained occasional parakeratotic cells. Overall, the granular layer was somewhat thinner or less regular than that seen *in vivo*. Neo-epidermis grown in presence of calcipotriol (1 μ M) contained slightly larger nuclei and nucleoli, whereas the opposite was observed in cultures exposed to tazarotene. Otherwise, no drug-related alterations in tissue architecture or histological signs of toxicity were observed.

Re-epithelialization

Fluorescence imaging of skin cultures by FIRE technique revealed that re-epithelialization of the DED was visible on day 3 or 4 and increased continuously until the peripheral edge of the DED was reached (Fig. 2). The outgrowth curves for tazarotene, dithranol, tacrolimus and betamethasone (all 1 μ M) were close to vehicle whereas cultures exposed to PKI166 and calcipotriol produced smaller outgrowth areas. The radial outgrowth rates were approximately linear for all compounds tested (calculated for days 3–9), although occasional growth curves levelled off or declined shortly before culture termination (Fig. 3). In a few instances, this resulted from rapidly growing segments of neo-epithelium passing the edge of the DED, thus escaping detection by microscopy on day 8 or 9. In general, however, neo-epidermis expanded in a concentric fashion around the explant.

Although the starting points (i.e. intercepts) for individual specimens differed slightly, the growth rates (given by the slope of the growth curves) were consistent both intra- and inter-experimentally (Fig. 3). The radial growth rate of samples cultured with PKI166 or calcipotriol at different concentrations (10 nM, 100 nM and 1 μ M) seemed to be dose-dependent. As expected, markedly growth inhibitory effects relative to control cultures occurred at the highest drug concentration (Table I). PKI166 and calcipotriol at 1 μ M concentration reduced the rates of dermal re-surfacing (means: 393 and 411 μ m/day, respectively) significantly compared to vehicle-exposed explants (mean 485 μ m/day, $p < 0.0001$). In addition, 1 μ M betamethasone slightly inhibited neo-epidermal growth, whereas tazarotene at 1 μ M promoted epidermal growth. Since dithranol is poorly soluble in ethanol, a control experiment was made using dimethyl sulphoxide as alternative vehicle. However, the same results were obtained with either solvent (data not shown).

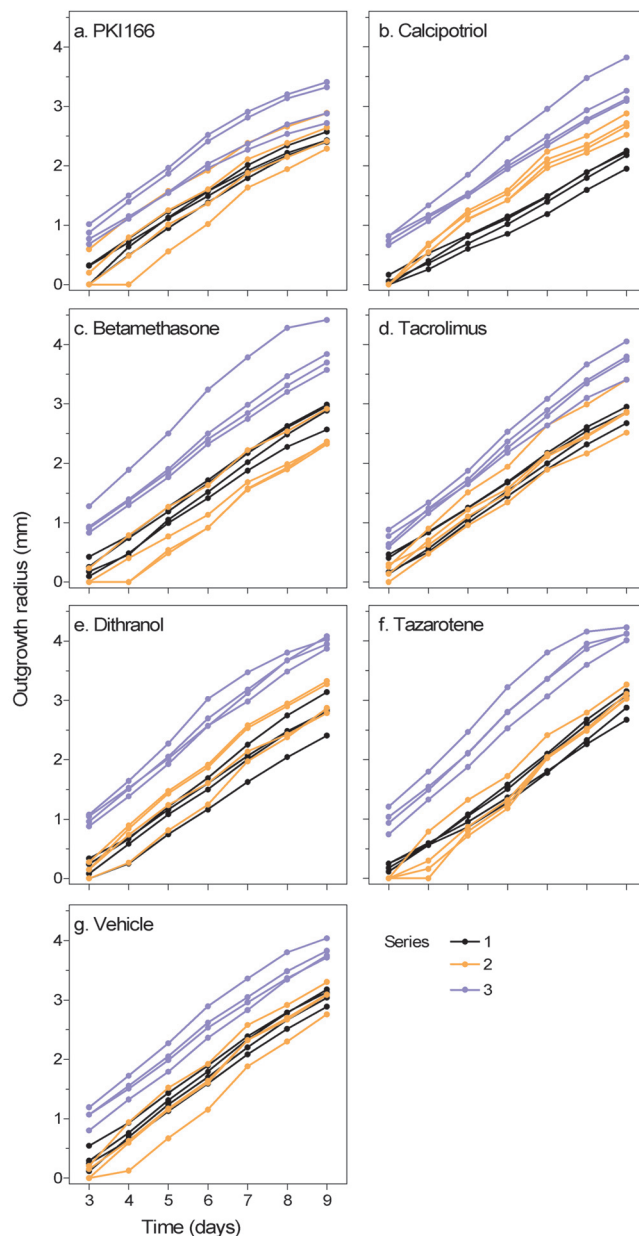


Fig. 3. Radial outgrowth courses of neo-epidermis exposed to 1 μ M drug concentration. Data from three separate experiments (series 1–3) are given. Radial growth curves for individual samples exposed to each drug (a–f) and vehicle (g).

Proliferation and histomorphometry

To evaluate any anti-proliferative effects on neo-epidermal keratinocytes, the specimens were examined by BrdU-incorporation assay. Furthermore, neo-epidermal thickness, papillomatosis index and gross morphology were assessed. As shown in Fig. 4a, over the three series, the mean proliferative cell density was reduced in neo-epidermis grown in presence of 1 μ M PKI166 (16.3 BrdU-positive cells/mm basal membrane) compared to vehicle (25.8, $p = 0.016$). No other drug affected the proliferative cell density significantly. Although

Table I. Estimated radial growth rates ($\mu\text{m}/\text{day}$) for drugs tested at three different concentrations. Mean values $\pm$ SEM from three independent experiments are given. $n=12$ for each group

Drug	1 μM		100 nM		10 nM	
	Growth rate	p^a	Growth rate	p^a	Growth rate	p^a
PKI166	393 $\pm$ 9	<0.0001	466 $\pm$ 8	0.168	475 $\pm$ 5	0.459
Calcipotriol	411 $\pm$ 16	<0.0001	495 $\pm$ 15	0.419	510 $\pm$ 9	0.051
Betamethasone	456 $\pm$ 12	0.033	459 $\pm$ 8	0.054	478 $\pm$ 10	0.625
Tacrolimus	470 $\pm$ 15	0.274	457 $\pm$ 9	0.035	460 $\pm$ 12	0.064
Dithranol	486 $\pm$ 12	0.932	476 $\pm$ 10	0.494	482 $\pm$ 13	0.848
Tazarotene	517 $\pm$ 13	0.015	501 $\pm$ 11	0.209	501 $\pm$ 11	0.223
Vehicle	485 $\pm$ 7					

^aStatistical test of difference in radial growth rate between drug and vehicle as described in the materials and methods section.

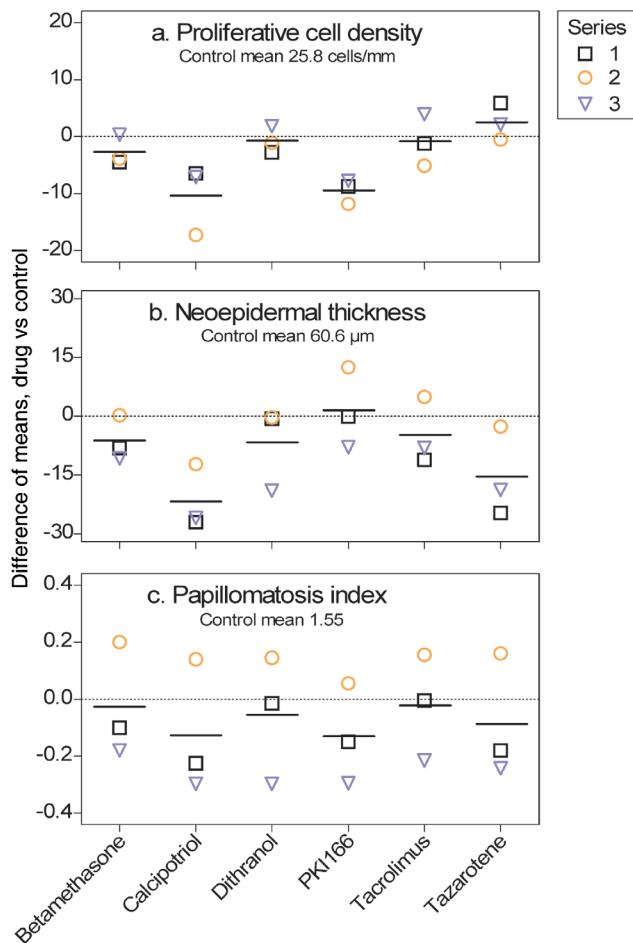


Fig. 4. Proliferative and histometric analysis of skin cultures grown in the presence of test compounds (1 μM) relative to vehicle (control). Differences of means between drug and control are given for each series, normalizing the control to zero (dotted line). Mean absolute values of controls are given and mean differences for individual drugs are indicated by solid lines. (a) Proliferative cell density (BrdU-positive cells in neo-epidermis/mm basal membrane). (b) Neo-epidermal thickness (μm , area of viable neo-epidermis/horizontal length of neo-epidermis). (c) Papillomatosis index (length of basal membrane/horizontal length of neo-epidermis).

calcipotriol seemed to reduce the mean BrdU-labelling density (15.5), the effect was statistically insignificant due to high variability ($p=0.099$). Mean neo-epidermal thickness was reduced in cultures with calcipotriol (38.8 μm), compared to vehicle (60.6 μm , $p=0.045$). The other test compounds, including PKI166, did not significantly affect epithelial thickness (Fig. 4b). None of the drugs altered the papillomatosis index of neo-epidermis (Fig. 4c).

Migration

Cytoplasmic laminin 5 was used as indicator of migrating keratinocytes (12) in the groups with small (PKI166 and calcipotriol) and large (vehicle and tazarotene) outgrowth areas (see Fig. 2). The number of keratinocytes displaying strong laminin 5 immunostaining correlated well with the length of neo-epidermis, since both PKI166 and calcipotriol reduced the amount of positive cells, whereas tazarotene did not. Compared to the vehicle (100%), the number of laminin 5-positive cells was 55% ($p=0.040$) in the PKI166 group, 60% ($p=0.046$) in the calcipotriol group and 103% ($p>0.05$) in the tazarotene group (Fig. 5). The clear staining of extracellular laminin 5 (Fig. 5, arrowheads) and type IV collagen (Fig. 1d, arrowheads) along the papillary surface of the DED indicates that these basement membrane components are well preserved.

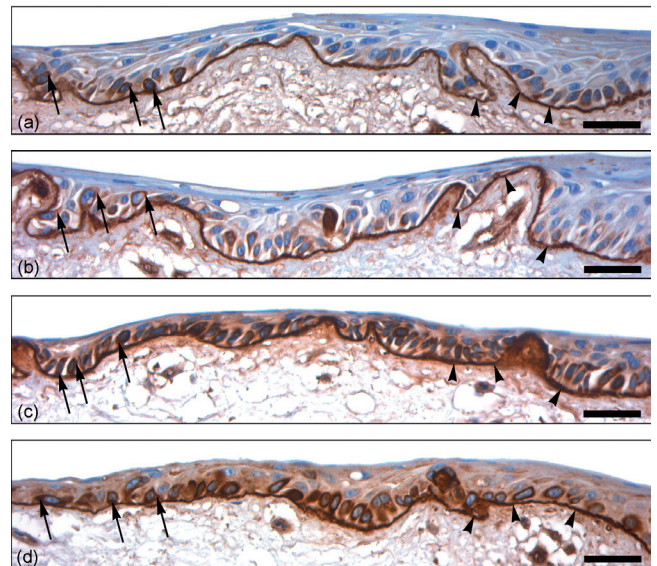


Fig. 5. Laminin 5 immunostaining of neo-epidermis exposed to 1 μM of (a) PKI166, (b) calcipotriol, (c) tazarotene and (d) vehicle. The samples were selected as representatives of small (PKI166- and calcipotriol-exposed, mean 23.3 and 24.2 mm^2 , respectively) or large (tazarotene- and vehicle-exposed, mean 37.0 and 35.1 mm^2) outgrowth areas. Cytoplasmic (arrows) and basement membrane (arrowheads) laminin 5 stainings are indicated. Bars = 50 μm .

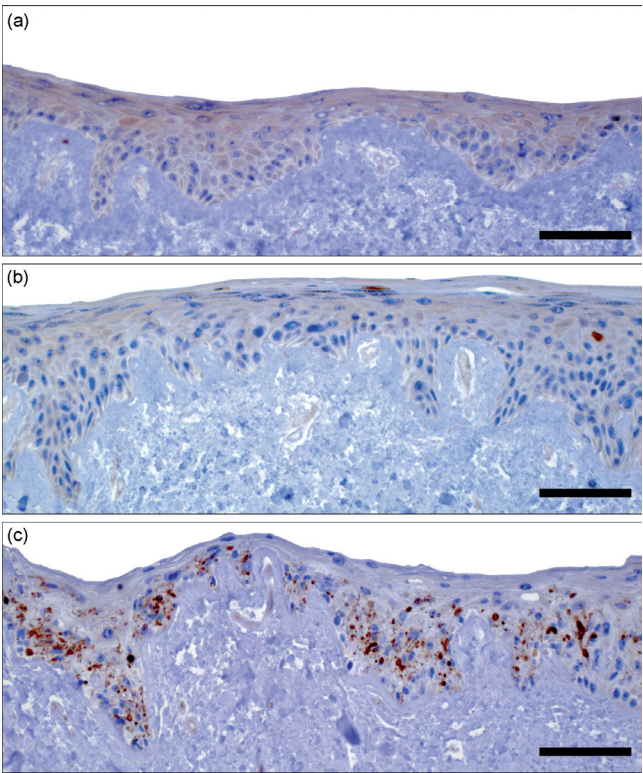


Fig. 6. Caspase-3 immunostaining of neo-epidermis exposed to 1 μ M of (a) PKI166, (b) vehicle and (c) staurosporine (positive control) as described in the materials and methods section. Bars = 100 μ m.

Apoptosis

To evaluate signs of apoptosis, paraffin-embedded tissue was sectioned and immunostained for cleaved caspase-3. Staurosporine-exposed (1 μ M, 24 h) tissue served as positive control. Staurosporine generated a high proportion of cleaved caspase-3-positive keratinocytes (92 positive cells/mm), whereas all samples exposed to test drugs were immuno-negative or contained very few caspase-3-positive cells (0–3 cells/mm), as exemplified in Fig. 6 (see also Table II).

DISCUSSION

Pre-clinical evaluation of topical drugs *in vitro* is traditionally based on cell or skin explant cultures attached to plastic that supports growth of keratinocytes or fibroblasts in monolayer. Despite a continuing trend towards complex skin reconstructs, simple *in vitro* models without all native constituents may still be appropriate, e.g. when studying direct drug responses in selected cells or tissue compartments. In this study, skin explants were adhered onto acellular DED and cultured on rafts to encourage formation of *in vivo*-like epidermis. Human skin biopsies served as source of regenerating keratinocytes, whereas dermal fibroblasts diminished in number and were eventually

Table II. Summary of dynamic and histometric results of neo-epidermis exposed to drug (1 μ M) compared to vehicle (control). Mean values from three independent experiments are given. Absolute values for vehicle-exposed samples are included for reference. Apoptosis is expressed semi-quantitatively using staurosporine-exposed samples as positive control

Exposure	Growth rate	Prolife- ration	Neo- epidermal thickness	Papillo- matosis index	Migra- tion	Apopto- sis
	% change vs. vehicle					
PKI166	–19**	–37*	+5	–7	–45*	(–)
Calcipotriol	–15**	–38	–35*	–6	–40*	(–)
Betamethasone	–6*	–10	–9	0	NA	(–)
Tacrolimus	–3	–1	–6	0	NA	(–)
Dithranol	0	–2	–10	–2	NA	(–)
Tazarotene	+7*	+11	–23	–4	3	(–)
Staurosporine			NA			(++)
Vehicle	485 ^a	28.5 ^b	60.6 ^c	1.55 ^d	28.7 ^e	(–) ^f

^aRadial growth rate in μ m/day

^bNumber of BrdU-positive cells/mm basement membrane

^cNeo-epidermal thickness in μ m (total area of viable neo-epidermis/horizontal length of neo-epidermis).

^dPapillomatosis index (length of neo-epidermal basement membrane zone/horizontal length of neo-epidermis).

^eNumber of laminin 5-positive cells in the outermost neo-epidermal front.

^fDensity of caspase-3-positive (pos) cells in neo-epidermis. (–): 0–3 pos cells/mm, (++) > 50 pos cells/mm.

* $p < 0.05$, ** $p < 0.01$, statistical test of difference between drug and vehicle as described in the materials and methods section.

NA, not analysed.

encapsulated due to epibolia. Likewise, Langerhan's cells and melanocytes disappeared during culturing, probably due to unfavourable medium conditions (4). Thus, an expanding disc of multilayered keratinocytes was generated, allowing pharmacodynamic studies of re-epithelialization. Modified versions of the DED-based explant model have emerged to elucidate different aspects of epidermalization, e.g. DEDs with denuded BMZ may be prepared to obtain a more ulcer-like substrate (13), or cultured fibroblasts may be incorporated to study dermal-epidermal cell interactions (14, 15). Since our ambition was to study keratinocyte-directed drug effects on epithelialization under non-ulcer conditions, we chose cell-free, but otherwise intact human dermis as substrate. The DED has preserved basement membrane components, as shown previously (16, 17) and confirmed by distinctive linear immunostainings of collagen type IV and laminin 5. Collagen IV is a major component of lamina densa, whereas laminin 5 associates with lamina lucida of the BMZ and facilitates migration of epidermal cells (18, 19).

The course of re-epithelialization was studied under influence of drugs used in management of psoriasis, dithranol (20) and betamethasone (21), representing the two first generations of anti-psoriatic remedies (22). From third-generation drugs, three examples were included: calcipotriol, a potent vitamin D3 analogue (23);

tacrolimus, a calcineurin inhibitor mainly used in atopic eczema (24); and tazarotene, a receptor-specific retinoid (25). Although these drugs affect diverse processes and cell types of the skin, they all have more or less anti-proliferative activity on epidermal cells (26–30). The established drugs were compared with a more recent anti-proliferative substance, PKI166, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, not yet launched as drug for clinical application in dermatology (31, 32). In the present study, only PKI166 and calcipotriol displayed marked inhibitory effects on radial outgrowth rates.

Morphologically, the overall architecture of neo-epidermis was characterized by a mixture of proliferative and differentiating features as a consequence of both mitogenic (EGF, insulin) and differentiating (serum, air) stimuli. The general histological pattern, resembling that of hyperproliferative epidermis, was not affected by drug exposure. However, calcipotriol reduced the average thickness of viable neo-epidermis and the keratinocytes displayed slight increments of their nuclei and nucleoli. The significance of the latter finding is unclear, but a previous report claimed that keratinocytes exposed to 1 µM calcipotriol had enlarged nuclei (28). No obvious signs of neo-epidermal toxicity, such as single cell necrosis, were found in our samples, but we did not assay highly sensitive markers of drug cytotoxicity.

Since re-epithelialization is a result of closely associated cellular activities, we tried to relate drug effects to apoptosis, proliferation and migration in neo-epidermal cells (summarised in Table II). Apoptosis was unlikely as an outgrowth-reducing factor for PKI166 and calcipotriol since neither drug was associated with cleaved caspase-3 expressing keratinocytes. By contrast, PKI166 clearly reduced the density of cycling keratinocytes in neo-epidermis, whereas calcipotriol did not. This was an unexpected finding since topical application of calcipotriol – albeit at 10 to 100-fold drug concentration – is known to markedly inhibit keratinocyte proliferation in lesional psoriatic epidermis (33, 34). To investigate a possible correlation between neo-epidermal outgrowth and cell migration, laminin 5 immunostainings were performed. Cytoplasmic staining for laminin 5 reflects active synthesis of this protein and is usually encountered in migrating keratinocytes of suction blisters and acute wound edges (12, 35). We selected four sample groups to judge laminin 5 as a migration marker: PKI166 and calcipotriol (representing small outgrowths), tazarotene (representing large outgrowths) and vehicle (control). As expected, the length of the neo-epidermal tongue by day 9 strongly correlated with the frequency of laminin 5-positive cells. Both PKI166 and calcipotriol reduced the number of laminin 5-positive cells significantly, supporting the idea that inhibition of re-epithelialization by these drugs is

linked to anti-mitotic effects and/or reduced migration of keratinocytes. Tazarotene, which had minor stimulatory effects on neo-epidermal outgrowth, did not differ from vehicle-exposed cultures in terms of intracellular laminin 5 staining.

Selective inhibition of EGFR tyrosine kinase with PKI166 strongly affected re-epithelialization. EGFR (HER-1, ErbB1) is the prototypic member of the EGFR family of receptor tyrosine kinases and widely distributed in epithelial tissues. Epidermal keratinocytes express both the receptor and several of its ligands, including transforming growth factor- α , amphiregulin and heparin binding-EGF (HB-EGF). Signalling through EGFR regulates essential processes in epithelia, e.g. by promoting cell proliferation and migration, regulating differentiation and preventing apoptosis (36–38). In normal adult skin, EGFR is mainly expressed by keratinocytes within the basal compartment of the epidermis (39). Squamous cell carcinomas and psoriatic plaques overexpress EGFR (40–42) and a putative role for EGFR in psoriasis hyperplasia has been suggested (43–45). High expression of EGFR and HER-2 (ErbB2) has also been observed in a variety of solid tumours (46). Accordingly, selective tyrosine kinase inhibitors and receptor antibodies directed against members of the EGFR family have been developed as cancer drugs (31, 32, 46, 47). Like many other low molecular weight EGFR inhibitors, PKI166 interacts with the intracellular ATP-binding site of the receptor, thus inhibiting autophosphorylation and downstream EGFR signalling (32). In phase I clinical trials, oral administration of PKI166 resulted in unexpectedly high liver toxicity and the drug was therefore stopped from further development (31). In our study, where PKI166 was selected as model compound for EGFR/HER-2 inhibition of epidermis *in vitro*, the drug efficiently inhibited regeneration of neo-epidermis. This agrees with the general anti-proliferative response to EGFR inhibition in keratinocytes in monolayer culture (45, 48–50) and skin transplant models in mice (43). Better insight into the pathobiology of skin diseases, and advancements in *in vitro* modelling, will help to identify therapeutic targets among EGFR family members and other receptor tyrosine kinases in hyperproliferative epidermal disorders.

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