Novel insights into transcriptional changes during keratinocyte differentiation and ultraviolet radiation response

Doctoral theses

Gyorgy Paragh, MD

Semmelweis University
Molecular Medical Chemistry School of Doctoral Studies

Mentor: Norbert Wikonkál, MD, PhD

Official reviewers:
Csík Gabriella, PhD
László Mátyus, MD, DSc
Györgyi Rontó, MD, DSc

Comprehensive exam committee:
President: László Kopper, MD, DSc
Members: Judit Demeter, MD, DSc
Péter Antal-Szalmás, MD, PhD

Budapest
2009

INTRODUCTION

The epidermis is the major interface between the human body and the environment. It protects against dehydration, chemical agents, infection and physical environmental effects. Keratinocytes accumulate and produce necessary components of the stratum corneum the forefront and most important requisite of these barrier functions during their elaborate differentiation process. Therefore intact barrier function relies on undisturbed differentiation of keratinocytes. Conditions affecting keratinocyte differentiation such as dermatological diseases (atopic dermatitis, psoriasis and various genodermatoses ect.), acute UV damage and drug treatment have all been found to alter the barrier functions of the epidermis, while barrier disruption by organic solvents is known to alter keratinocyte differentiation. Although the inter-reliance of these processes and the importance of undisturbed keratinocyte differentiation are well understood and have been extensively studied, many aspects of keratinocyte differentiation are still obscure. Only a relatively small set of genes regulated during keratinocyte differentiation have so far been identified and mostly via hypothesis driven approaches. Furthermore regardless of the increasing attention to regulatory circuits governing keratinocyte differentiation (vitamin D receptor, retinoid receptor, p63 ect.) many of the underlying transcriptional regulatory mechanisms are not well understood.

Keratinocyte differentiation involves a complete restructuring of cellular lipid composition besides leading to changes in the cellular proteome. The produced lipids are major constituents of the stratum corneum lipid lamellae, and create the permeability barrier. One of the major lipid classes are ceramides. As many other lipophilic molecules these sphingolipids are also known to regulate cellular functions. Some ceramides have been implicated in apoptosis initiation and cellular differentiation promotion in keratinocytes. Despite of the potential therapeutic value of this latter effect a comprehensive comparison of ceramide species was lacking.

A proper model is necessary to study keratinocyte differentiation. To date most studies used a few known keratinocyte differentiation genes to assess the differentiation effect of tested molecules, in either two dimensional cultures of keratinocytes or differentiated three dimensional in vitro epidermal models. Unfortunately neither approach provides a well suited system for comparison of molecule effects. Two dimensional models with only a few keratinocyte differentiation genes tested can show large bias due to gene specific compound effects. Whereas the three dimensional model is incapable of identifying effects in early differentiation and also poses compound delivery problems, besides the obvious limitations and bias caused by the assessment of expression changes in only a few genes. Therefore a better system for analyzing differentiation effects of sphingolipids had to be developed.

ATP binding cassette transporter 12 (ABCA12) mutations are thought to result in greatly altered epidermal structure in the form of lamellar and in more serious cases harlequin ichthyoses due to the abrogated transportation of sphingolipids and other barrier building blocks accumulated in lamellar bodies. Nuclear receptors have been shown to stimulate lamellar body secretion and epidermal lipid metabolism, as well as cholesterol sulfate synthesis. Liver X receptor, peroxisome proliferator-activated

receptor (PPAR) alpha, PPAR-gamma and PPRA-delta activation have been shown to improve permeability barrier homeostasis; nevertheless their role in the regulation of ABCA12 expression was unknown.

Ultraviolet B (UVB) radiation is undoubtedly one of the most important environmental stressors affecting the epidermis. Besides its direct genotoxic effect it influences most epidermal homeostatic networks. It is known to abrogate epidermal lipid synthesis, lamellar body secretion and disrupt the epidermal lipid barrier, and it has also been shown to directly affect the expression of ABC transporters. Moreover pronounced vascular changes also take place in the skin after UV irradiation. The pathogenic role of several hypoxia regulated genes has been proposed in these UVB induced skin changes (e.g. endothelial growth factor [VEGF] and hemoxygenase-1 [HMOX1]). However the role of hypoxia signaling in the UVB response has not been studied.

Our current work focused on the transcriptional changes taking place during keratinocyte differentiation, the transcriptional effects of sphingolipid derivatives and specific transcriptional regulatory networks in the epidermis (ABCA12 regulation, and UVB hypoxia) to obtain novel insight into the keratinocyte differentiation process and find potential new tools and targets for the treatment dermatological diseases.

AIMS

During our studies we initially aimed to set up, and validate a fast and easy to use system, enabling quantitative comparison of compound effect, for testing suspected keratinocyte differentiation modifying sphingolipid compounds. Moreover we also aimed to identify previously undescribed novel keratinocyte differentiation genes, to better understand the homeostatic system guiding keratinocytes through the differentiation process, and to provide a more complete list of transcriptional changes during the process. Focusing on the effects specific regulatory networks of keratinocyte homeostasis, we studied the potential for the role of the hypoxia system in mediating some of the effects of UVB radiation in keratinocytes, and studied the nuclear receptor regulation of the recently identified keratinocyte ceramide transporter, ABCA12.

Specific aims

1. To set up a fast, efficient system for testing compounds with suspected keratinocyte differentiation modifying potential.
    a. To verify the ability of the keratinocyte differentiation system to effectively signal pro- or anti-differentiation effects of known keratinocyte differentiation modulator compounds.
        i. To validate the ability of the model
        ii. To find new potential keratinocyte differentiation genes in the model by studying the gene expression profile.
        iii. To validate interesting keratinocyte differentiation induced genes within a longer term calcium induced *in vivo* keratinocyte differentiation model.
    b. To test the keratinocyte differentiation modifying potential of various sphingolipid compounds by studying the gene expression changes of a high number of keratinocyte differentiation genes, in order to select the most promising compounds for further use.
2. To study the transcriptional regulation of ABCA12, the epidermal sphingolipid transporter and identify the effects of nuclear receptor ligands on ABCA12 gene expression in keratinocytes.
3. To analyze the effect of UVB radiation on hypoxia signaling in keratinocytes.
    a. To determine the timeline of HIF-1alpha protein expression regulation *in vitro* after low dose broadband UVB treatment.
    b. To determine the mRNA expression of HIF-1alpha and HIF-1alpha target genes after UVB.
    c. To study the potential regulatory mechanism responsible for the UVB regulation of HIF-1alpha.

METHODS

Reagents, chemicals and technical equipment

The most commonly used chemicals and inhibitors were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Fairlawn, NJ) unless otherwise stated. Troglitazone (Tro), ciglitazone (Cig) and TO901317 (TO) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). PPAR-gamma activator GI 251929X (GI) and PPAR-delta activator GW 610742X (GW) were obtained from GlaxoSmithKline (Brentford, UK), while 1α,25-Dihydroxyvitamin D_3 (VD3) was purchased from BIOMOL International (Plymouth Meeting, PA). 22(R)-OH-cholesterol (22R), clofibric acid (CLO), WY14643 (WY), all-*trans*-retinoic acid (ATRA), and 9-*cis*-retinoic acid (9-*cis*-RA) were purchased from Sigma (St Louis, MO). Molecular grade chemicals such as TRI Reagent were obtained from either Sigma or Fisher Scientific (Fairlawn, NJ). All reagents and supplies for Real-time PCR were purchased from Applied Biosystems (Foster City, CA). Sphingolipid compounds were provided by Degussa GmbH (currently known as Evonic GmbH, Düsseldorf, Germany).

Cell culture

HaCaT cells

HaCaT immortalized human keratinocyte cell line was obtained from Norbert E. Fusenig. Cells were grown at 37°C under normoxic conditions (5% CO_2, 95% air) in D-MEM supplemented a suggested by the literature. For UVB and hypoxia experiments HaCaT cells were grown to 70-90% confluence. Fresh medium was added 1 day before starting the experimental procedure. Cell treated with 4 hours of hypoxia in a hypoxia chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) in a gas mixture containing 1% O_2, 5% CO_2 and 94% N_2 (data not shown) served as positive controls.

Normal human epidermal keratinocyte (NHEK) isolation and culture

Skin samples were obtained from female donors undergoing plastic surgery. Keratinocytes were isolated after overnight by dermo-epidermal separation in Dispase II (2.4 U/ml, Roche Diagnostics, Germany) and by subsequent trypsin digestion. Cells were seeded and propagated in KBM media (Cambrex Bioscience, Walkersville, Maryland) with 0.06 mM $CaCl_2$ and fully supplemented according to the manufacturers protocol and. Cultures of normal human keratinocytes (NHEK) were split at 60-70% confluence. Medium was replaced every 2-3 days and cell morphology evaluated by phase contrast microscopy. In nuclear receptor ligand ABCA12 mRNA expression studies second passage human keratinocytes from foreskin were seeded and maintained in 0.07mM Ca++ serum-free keratinocyte medium (154CF) with growth supplement (Cascade Biologies, Inc., Portland, OR). After cells attached to the culture dish, the medium was switched to either low (0.03 mM) or high calcium (1.2 mM) medium. In a typical experiment, cells were treated with reagent at optimized concentration at pre-confluence (60%-70%) in either low or high calcium conditions, and were harvested at 80%-100% confluence. Cells were either treated with nuclear receptor ligands or control keratinocytes were treated with vehicle (0.05% ethanol or DMSO).

NHEK differentiation models

Confluence induced differentiation was carried out by growing second passage cells to 80-90% confluence. The experiment was initiated before the appearance of any visual signs of keratinocyte differentiation (day 0). After media change NHEK cells were either administered control or test compounds or were only administered empty

vehicle (ethanol) and were allowed to grow to confluence. Cells were harvested on day 0 (controls), day 1 and day 4. At each time point and treatment modality three 60cm^2 culture dishes were harvested from keratinocytes of each of the skin donors to enable isolation of enough RNA for microarray studies, and real time quantitative PCR. Calcium induced differentiation experiments were carried out by switching growth media (0.06mM Ca^{2+}) to 1.2mM Ca^{2+} containing fully supplemented Keratinocyte Basal Medium (KBM; Cambrex Bioscience, Walkersville, Maryland). Cells were harvested every second day until 10 days after differentiation induction.

Cytotoxicity study

Keratinocytes were cultured in 24-well plates until confluence, and test substances were administered subsequently along with controls (solvent control, SDS and H_2O_2). Cells were harvested at the time of treatment (day 0), 24 hours later (day 1) and 96 hours later (day 4). Lactate-dehydrogenase (LDH) activity was measured in the culture supernatant (S) and the remaining cell monolayers (M). Adenylate kinase release and cellular ATP content were used to determined ToxLight and ViaLight plus assays (Cambrex, Rockland, ME, USA) respectively. Assays were carried out according to the manufacturer's suggestions. Compounds showing at least intermediate cytotoxicity with more than 2 assays or high cytotoxicity in one assay and low in at least one were considered cytotoxic at the given time point. Results were used to determine safe doses of molecules for the keratinocyte differentiation studies.

Cell harvesting

For protein studies cells were harvested in 200µl lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 1% NP40) supplemented with protease and phosphatase inhibitors (1 mM Na_3VO_4, 1 mM PMSF, 10 mM benzamidine, 20 mM NaF, 1 mM para-nitrophenyl-phosphate, 10 µM lactacystin and 1:100 diluted SIGMA Inhibitor Cocktail). Samples were sonicated for 20 seconds and centrifuged (10 000 rcf, 10 min, 4°C) and aliquots of the supernatant were stored at -20°C until protein-measurement (DC Protein Assay reagents, BioRad, Hercules, CA, USA) and for gel-electrophoresis.

UV irradiation

For broad-band UVB irradiation an FS-20 fluorescent lamp (Westinghouse Electric, Pittsburgh, PA, USA) was used. Prior to experiments, regular UVB dosimetry was performed by a VLX-3W radiometer equipped with a CX-312 sensor (Vilber Lourmat, France). Mock-treatments were carried out the same way with the exception of UV irradiation.

Apoptosis assay

In order to determine biological efficacy of UV irradiation the percentage of apoptotic cells was determined by flow-cytometry using the Annexin V, propidium iodide (PI) method (Roche, Indianapolis, USA). Staining was carried out according to the manufacturer's protocol. Flow-cytometry was carried out on a Becton-Dickinson FACScan (Franklin Lakes, NJ, USA) flow-cytometer.

Western bloting

SDS polyacrylamide gel-electrophoresis was carried out using standard equipment and reagents BioRad (Hercules, CA, USA). Anti-HIF-1α antibody was purchased from BD Transduction Laboratories (Lexington, KY, USA), whereas the anti-phospho-AKT (Ser 473), and anti-AKT antibodies were from Cell Signaling Technology (Beverly, MA, USA). Samples were boiled in Laemmli loading buffer for 5 min and equal amounts of proteins were separated on a 7.5% polyacrilamide gels. The proteins were transferred to nitrocellulose or PVDF membrane, and immuno-detected with specific primary and horseradish-peroxidase labeled secondary antibodies. For ABCA12 detection 80-100µg protein was fractioned on pre-cast gels (3-8%) and transferred to PVDF membranes using the Enhanced Chemiluminescence (ECL) Western Blotting Detection System Kit.

RNA isolation

For microarray experiments and real-time PCR assays total RNA was extracted from cultured cells using the RNeasy Protect midi Kit (Qiagen, Hilden, Germany). Purity and integrity of the RNA was determined by Agilent 2100 bioanalyzer (Agilent Technologies, USA). RNA concentration was measured spectrophotometrically and samples were stored at -80 °C. During HIF-1alpha and hypoxia experiments RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA).

Real-time RT-PCR (TaqMan™) analysis

First-strand cDNA synthesis was performed with the Reverse Transcription System from Promega (Madison, WI, USA). Real-time RT-PCR analysis was performed with an ABI7900HT machine (Applied Biosystems, Chicago, IL, USA). Relative quantitation was performed according to the delta-delta-Ct method with 18S ribosomal RNA (rRNA) used as normalizer. Alternatively in HaCaT UVB studies reverse transcription was carried out with the "RETROscript" (Ambion) kit. Real time quantitative PCR was carried in an iCycler Real Time PCR machine (Bio-Rad) using TaqMan universal PCR master mix or SYBR green PCR Master Mix (Applied Biosystems). In these studies human beta-actin mRNA levels served as normalizer. Samples were run in triplicates and at least 3 independent experiments were preformed to enable thorough verification of results. Depending on sample number Mann–Whitney U or t-test were used for statistical assesment of results. A value of $p<0.05$ was considered significant. In studies aiming to identify the effect of nuclear receptor ligand stimulation on ABCA12 expression first strand cDNA for PCR was synthesized using an iScript™cDNA Synthesis Kit (BioRad, Hercules, CA, USA) following manufacturer's protocol.

DNA microarray and microarray data analysis

Whole transcriptome gene expression profiles were determined using Affymetrix HGU133 plus 2.0 GeneChips (Affymetrix, Santa Clara, CA). RNAs were processed based on Affymetrix GeneChip protocols and run on Affymetrix GeneChip Fluidics Station 400. Microarrays were scanned using the GeneArray Scanner (Agilent Technologies). Expression signals for each transcript and comparisons between different samples were calculated with the Affymetrix Gene- Chip software MAS5.0 and Microsoft Excel (Microsoft Corp., Redmond, WA). All data are expressed as fold changes in transcript levels relative to the control group.

Lists of genes that underwent greater than 2.5 lineal up- or downregulation from day 0 through day 1 to day 4 were generated and subjected to a search using Cytoscape 2.5 and the Bingo 2.0 plugin and significantly overrepresented Go annotations were listed.

To enable comparison of compound effects, first literature search was carried out and a list of 150 known keratinocyte differentiation related genes were assembled. The subset of these genes, which showed regulation in our confluence induced differentiation model was determined. 53 of these 150 genes were found to be linearly regulated by at least 1.9 fold in the our differentiation model. The geometric mean expression levels of these genes were therefore used to assess alterations in the differentiation state of the keratinocytes and were used to assess the effects of compounds on keratinocyte differentiation. The Wilcoxon matched-pair signed-ranks test was used to assess the differences between control and compound treated samples.

To compare the proposed keratinocyte differentiation gene lists with genes regulated in psoriasis, we reanalyzed the gene expression data of uninvolved and lesional skin of 13 patients with plaque-type psoriasis (GDS2518) obtained from the Gene Expression Omnibus by Significance Analysis of Microarrays (SAM, http://www-stat.stanford.edu/~tibs/SAM/). The overrepresentation of the resultant keratinocyte differentiation lists in the similarly regulated psoriasis datasets were statistically assessed by Pearson's chi-square test (http://faculty.vassar.edu/lowry/VassarStats.html).

Statistical analysis

Statistical analysis of experiments was carried out as noted in the respective sections of the materials and methods. All experiments were carried out in triplicates unless otherwise noted.

RESULTS

Establishing the keratinocyte differentiation model

The messenger RNA expression of four established keratinocyte differentiation markers, transglutaminase 1, filaggrin, keratin 10, and corneodesmosin were upregulated 4 days after induction of differentiation by confluence. Retinol, a known keratinocyte differentiation inhibiting agent, prevented the induction of transglutaminase 1, filaggrin, keratin 10, and corneodesmosin, while vitamin D3 (VD3), a known inducer of differentiation enhanced the expression of these differentiation makers even further. These suggested that confluence induced differentiation provides a suitable model system for studying keratinocyte differentiation.

From the 150 keratinocyte differentiation related genes assembled by literature search, 53 were found to be regulated by at least 1.9 fold four days after induction of differentiation. From this set of 53 genes only 2 were down regulated and 51 were upregulated by confluence induced keratinocyte differentiation. The expression levels of these 53 genes were used to calculate the geometric means of pro-keratinocyte differentiation changes and thereby enable comparison of alterations in the differentiation state of the keratinocytes treated with control compounds and sphingolipids. Retinol and VD3 were found to modify the expression of the geometric mean of these gene expression changes as expected from their known respective inhibitory and stimulatory effects on keratinocyte differentiation. The geometric mean of the gene expression changes for retinol treatment showed a 29.38 % ($p = 0.0995$) and a 59.04 % ($p < 0.0001$) decrease at day 1 and day 4, respectively, whereas VD3 treatment resulted in increased pro-differentiation change in gene expression of these marker genes (day 1: 13.76 % ($p = 0.015$); day 4: 19.77 % ($p < 0.001$)), suggesting the ability of this method to signal keratinocyte differentiation modifying potential of molecules.

In order to further test the ability of the model to identify keratinocyte differentiation genes and to therefore potentially enable the identification of genes not yet described in keratinocyte differentiation we subjected the more than 2.5 fold up and down regulated keratinocyte confluence induced differentiation gene lists established by microarray to analysis by the Bingo tool in the Cytoscape software environment. This method identified the marked overrepresentation of epidermal differentiation related gene ontology terms indicating the ability of the confluence induced *in vitro* keratinocyte differentiation system to model *in vivo* keratinocyte differentiation.

Validating novel keratinocyte differentiation genes

Based on the previous findings, showing the significant overrepresentation of genes related to keratinocyte differentiation in the dataset we assumed that the lists created during the microarray analysis of the confluence induced keratinocyte differentiation model could also contain novel keratinocyte differentiation genes. Therefore from the list of upregulated genes eight identified as potential keratinocyte differentiation genes by microarray were selected after literature review of potential candidates. These 8 genes showed 3.7 – 24.3 fold expression upregulation during the 4 day confluence induced differentiation experiment. In order to validate the

differentiation related upregulation of the selected 8 potential keratinocyte differentiation genes (ring finger protein 39, [RNF39], plakophilin 3 [PKP3], heme oxygenase 1 [HMOX1], ICEBERG caspase-1 inhibitor [ICEBERG], forkhead box Q1 [FOXQ1], ELMO/CED-12 domain containing 1 [ELMOD], family with sequence similarity 43, member A [FAM43A], phospholipase A2, group IVB [PLA2G4B]) their regulation along with the gene expression of the known keratinocyte differentiation genes (TGM1, PI3) were analyzed during calcium induced 10 day NHEK differentiation. TGM1 and PI3 were both found to be markedly upregulated (at least 2.5 fold by day 10) along with six of the tested eight potential differentiation genes (75%) in the longer term Ca^{2+} induced differentiation model. These findings not only validate the role of the selected proteins in the keratinocyte differentiation process but also verify the ability of the initial short term confluence induced keratinocyte differentiation study to identify genes that were previously not described to play a role in keratinocyte differentiation.

Testing the effects of sphingolipid derivatives on keratinocyte differentiation

As the geometric mean of gene expression changes of the 53 known keratinocyte differentiation genes were shown to signal differentiation related changes this was determined in case of sphingolipid compound treatment and compared to differentiated control cells of the same respective days. Hexanoyl-phytosphingosine(PS-C6), sphinganine (SA), phytosphingosine-salycilate (PS-SLC), and sphingosine-salycilate (SO-SLC) were the most potent promoters of differentiation after one day treatment. Although, the difference vs. untreated controls was no longer as marked 4 days into the differentiation process, differentiation-associated gene expression was still stimulated significantly as majority of keratinocyte differentiation genes showed mRNA upregulation compared to same day non compound treated controls. A less marked stimulation with similar kinetics was seen after incubation with PS, SO, and Stearoyl(C18)-SA, with SO showing a non-significant decrease compared to controls on day 4. SO-C6, SA-SLC, and salycilate treatment showed different kinetics with increasing pro-differentiation effect from day 1 to day 4. No significant effect on keratinocyte differentiation was seen with PS-C18 and SO-C18, whereas C12-SLC was the only compound tested that negatively regulated differentiation-associated gene expression. Interestingly SO-C6, PS-SLC, and SO-SLC were capable of more powerfully promoting KC differentiation than VD3 at both examined time points (day 1 and day 4 after differentiation induction). Furthermore, C12-SLC steadily inhibited expression of keratinocyte differentiation genes in our experimental system, with an effect slightly less potent than the anti-differentiation effect of retinol.

ABCA12 regulation in human keratinocytes

In NHEK undergoing 4-day confluence induced differentiation ABCA12 mRNA underwent significant 2 fold upregulation. In both HaCaT keratinocytes and NHEK undergoing 10 day Ca^{2+} induced differentiation ABCA12 mRNA levels were markedly elevated compared to non-differentiated (0 day) controls.

The effect of well known regulators of keratinocyte lipid metabolism on ABCA12 expression was assessed. ABCA12 mRNA level increased markedly following treatment with PPAR-gamma activators (Ciglitazone: 11.8-fold; Troglitazone:

12-fold; GI 251929X: 6.4-fold) and PPAR-delta activator (GW610742: 5.1-fold), after 24h incubation. In addition, while the LXR activator TO901317 modestly increased ABCA12 mRNA level (3.3-fold), activators of PPAR-alpha, RAR, RXR, or VDR had no effect. Moreover the up-regulation of ABCA12 by PPAR-gamma ligand Ciglitazone occurred rapidly and was sustained over an extend period of time showing clear dose-dependence and similar time- and dose-dependent up-regulation of ABCA12 mRNA was induced by PPAR-delta activation.

The expression of ABCA12 splice variants was also studied in real-time quantitative RT-PCR experiments. Neither the long variant (ABCA12-L) nor the short isoform (ABCA12-S) were found to be regulated by 24 hour calcium switch in NHEK. Threshold cycle values (mean) for the basal levels of these two transcripts, ABCA12-L and -S were 25.4 and 30.9 respectively in quantitative PCR. PPAR-gamma activator Ciglitazone increased both transcripts by roughly 5 fold. Similarly, PPAR-delta (GW) or LXR activator (22R) increased both transcripts by 2- and 2.5-fold, respectively, while activation of PPAR-alpha had no effect. Western blot performed by polyclonal antibodies against human ABCA12 recognized both 293.3 kDa (upper) and 257 kDa (lower bands) isotype of the protein, corresponding to gene products of ABCA12-L and –S. PPAR-gamma activation of by Cig increased ABCA12 protein expression in independent batches of NHEK. Furthermore, activation of PPAR-delta by GW also caused an increase in ABCA12 protein levels in these cells. Thus, PPAR-γ and –β/δ activator up-regulate both known isoforms of ABCA12 at mRNA and protein levels. The function of these isofroms, however, remains unknown.

Effect of UVB irradiation on HIF-1alpha expression in keratinocytes

Cytotoxicity after broadband UVB irradiation was assessed in HaCaT cells at approximately 70-90% confluence. The doses employed ranged from 5 to 67 mJ/cm^2. To assess the viability of the cultures light microscopy, and flow cytometry after staining by propidium iodide and annexin-V-FLUOS were performed 24 hours after UVB treatment. Both examinations indicated that 20 mJ/cm^2 UVB can be safely applied in our experiments without marked apoptosis.

HaCaT cells irradiated with 20 mJ/cm^2 UVB were harvested 1 to 24 hours after treatment and the hypoxia signaling master regulator hypoxia induced factor 1-alpha (HIF-1alpha) protein levels were determined by western blot. We found that 3 hours after the UVB irradiation HIF-1alpha protein levels decreased and became barely detectable. However, 12 hours after UVB a significant increase in HIF-1alpha became apparent compared to control samples. This elevated HIF-1alpha level then showed steady decrease towards baseline levels during the second 12 hours. When studying dose dependence the decrease in HIF-1alpha protein was clearly visible 1 hour after UVB treatment at all doses (6.7-50 mJ/cm^2) and the lowest value was seen at 50 mJ/cm^2. However, at the greatest dose instead of an increase seen at lower doses a decrease in the HIF-1alpha signal occurred 12 hours after UVB irradiation, which is most likely due to the overwhelming toxic effects of higher doses of UVB seen also in the cytotoxicity study. Given these observations, the best comparison appeared to be at the 20 mJ/cm^2 dose-point, which dose is also comparable to the UVB dose basal keratinocytes are subjected to *in vivo* during sun exposure.

The effect of UVB on the mRNA expression of VEGF and HMOX1 was determined by real-time quantitative PCR to study the mRNA expression of these two

well known HIF-1 target genes parallel to the observed HIF-1alpha changes. Moreover HIF-1α mRNA levels during the experiments were also analyzed. We found that UVB treatment led to upregulation of both VEGF and HMOX1 mRNA levels 12 and 24 hours after UVB irradiation while HIF-1alpha mRNA showed a significant decrease 3, 12, and 24 hours after UVB treatment.

Previous studies have shown that the PI3K is an important positive regulator of HIF-1alpha in various model systems, and UV irradiation was shown to result in increased PI3K activation. Therefore, the effects of wortmannin, a specific inhibitor of the PI3K enzyme, were analyzed in our experimental setup. Wortmannin was added to the cell suspension immediately after UVB irradiation. Cells were harvested 3, 12 and 24 hours after the inhibitor treatment. Western-blot analysis showed that wortmannin effectively decreased the elevation of the HIF-1alpha protein 12 hours after UVB treatment. Akt phosphorylation was also studied by specific anti-phospho-serine AKT antibody. In case of UV-treated samples increased Akt phosphorylation was found.

CONCLUSIONS

The altered differentiation of keratinocytes is an important contributor to epidermal pathogenesis. Regulation of keratinocyte differentiation and transcriptional changes taking place during this process are of paramount dermatological importance. Therefore during our studies we focused on the transcriptional changes taking place in keratinocytes on several levels of the regulatory process. We identified previously undescribed genes as keratinocyte differentiation genes, found molecules capable of broadly increasing keratinocyte differentiation genes expression and focusing on ABCA12 and the UV response we described the involvement of specific regulatory networks in the transcriptional regulation of epidermal homeostasis.

Molecules capable of modifying keratinocyte differentiation are of potential dermatological and cosmetic value. To date most studies used a few known keratinocyte differentiation genes to assess the differentiation effect of tested molecules, in either two dimensional cultures of keratinocytes or differentiated three dimensional in vitro epidermal models. Unfortunately neither approach provides a well suited system for comparison of molecule effects in the first stages of drug development due to their inherent limitations. Two dimensional models with only a few keratinocyte differentiation genes tested can show large bias due to gene specific compound effects. Whereas the three dimensional model is incapable of identifying effects in early differentiation and also poses compound delivery problems, besides the obvious limitations and bias caused by the assessment of expression changes in only a few genes. Therefore we set up a new system for analyzing the effects of sphingolipid compounds on keratinocyte differentiation. Assessment of the expression of more than 50 keratinocyte differentiation genes in confluence induced differentiation based on microarray measurements proved to be a valuable tool for comparing both keratinocyte differentiation promoter and inhibitor effects of control and test compounds. These findings provided evidence for the usefulness of whole genome transcriptional profiling in keratinocyte differentiation modulating compound testing and suggested that PS-SLC and SO-SLC are potent novel keratinocyte differentiation promoting agents. Moreover our results provide further evidence to the transcriptional effects of sphingolipids in epidermis, showing that structural molecules or their derivatives possess transcriptional regulatory role modifying expression of epidermal structural and metabolic genes.

Despite the obvious importance of keratinocyte differentiation in regulating epidermal homeostasis, the transcriptional regulation and gene expression changes during this process are not well understood. Therefore we analyzed microarray data from keratinocytes undergoing confluence induced differentiation and identified several novel keratinocyte differentiation genes (RNF39, PKP3, HMOX1, ICEBERG, FOXQ1, ELMOD), which were also verified in a longer calcium induced differentiation model of normal human keratinocytes using TaqMan gene expression analysis. These genes provide evidence for the involvement of previously unrecognized pathways in epidermal differentiation and may prove to be suitable targets for future drug development.

Sphingolipids are not only potential regulators of epidermal homeostasis they also serve as building blocks of the epidermal permeability barrier. ABCA12 mutations are thought to result in greatly altered epidermal structure in the form of lamellar and in more serious cases harlequin ichthyoses due to the abrogated transportation of

sphingolipids, and deficient formation and secretion of lamellar bodies. We and others have found ABCA12 to show keratinocyte differentiation depended gene expression. Many other members of the ABC transporter protein family are regulated by nuclear hormone receptors. Moreover nuclear receptors such as LXR, PPAR-alpha, PPAR-delta and PPAR-gamma activation have been found to improve permeability barrier homeostasis by both effecting the protein and lipid constituents of the epidermal permeability barrier. The findings presented here demonstrate that PPAR and LXR activators directly increase ABCA12 expression, which may enhance sphingolipid trafficking. This highlights another mechanism by which activators of these nuclear receptors promote epidermal permeability barrier integrity. Furthermore the regulation of ABCA12 by nuclear receptor lignads suggests the possible therapeutic effects of these nuclear receptors ligands in lamellar ichthyosis patients with residual activity of the transporter.

UVB radiation is one of the most important environmental stressors affecting the epidermis. Besides its widely studied mutagenic potential, it is known to abrogate epidermal lipid synthesis, lamellar body secretion and disrupt the epidermal lipid barrier. Although vascular and gene expression changes (VEGF, HMOX1) after UV irradiation pointed towards the possible involvement of hypoxia signaling in the UV response proof of this was lacking. HIF-1alpha, the master regulator of hypoxia signaling, was seen to undergo a biphasic response to UVB in keratinocytes, and its upregulation coincided with the transcriptional upregulation of VEGF and HMOX1 mRNA providing evidence for the involvement of HIF-1alpha regulation in the UVB response. Given HIF-1alpha's ability to convey cellular metabolism from an energy rich oxidative to a low energy hypoxic state, its role in downregulating the highly energy and thus oxygen dependent cellular metabolism of lipids after UVB can be suspected, potentially also explaining some of the lipid barrier changes taking place after sun exposure. HIF-1alpha may later also provide a therapeutic target for minimizing the epidermal effects of UV radiation.

Our findings on keratinocyte transcriptional changes and regulatory processes provide improved tools for studying keratinocyte differentiation and identified novel regulatory mechanisms that broaden our understanding of the processes underlying epidermal homeostasis and differentiation. Furthermore they also provide new potential targets for treating dermatological diseases.

LIST OF OWN PUBLICATIONS

Publications related to theses

1. **Paragh G,** Schling P, Ugocsai P, Kel AE, Liebisch G, Heimerl S, Moehle C, Schiemann Y, Wegmann M, Farwick M, Wikonkál NM, Mandl J, Langmann T, Schmitz. Novel sphingolipid derivatives promote keratinocyte differentiation. Exp Dermatol. 2008 Dec;17(12):1004-16. (IF=3.259)
2. Wunderlich L*, **Paragh G***, Wikonkal NM, Banhegyi G, Karpati S. and Mandl J. UVB induces a biphasic response of HIF-1α in cultured human keratinocytes. Exp Dermatol. 2008 Apr;17(4):335-42. **Authors contributed equally* (IF=3.259)
3. Jiang YJ, Lu B, Kim P, **Paragh G,** Schmitz G, Elias PM and Feingold KR. PPAR and LXR Activators Regulate ABCA12 Expression in Human Keratinocytes. J Invest Dermatol. 2008 Jan;128(1):104-9. Epub 2007 Jul 5. (IF=5.251)

Publications not related to theses

1. **Paragh G**, Kumar SM, Rakosy Z, Choi SC, Xu X, and Acs G. RNA Interference-Mediated Inhibition of Erythropoietin Receptor Expression Suppresses Tumor Growth and Invasiveness in A2780 Human Ovarian Carcinoma Cells. Am J Pathol. 2009 Apr;174(4):1504-14. Epub 2009 Mar 5. (IF=5.697 (2008))
2. Acs G, **Paragh G**, Chuang ST, Laronga C, Zhang PJ. The Presence of Micropapillary Features and Retraction Artifact in Core Needle Biopsy Material Predicts Lymph Node Metastasis in Breast Carcinoma. Am J Surg Pathol. 2009 Feb;33(2):202-10. (IF=4.020 (2008))
3. Balogh A, **Paragh G Jr,** Juhasz A, Kobling T, Torocsik D, Miko E, Varga V, Emri G, Horkay I, Scholtz B, Remenyik E. Reference genes for quantitative real-time PCR in UVB irradiated keratinocytes. J Photochem Photobiol B. 2008 Dec 11;93(3):133-9. Epub 2008 Aug 14. (IF=1.838)
4. Balogh Z, Fóris G, Kosztáczky B, **Paragh G Jr,** Seres I, Zsiros E, Kónya G, Paragh G. The concentration dependent biphasic effect of leptin on endogenous cholesterol synthesis in human monocytes. Peptides. 2007 Oct;28(10):2081-3. Epub 2007 Jul 12. (IF=2.368)
5. Kosztáczky B, Fóris G, **Paragh G Jr,** Seres I, Zsiros E, Koncsos P, Balogh Z, Paragh G. Leptin stimulates endogenous cholesterol synthesis in human monocytes: New role of an old player in atherosclerotic plaque formation. Int J Biochem Cell Biol. 2007;39(9):1637-45. (IF=4.009)
6. Remenyik E., Torocsik D., Balogh A., **Paragh G.,** Dózsa A., Marko L., Emri G., Horkay I. Nuclear receptros (in Hungarian). Hungarian Journal of Dermatology and Venereology 2007. Jun (no IF)
7. Seres I, Foris G, Pall D, Kosztaczky B, **Paragh G Jr**, Varga Z, Paragh G. Angiotensin II-induced oxidative burst is fluvastatin sensitive in neutrophils of patients with hypercholesterolemia. Metabolism. 2005 Sep;54(9):1147-54. (IF=2.294)

8. Paragh G, Foris G, **Paragh G Jr**, Seres I, Karanyi Z, Fulop P, Balogh Z, Kosztaczky B, Teichmann F, Kertai P. Different anticancer effects of fluvastatin on primary hepatocellular tumors and metastases in rats. Cancer Lett. 2005 May 10;222(1):17-22. (IF=3.049)
9. Petrohai A, Nagy G, Bosze S, Hudecz F, Zsiros E, **Paragh G**, Nyarady Z, Nemeth P, Berki T. Detection of citrate synthase-reacting autoantibodies after heart transplantation: an epitope mapping study. Transpl Int. 2005 May;17(12):834-40. Epub 2005 Feb 15. (IF=1.797)
10. Paragh G, Kertai P, Kovacs P, **Paragh G Jr**, Fulop P, Foris G. HMG CoA reductase inhibitor fluvastatin arrests the development of implanted hepatocarcinoma in rats. Anticancer Res. 2003 Sep-Oct;23(5A):3949-54. (IF=1.347)
11. **Paragh G**, Torocsik D. Effect of nuclear receptor activators on the expression of ENC-1 (in Hungarian) Student thesis, University of Debrecen, 2003 (no IF)
12. Torocsik D. **Paragh G**, Role of steroid hormone receptors in myeloid differentiation in different myeloid cell lines (in Hungarian) Student thesis, University of Debrecen, 2003 (no IF)
13. **Paragh G**, Torocsik D. Expression of nuclear receptors in human placental macrophages (in Hungarian). Student thesis, University of Debrecen, 2002 (no IF)
14. Torocsik D. **Paragh G**, Effect of nuclear receptor ligands on Retinoic acid sensitive and resistant acute promielocytic leukemia cells (in Hungarian) Student thesis, University of Debrecen, 2002 (no IF)