SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Examination of factors playing role in skin barrier function on human keratinocyte cell lines

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The Examination takes place at Division of Clinical Immunology, Faculty of Medicine, University of Debrecen, 26 Jun 2017

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The PhD Defense takes place at the Dermatology Department, University of Debrecen, 29 Jun 2022, 01:00 pm.
Live online access will be provided. If you wish to take part in the discussion, please send an e-mail to payer.edit@med.unideb.hu not later than 2 p.m. on 28th of June 2022. After the deadline, for technical reasons, it is no longer possible to join in to the defense.

INTRODUCTION

Skin diseases not only cause aesthetic problems, but also a number of unpleasant symptoms (pain, itching) and their treatment can lead to additional complications (infections, osteoporosis, kidney failure, etc.). For these reasons, there is a growing effort to understand the pathogenesis of these diseases and to use more effective targeted therapies with fewer side effects and a better quality of life for patients.

The main aim of our work was the investigation of barrier function and regeneration signalling processes, which play an important role in the pathogenesis of pemphigus vulgaris and atopic dermatitis. We first investigated the processes that influence the expression of desmoglein (dsg)-1, dsg-3 and P-cadherin (P-cad), adhesion molecules that are important in maintaining mechanical integrity and that form the intercellular adhesion units between keratinocytes, with particular attention to the protein kinase C (PKC) isoenzyme family that regulates the proliferation and differentiation processes of these cells. As keratinocytes undergo the certain differentiation phases during epidermis formation, in which different adhesion molecules are expressed, the question arose whether the PKC enzyme family has an effect on these proteins.

Glycerol and xylitol with similar structures have been successfully used in dermatological externals to reduce irritation and restore barrier function. Thus, in the second part of our experiments, we focused on whether these two polyols alter the expression of differentiation and inflammatory markers in keratinocytes and affect related signalling processes.

AIMS

My main aim in my PhD work was to investigate the factors that influence skin barrier function. For this purpose, I conducted research in two areas.

1. In the first part of our experiments, we analysed the effect of PKC isoenzymes on the expression of adhesion molecules (dsg-1, dsg-3, P-cad) on human epidermal HaCaT keratinocytes in relation of culturing time, i.e. proliferation and differentiation status; in HaCaT cells are stably overexpressing specific PKC isoenzymes; and finally by pharmacological methods (selective PKC inhibitors) and RNA interference (RNAi) techniques.

2. In the second part of our studies, we investigated the effects of barrier repair agents (glycerol, xylitol) on mechanisms involved in barrier formation, such as keratinocyte proliferation, expression of differentiation markers, intracellular calcium levels and signalling processes, including PKC isoenzymes and the MAPK pathway, and their anti-inflammatory effects in inflammatory models.

MATERIALS AND METHODS

Cell culture

HaCaT

Human immortalized HaCaT keratinocytes were cultured in 25 or 75 cm^2 tissue culture flasks (unless otherwise indicated) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U⁄ml penicillin, 50 µg⁄ml streptomycin, 1.25 µg⁄ml Fungizone at 37ºC in a 5% CO_2 atmosphere.

NHEK

Human skin samples were obtained after written informed consent from healthy individuals, undergoing dermatosurgery, adhering to Helsinki guidelines and after obtaining Institutional Research Ethics Committee's and National Public Health and Medical Officer Service-Office of the Chief Medical Officer's permission (protocol No.: DE OEC RKEB/IKEB 3724-2012; document No.: IX-R-052/01396-2/2012). NHEKs were isolated after overnight dermo-epidermal separation in 2.4 U/ml dispase by short trypsin (0.05%) digestion. Cells were cultured in EpiLife serum free medium supplemented with 1 µM insulin, 1 µM cortisol, 100 µg/ml streptomycin, 100 U/ml penicillin, 50 ng/ml amphotericin B, 0.4% bovine pituitary extract and 0.06 mM $CaCl_2$, at 37ºC in a 5% CO_2 atmosphere.

Generation of PKC-overexpressing HaCaT cells

Stable overexpression of various PKC isoforms in HaCaT keratinocytes was performed as described by Papp et. al. Experiments were routinely carried out on pools of transfected cells, but the results were confirmed on at least three individual clones for each isoform. As described before, the efficacy of recombinant overexpression was monitored by Western blotting and kinase assays (not shown).

RNA interference (RNAi)

Cells were seeded in six-well tissue culture plates in DMEM containing serum but lacking antibiotics. At 40–50% confluence, cells were transfected with RNAi probes against cPKCα and β and nPKCδ and ε, with scrambled RNAi probes (used as control), or with fluorescein-labelled control siRNA previously mixed (and incubated at room temperature for 25 min) with transfection medium also containing the transfection reagent. The efficacy of siRNA-driven

'knock-down' of the PKCs was daily evaluated by Western blot and quantitative 'real-time' Q-PCR techniques for 4 days.

Western blot analysis

The Western blot technique was employed to assess expression of adhesion molecules and to verify the efficacy of RNAi suppression of the PKC isoforms and the activation of the MAPK Erk1/2 at the protein level. In brief, cells were washed with ice-cold phosphate-buffered saline (PBS), harvested in homogenization buffer and disrupted by sonication on ice. Protein content of samples was measured by a modified bicinchonic acid protein assay. Total cell lysates were mixed with SDS-PAGE sample buffer and boiled for 10 min at 100ºC. The samples were subjected to SDS-PAGE (8% gels were loaded with 20–30 μg protein per lane) and transferred to nitrocellulose membranes. Membranes were then blocked with 5% dry milk in PBS and probed with the appropriate primary antibodies against the given protein, incubatei overnight at 4ºC. After washing with PBST peroxidase conjugated goat anti-rabbit, anti-mouse and horseradish peroxidase-conjugated IgG antibodies were used as secondary antibodies (1:1000 dilution), and the immunoreactive bands were visualized by an ECL Western blotting detection kit. Immunoblots were subjected to densitometric analysis using an Intelligent Dark Box and the Image Pro Plus 4.5.0 software, and then normalized densitometric values of the individual lanes of several independent experiments were determined and expressed as mean ± SEM. To assess equal loading, membranes were stripped in 200 ml of 50 M Tris–HCl buffer (pH 7.5) containing 2% SDS and 0.1 β-mercaptoethanol at 65ºC for 1 h and were re-probed with a mouse β-actin antibody followed by a similar visualization procedure as described above.

Immunocytochemistry

Human keratinocytes, seeded and cultured on sterile coverslips in 24-well plates, were fixed in acetone, permeabilized by 0.1% Triton-X-100. After washing with PBS the aspecific binding sites were blocked with 5% albumin containing PBS (30 min, room temperature).

Afterthat slides were incubated with rabbit primary antibodies against PKCα or PKCδ (1:200 dilution in both cases)(overnight, 4 ºC). After washing, for fluorescence staining, slides were then incubated with fluorescein-isothiocytanate (FITC) conjugated secondary antibodies (dilution 1:200) and the nuclei were visualized using DAPI. As negative controls, the appropriate antibody was either omitted from the procedure or was pre-incubated with synthetic blocking peptides.

To assess the translocation of the PKC isoforms (reflecting activation of the enzymes), the above labeling was performed on cells which were previously treated by 0.27% glycerol or 0.45% xylitol for various time intervals. FITC-labeled cells were then subjected to a visualization procedure using a Zeiss LSM 510 confocal microscopy.

Quantitative 'real-time' Q-PCR

To assess the efficacy of RNAi of the PKC isoforms at the gene level, Q-PCR was carried out on an ABI PRISM 7000 Sequence Detection System by using the 5' nuclease assay. Total RNA was isolated using TRIzol and then 2–3 μg of total RNA were reverse transcribed into cDNA by using 15 units of AMV reverse transcriptase and 0.025 μg/μl random primers. PCR amplification was carried out by using the TaqMan primers and probes, using the TaqMan Universal PCR Master Mix Protocol. As internal controls, transcripts of β-actin or cyclophilin A (PPIA) were determined.

Determination of viable cell numbers and proliferation (MTT assay)

The number of viable cells (hence the rate of proliferation) was determined by measuring the conversion of the tetrazolium salt MTT (Methylthiazolyldiphenyl-tetrazolium bromide) to formazan by mitochondrial dehydrogenases. Cells were plated in 96-well multiter plates (10,000 cells/well density) in quadruplicates and were treated with xylitol (0.0045-0.45%) or glycerol (0.0027-0.27%) for various time intervals. Cells were then incubated with 0.5 mg/ml MTT for 2 hrs, and concentration of formazan crystals was determined colorimetrically at 565 nm using a FlexStation 3 Fluorescence Image MicroPlate Reader (FLIPR). The maesured absorbancy was proportionate with the number of alive cells.

Determination of apoptosis (MitoProbe™)

A decrease in the mitochondrial membrane potential is one of the earliest markers of apoptosis. Therefore, to assess the process, mitochondrial membrane potential was determined using a MitoProbe™ DiIC_1(5) Assay Kit. The used dye (1,1′,3,3,3′,3′-hexametil-indodicarbocianin-iodid; „DilC_1 (5)") accumulates in mitochondria in related to membrane potential, so in apoptotic cells the intensity of the sign decreases. Cells (20,000 cells/well) were cultured in 96-well black-well/clear-bottom plates in quadruplicates and were treated with various compounds. After removal of supernatants, cells were incubated for 30 minutes with DilC_1(5) working solution (50 μl/well), then washed with PBS, and the fluorescence of DilC_1(5) was

measured at 630 nm excitation and 670 nm emission wavelengths using a FlexStation 3 Fluorescence Image MicroPlate Reader (FLIPR).

Determination of necrosis (SYTOX Green)

Necrotic processes were determined by SYTOX Green staining. The dye is able to penetrate (and then bind to the nucleic acids) only to necrotic cells with ruptured plasma membranes, whereas healthy cells with intact surface membranes show negligible SYTOX Green staining. In the nucleus then bind to the dsDNS. The fluorescens intensity of the dye correlates with cytotoxic mechanisms. 20.000 cells/well were cultured in 96-well black-well/clear-bottom plates, and treated with polyols. Supernatants were then discarded, and the cells were incubated for 30 minutes with 1 μM SYTOX Green dye. Following incubation, cells were washed with PBS, the culture medium was replaced, and fluorescence of SYTOX Green was measured at 490 nm excitation and 520 nm emission wavelengths using FLIPR.

SYTOX Green and $DilC_1(5)$ both were added to the cells to invesigate the mechanisms at the same time.

Ca^{2+}-imaging

Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) upon applications of the polyols were detected by fluorimetric Ca^{2+}-imaging. A Ca^{2+} sensitive fluorescent dye, the Fluo-4 AM was used for the experiments. The dye can be transported into the cell with its AM (acetoxi-metil estere) group which will be cloven by intracellular esterases. Fluo-4 will be captured and its fluorescence intensity increases in the presence of Ca^{2+}. Cells were seeded in 96-well black-well/clear-bottom plates at a density of 10,000 cells/well and then were incubated with culturing medium containing the cytoplasmic calcium indicator 2 μM Fluo-4 AM at 37°C for 40 min. The cells were washed four times with and finally cultured in Hank's solution containing 1% bovine serum albumin and 2.5 mM Probenecid for 30 min at 37 °C. The plates were then placed to FLIPR and changes in $[Ca^{2+}]_i$ (reflected by changes fluorescence; EX=494 nm, EM=516 nm) induced by various concentrations of the drugs were recorded in each well. We used ATP as positive and calcium free medium as negative control.

Statistical analysis

When applicable, data were analyzed using a two-tailed, two-sampled t-test and $P<0.05$ values were regarded as significant differences.

RESULTS

1. *The effect of PKC isoenzimes ont he expression of adhesion molecules in human epidermal HaCaT keratinocytes*

Expression of adhesion molecules changes during the high cell density-induced differentiation of HaCaT keratinocytes

We have previously shown that HaCaT cell cultures start to differentiate upon reaching confluence (high cell density-induced differentiation). Hence, we first defined the alterations in the expression patterns of the adhesion molecules Dsg1, Dsg3 and P-cad during culture of HaCaT keratinocytes. As revealed by Western blotting, the expression of the molecules differentially altered with days in culture. The level of Dsg1 (which is mostly expressed in the suprabasal layers of the human epidermis in vivo) was very low in the subconfluent, proliferating cultures. However, as expected, the expression of the molecule markedly increased in parallel with the onset of differentiation. In contrast, the expression of Dsg3 first increased with time in the highly proliferating cultures and then dropped to practically undetectable levels in the postconfluent (differentiating) cells. Partly similar to these data, the level of P-cad was highest in the preconfluent cultures and significantly decreased with the onset of the high cell density-induced differentiation programme. These findings were in good accord with previous results demonstrating that Dsg3 and P-cad are chiefly expressed in vivo in the highly proliferation basale layer of the human epidermis. It appears, therefore, that the proliferating and differentiating HaCaT cell culture provides a model for the in vivo like differentiation-dependent alterations in the expression of certain adhesion molecules.

Expression of adhesion molecules differentially alters in HaCaT keratinocytes overexpressing certain PKC isoforms

We have previously also shown that recombinant overexpression of certain PKC isoforms significantly affected the in vitro and in vivo proliferation and differentiation of HaCaT keratinocytes. Comparison of various PKC overexpresser HaCaT keratinocytes at similar proliferation and differentiation stages revealed that the overexpression of cPKCα or nPKCδ decreased cellular proliferation rate but, of great importance, markedly increased the expression of differentiation markers (e.g. involucrin, filaggrin, transglutaminase-1). In contrast, overexpression of cPKCβ or nPKCε resulted in hyperproliferative transformation of the cells and the suppression of differentiation. In the next phase of our study, we therefore investigated the expression of the above adhesion molecules inthe PKC overexpresser HaCaT keratinocytes.

In the course of these experiments, it was also of importance to compare the levels of the adhesion molecules on cells with similar proliferation/differentation stages. Since the growth rates of the PKC overexpresser cells were markedly different, the expression of the adhesion molecules was determined on cells harvested at 80–85% confluence, as described before. Western blot analysis revealed that the expression pattern of the adhesion molecules depends not simply on the differentiation status of the cells but also on the specific PKC isoform whose overexpression induced the altered differentiation of the cells. In the cPKCβ-HaCaT cells, which were hyperproliferative, the level of P-cad markedly and significantly ($P < 0.05$, $n = 5$) increased compared with control (empty vector transfected) keratinocytes (Fig. 2). However, we observed no alteration in the expression of Dsg1 or Dsg3. In contrast to these finding, in the nPKCε overexpresser (also characterized by decreased differentiation and increased proliferation), the expression of Dsg3 increased ($P < 0.05$, $n = 6$), that of Dsg1 decreased ($P < 0.05$, $n = 5$), whereas the level of P-cad did not change (when compared with control). Although to a lesser extent, a differentially modified expression pattern of the adhesion molecules was also observed in those PKC overexpresser HaCaT cells which were characterized by accelerated differentiation and suppressed proliferation. In cPKCα-HaCaT cells, the expression of Dsg3 and P-cad ('markers' of the proliferating keratinocytes of stratum basale) significantly decreased (in both cases, $P < 0.05$, $n = 5$) whereas that of Dsg1 was negligibly altered compared with control. In the nPKCδ overexpresser keratinocytes, levels of Dsg3 and P-cad were likewise suppressed (in both cases, $P < 0.05$, $n = 6$). A difference, however, was that Dsg1 (a 'marker' of the suprabasal, differentiating epidermal layers) was appreciably elevated ($P < 0.05$, $n = 5$).

Expression of adhesion molecules also differentially alters in HaCaT keratinocytes in which the levels of certain PKC isoforms were suppressed by RNAi

To complement the above studies measuring the effect of recombinantly overexpressed PKC isoenzymes, in the next phase of our experiments we selectively 'knocked-down' individual PKC isoforms using the RNAi technique and then investigated the effect of such interventions on the expression of the adhesion molecules. First, we assessed the efficacy of RNAi treatment for suppression of the expression of individual PKC isoforms. Using Western blot, followed by densitometry analysis, as well as Q-PCR techniques at various time-points after RNAi transfection, we found that the levels of all PKC isoforms investigated were markedly (approximately 20% of the scrambled RNAi probetransfected control) and, of great importance, selectively suppressed by the RNAi probes 48 h after transfection. The selectivity of the intervention was supported by the fact that in no case did an RNAi probe affect the levels of

the other (i.e. 'non-targeted') PKC isoforms or of the endogenous control β-actin; further, the scrambled RNAi probes did not modify the expression of the 'targeted' PKC isoenzyme. We therefore measured the expression of the adhesion molecules 48 h after transfection. Western blot analysis of the RNAi-mediated 'knockdown' cells revealed that suppression of individual PKC isoforms had effects opposite of those when the same individual isoforms were overexpressed, with only minor differences. Thus, suppression of cPKCα resulted in elevation of Dsg3 and P-cad (in both cases, $P < 0.05$, $n = 5$) whereas suppression of nPKCδ resulted in elevation of Dsg3 and P-cad (in both cases, $P < 0.05$, $n = 6$), together with a decrease in Dsg1 ($P < 0.05$, $n = 5$). In cPKCβ 'knock-down' cells, the expression of P-cad decreased ($P < 0.05$, $n = 5$), with no change in Dsg1 and Dsg3 whilst in nPKCε 'knock-down' keratinocytes, Dsg1 levels were elevated and Dsg3 and P-cad levels were reduced (in all cases, $P < 0.05$, $n = 5–6$). Other than for the reduction in P-cad in the nPKCε 'knock-down' keratinocytes, whereas P-cad was unaffected in the nPKCε overexpressers, the effects of the suppression of the individual PKC isoforms was opposite of those for their overexpression.

Selective inhibition of nPKCδ significantly alters the expression of adhesion molecules in HaCaT keratinocytes

Finally, we examined the effect of rottlerin, a compound often used as a selective nPKCδ inhibitor. Consistent with the role of PKCδ as an inhibitor of HaCaT proliferation, previous studies have shown that the treatment of HaCaT cells with rottlerin caused enhanced proliferation. Here, we determined its effect on adhesion molecules. As was expected, treatment of (control, non-transfected) HaCaT keratinocytes with rottlerin altered the expression pattern of the adhesion molecules in a dose-dependent fashion. In full agreement with the results from overexpression and RNAi treatment, the inhibition of nPKCδ with rottlerin significantly suppressed the level of Dsg1 (a 'marker' of differentiating keratinocytes) whilst it elevated the expression of Dsg3 and P-cad ('markers' of proliferating epidermal cells) (in all cases, $P < 0.05$, $n = 5–6$). Although confidence in the interpretation is limited because of possible effects of rottlerin (especially at higher concentrations) in systems other than PKC, these findings further support the concept that the endogenous nPKCδ activity plays a key role in the regulation of the adhesion molecules Dsg1, Dsg3 and P-cad.

2. *The effect of xylitol and glycerol on mechanisms and signal transduvction pathways which play role in barrier formation in normal human epidermal keratinocytes*

Polyols do not affect viability of NHEKs.

We first assessed the effects of polyols on cellular viability. Cultured NHEKs were treated with various concentrations of glycerol (0.0027%-0.27%) and xylitol (0.0045%-0.45%), establishing identical osmolarities, for up to 72 hours. Neither polyol affected viability (MTT assay). The lack of cytotoxicity was also verified by fluorimetric DilC1(5) and SYTOX Green labelling. Therefore, in the further experiments, the highest polyol concentrations were utilized.

Polyols differentially alter expressions of various differentiation/barrier markers

Next, we studied the effects of polyols on the expression of such molecules (filaggrin, involucrin and loricrin) which play key roles in the differentiation programme in epidermal keratinocytes and hence participate in the establishment of the epidermal barrier. As the differentiation programme of cultured NHEKs is automatically initiated upon reaching confluence (high-cell density-induceddifferentiation), we first investigated the putative "pro-differentiating" effects of polyols on preconfluent cultures of NHEKs. Importantly, as revealed by Q-PCR analyses, both polyols significantly increased the mRNA level expression of the differentiation/barrier markers filaggrin and loricrin after 24-and/or 48-hour treatments. Notably, only tendencies of up-regulation were observed in the case of involucrin. Likewise, both agents increased the expressions of filaggrin and loricrin (after 48-hr treatment) on postconfluent (hence already differentiating) NHEKs, and xylitol (but not glycerol) was also able up-regulate the level of involucrin. In line with these findings, in a pilot experiment, we found that glycerol indeed greatly increased loricrin protein expression of differentiating NHEKs, whereas somewhat surprisingly, xylitol's effects were less pronounced (Western blot;). We also studied the effects of polyols on the expression of occludin, a key molecule of epidermal tight junctions also involved in barrier formation. Importantly, both polyols significantly increased the expression of occludin mRNA both in the pre-and postconfluent cultures (48 hours).

Polyols do not elevate $[Ca^{2+}]_i$ of NHEKs

We then intended to assess the effects of polyols on such signalling pathways, which were shown to involved in controlling epidermal differentiation. First, we measured the effects of polyols on the level of $[Ca^{2+}]_i$, whose elevation is one of the key events in initiating the cellular differentiation process. Importantly, neither polyol elevated the $[Ca^{2+}]_i$ of cultured NHEKs.

Xylitol selectively translocates PKCδ

Protein kinase C (PKC) isoforms are key intracellular signalling molecules fundamentally involved in regulating cellular proliferation, differentiation and survival. In human epidermal keratinocytes, activation of PKCα and δ isoforms was shown to promote differentiation; therefore, we measured the effects of polyols on these isoenzymes. As activation of PKCs can be followed by assessing their translocation (i.e. transition from a given cellular compartment to another one), we employed confocal microscopy following immunolabelling of the isoforms after treatment. The polyols did not alter the subcellular localization of the Ca^{2+}-sensitive PKCα. However, xylitol (but not glycerol) altered intracellular distribution of the Ca^{2+}-insensitive PKCδ, suggesting that xylitol may activate it.

Polyols activate the MAPK pathway in NHEKs

Another important signal transduction system that controls multiple keratinocyte functions is the mitogen-activated protein kinase (MAPK) system; therefore, we also measured the effects of polyols on the activity of this signalling pathway. As shown by Western blotting, both xylitol and glycerol activated the MAPK cascade, as reflected by the transiently increased phosphorylation of Erk1/2 (p42/44) MAPK.

Polyols exert differential anti-inflammatory effects in NHEKs

Next, we assessed whether glycerol and xylitol can modulate the inflammatory response of keratinocytes. For this, NHEKs were treated with activators of the toll-like receptor (TLR) pathways, that is with lipoteichoic acid (LTA; TLR2); polyinosinic:polycytidylic acid (poly-(I:C); TLR3); and lipopolysaccharide (LPS; TLR4), and the effects of the polyols on the TLR-mediated up-regulation of various inflammation markers—interleukins (IL), tumor necrosis factor-α (TNF-α), matrix metalloproteases (MMP)—were determined. Moreover, we also measured the expression changes in HLA-DR whose up-regulation in the skin was shown to be related to keratinocyte immune activation, inflammation and TLR signalling. In our hands, mRNA expression of HLA-DR exhibited some donor dependence; however, both polyols at least tended to prevent the TLR-induced up-regulation of HLA-DR in all cases when TLR stimulation elevated it (and when the elevation was great enough, xylitol could significantly suppress it). In addition, albeit exerting somewhat donor-dependent effects, both polyols were able to significantly suppress TLR activation-inducedup-regulation of IL-1α and IL-1β in the majority of the cases. Interestingly, although the polyols were less efficient in case of IL-6 and

IL-18, and could hardly decrease TLR activation-induced expression of IL-8, xylitol was quite effective in suppressing poly-(I:C)-induced up-regulation of TNF-α. Finally, while up-regulation of MMP1 and MMP9 was less efficiently induced by TLR activation, both polyols (but especially xylitol) were able to significantly suppress MMP9 expression. As elevation of the $[Ca^{2+}]_i$ can often lead to pro-inflammatory responses of human epidermal keratinocytes, finally, we tested how the polyols affect ATP-and bradykinin (BK)-induced Ca^{2+} signals of NHEKs. Importantly, we found that neither polyol suppressed ATP-or BK-induced elevation of $[Ca^{2+}]_i$ of NHEKs, suggesting that mechanism of their anti-inflammatory actions most probably does not involve modulation of the Ca^{2+} homeostasis.

DISCUSSION

Our skin is the largest organ and its most important function is to protect our body from physical, chemical and pathogenic agents. To do this, a number of well-coordinated mechanisms have evolved, the pillars of which are the keratinocytes themselves, immune cells and the micro-organisms that colonise the skin. Keratinocytes are involved in the defence process both through their epidermal structure and through their immunological properties. In the characteristic four layers of the epidermis, cells progress upwards from dividing cells above the basement membrane to the top, scarring layer by a series of differentiation processes. During the differentiation process, cell structure and cell-cell contacts change and a number of substances are produced that protect against mechanical and chemical attack, inhibit water loss through the epidermis and help maintain pH (cornified envelope). They also have direct immunological functions, as they express on their surface a number of immune activating proteins (PRRs), antimicrobial peptides (e.g. β-defensin, psoriasin, RNase 7), chemokines, cytokines (e.g. IL-1, 6, TNFα, TGF-β).

Various compensatory and regenerative processes take place in the epidermis to maintain the skin barrier. Depending on the component of the barrier that is damaged, different processes are triggered, such as keratinocyte proliferation, fat and protein production. However, persistent damage can upset the balance and the processes become uncontrolled, creating the characteristic symptoms of various barrier damage diseases such as keratinocyte hyperproliferation, dry skin, peeling, itching or erythema. In addition to aesthetic problems, these diseases can cause serious and even life-threatening conditions, which is why it is important to understand their pathogenesis in detail, as this is the only way to provide targeted, and effective treatment. It is also important to prevent, if possible, or reverse the processes at an early stage, even in the case of mild abnormalities.

In the experiments described in the present work, we have explored the effect of the PKC isoenzymes on the expression of adhesion molecules important in the formation of the physical barrier and the role of xylitol and glycerol, which are known to play a role in barrier repair, in addition to their beneficial physicochemical effects (water-binding capacity), which are thought to influence signal transduction.

The role of the PKC system in the regulation of adhesion molecules

The cell-cell coupling apparatus of the epidermis is responsible for maintaining the structural integrity of the epidermis, but also acts as a physical barrier against external agents. In our

experiments, we investigated the desmosome-forming proteins desmoglein-1 (dsg-1), desmoglein-3 (dsg-3) and P-cadherin (P-cad), which are among these apparatuses. It is known that dsg-1 and 3 are located in different epidermal cell layers depending on their differentiation. Dsg-1 is expressed to a higher extent in the upper, more differentiated layers, whereas dsg-3 is expressed to a higher extent in the lower, undifferentiated layers. These molecules play a role in the pathogenesis of pemphigus vulgaris and foliaceus, autoimmune blistering skin diseases, as antibodies against them produce the typical blisters. Another important coupling apparatus is the adherens junction, which is composed of cadherins. The focus of our studies was also on P-cad, which is more common in the basal and suprabasal layers. It is known from the literature that these adhesion molecules may be affected by a number of processes that also influence differentiation. Among these signal transduction systems, the PKC system, which is known to regulate differentiation and proliferation and to have opposing effects in different isoforms, was the focus of our interest.

In our experiments investigated the relationship between adhesion molecules and the PKC system, our first objective was to investigate whether the characteristic differentiation-dependent distribution of adhesion molecules observed in vivo is also observed in cell culture, i.e. whether more differentiated cells express dsg-1 and more proliferating cells express dsg-3 and P-cad to a greater extent. It is a known property of keratinocytes that during culture, when they reach confluency, their proliferation is reduced and their cellular processes shift towards differentiation due to inhibition by high cell density. Thus, HaCaT keratinocyte cultures expanded at the same cell density were 'harvested' on different culture days (days 1, 3, 5, 7, 9) and examined by Western blot for protein expression of adhesion molecules. We found that in pre-confluent (days 1, 3, 5) samples, dsg-3 and P-cad expression was significantly higher compared to dsg-1, whereas in contrast, after reaching confluence (days 7, 9), dsg-1 was significantly expressed in cells in the differentiation phase, while the other two adhesion molecules were expressed to a lesser extent. Based on our results, we conclude that in vivo conditions in the epidermis correlate well in vitro with cell culture cultures tested on different culture days with respect to the expression of adhesion molecules.

Our working hypothesis is that if there is a regulatory role of the PKC system on cell-coupling proteins, then their increased or even decreased expression/inhibition of their expression/function will also alter the expression of adhesion molecules. Therefore, in our subsequent series of experiments, we aimed to study the regulatory effects of PKC isoenzymes on adhesion molecules using HaCaT cells overexpressing PKC isoforms silenced by RNA interference and rottlerin, known as a specific inhibitor of PKCδ. We found that nPKCδ, which

promotes differentiation, increased the expression of dsg-1, a marker of differentiation, while it decreased the synthesis of dsg-3 and P-cad, which are more abundant on proliferating cells, on overexpressing cells, whereas our results were opposite for both the specific inhibitor and gene silenced cell cultures. In contrast, cPKCα had no effect on the expression of dsg-1, suggesting that although both isoforms play a role in differentiation, they affect the expression of adhesion molecules in different ways. Regarding the effect of nPKCε and cPKCβ, which regulate keratinocyte proliferation, the former decreased the expression of dsg-1 and increased that of dsg-3 and P-cad, whereas the latter isoenzyme only affected, i.e. increased, the expression of P-cad. Based on our experiments, it appears that, interestingly, calcium-independent PKC isoenzymes (nPKCδ and ε) are involved in the regulation of dsg-1 expression. In addition, calcium is known to enhance dsg-1 expression and keratinocyte differentiation, which is probably not directly via activation of calcium-dependent PKCs. Calcium-independent nPKCs also appear to be involved in the regulation of dsg-3, but cPKCα also has an effect, supported by the finding of Osada et al. that an anti-dsg-3 autoantibody isolated from patients with pemphigus vulgaris induced activation of cPKCα and nPKCδ. P-cad can be affected by both classical and novel PKC pairs (cPKCα/nPKCδ and cPKCβ/nPKCε) in opposite ways.

There are several data in the literature indicating a role for the PKC system in the development of desmosomes, but it is not yet known how each PKC isoenzyme individually affects it. Kimura et al. have shown that PKC activation/inhibition (isoform-dependent) alters the formation of calcium-independent, hyper-adhesive desmosomes, which in turn allows for a more mechanically resistant epidermis. The literature suggests that several PKC isoforms (PKC α and δ) are activated by pemphigus vulgaris IgG. In addition, Sanchez-Carpintero et al. were able to prevent pemphigus vulgaris IgG-induced blister formation in neonatal mice by using the PKC inhibitor bisindolylmaleimide. It is known that inhibition of PKC stabilizes dsg-3 in the desmosome and that the dsg-3 eliminating effect of PV-IgG was counteracted by inhibitors (Gö-6976- cPKC inhibitor, Safingol-PKCα inhibitor). In addition, it is known that PKCα plays a role in the phosphorylation of desmoplakin, its inhibition stabilizes the desmosome and prevents its disassembly. Our own results suggest that PKC isoenzymes have an effect on Dsg-3 expression depending on which isoform we are talking about, but I could not find any publication that investigated their effect depending on the isoform. The pathophysiological role of other signalling pathways is also known in pemphigus vulgaris, such as p38, MAPK, GTPase family Rho, c-myc and phospholipase C signalling, but their exact role remains to be elucidated. Egu et al. investigated the effect of PKC and Erk pathway regulation on dsg-1 expression and vesicle formation in the presence of PV-IgG. Using a non-selective inhibitor of PKC (Bim-X),

no changes were observed in either the inhibition of vesicle formation or the reduction of dsg-1 depletion, whereas inhibition of Erk succeeded in reducing them. The failure to activate the PKC pathway may be due to the fact that the non-selective PKC inhibitor inhibited PKC isoenzymes with opposite effects, resulting in a 'net ineffectiveness'. Isoform specific effects were investigated only for PKCα, no data were available for the other isoenzymes, which would be essential to elucidate the regulatory processes. Our results may provide a good basis for further research aimed at developing clinically useful therapeutic options based on the in vivo associations found.

Effect of polyols on signalling pathways regulating keratinocyte life processes

Polyols are sugar alcohol molecules that contain several hydroxyl groups. Glycerol (glycerol) is found in many pharmaceutical preparations. It is known to have antimicrobial activity, which is exploited in the treatment of wounds in the form of hydrogel-containing dressings. It is used as a moisturizer in cosmetics and dermatological externals due to its water-binding properties, as a constituent of syrups due to its sweet taste, and as a laxative in the form of rectal suppositories due to its hyperosmotic property. The other polyol of interest, xylitol, is less widely used. Most of us think of xylitol as a sugar substitute. However, it also has a wide range of dermatological uses, mainly as an ingredient in moisturising creams (e.g. Aquaxyl, Xylinep). There have been several studies on the effects of glycerol and xylitol on keratinocytes. They have been shown to play a role in the repair of barrier dysfunction through their physico-chemical action. In a mouse model, it has been demonstrated that the irritant, pro-inflammatory effect of sodium lauryl sulphate could be counteracted by a decrease in TEWL, reduction in IL-1β and TNF-α mRNA levels, reduction in white blood cell infiltration and preservation of epidermal thickness. Other groups have investigated the effect of glycerol on the epidermal barrier in AQP3 knock down mice. Aquaporin 3 is known as a water/glycerol transporter in the epidermis. Hara et al. found that in these mice, topical or oral glycerol improved SC hydration and TEWL was reduced. The same effect was not observed with other polyols (xylitol, erythritol, propanediol). Others have found an increase in intestinal permeability in the same mouse model, a decrease in the expression of claudin-1 and occludin on intestinal epithelial cells, suggesting a role for glycerol as a barrier regulator. Human studies have also been conducted on the combined use of the two polyols. After two weeks of topical application, various hydration and morphological parameters of the skin (Str corneum thickness, TEWL, roughness, epidermis thickness, dermal papilla echogenicity) and the presence of filaggrin in the epidermis were examined and it was found that the above parameters showed significant

improvement when the two substances were topically applied together. It is known that the osmotic stress, which plays a pathogenetic role in irritant contact dermatitis and dry skin syndromes, induces keratinocytes to produce inflammatory mediators. Both xylitol and glycerol have been shown to reduce this inflammatory response. Furthermore, it was shown that the combined application of a cream containing xylitol and farnesol in patients with atopic dermatitis reduced S. aureus colonization, which plays a pathogenetic role in AD, and also reduced TEWL and increased conductance, which shows SC hydration.

What is less well understood, however, is how these effects are mediated through signal transduction processes. In our experiments, we have demonstrated for the first time that the two polyols do not affect keratinocyte life processes at the doses we used (0.27% glycerol, 0.45% xylitol). Subsequently, we examined whether the expression of certain differentiation markers (filaggrin, loricrin, occludin) at the mRNA level is enhanced on NHEK in both proliferating and differentiating keratinocytes. Calcium is known to have a regulatory role in differentiation, but glycerol and xylitol did not achieve this effect by increasing intracellular calcium concentrations. To investigate how the increase in the expression of differentiation markers might have occurred, we examined other signalling pathways such as the MAPK/Erk and PKC pathways, which have also been shown to regulate proliferation-differentiation. We found that their effects activate the MAPK/Erk pathway and xylitol leads to PKCδ activation. This result is in correlation with the fact that calcium-dependent PKCα was not affected by the substances we tested. Furthermore, our data also suggest that the effect may be mediated through PKCδ signalling, which has been shown to play a role in keratinocyte differentiation. We also confirmed that xylitol and glycerol have different anti-inflammatory effects. Both substances were able to inhibit HLA-DR up-regulation, which is important in immune activation, during stimulation of TLR2 and 3 pathways. It was shown that while glycerol mainly affected the TLR3 pathway, xylitol had an effect on cytokine expression in all three (TLR2, 3, 4) inflammation models. However, it is necessary to note that the effect showed a significant donor dependence and that the changes we observed at the mRNA level need to be complemented and validated by further studies at the protein level.

Based on the literature data and our own results, we hypothesize that the combined topical application of the two agents, in addition to hydration, also has an effect on keratinocyte differentiation and reduction of inflammation, which may be confirmed by further human clinical studies in the future.

SUMMARY

The integrity of our skin is indispensable in the protection against harmful agents in our environment. One important factor in maintaining integrity is cell adhesion apparatus, whose structure proteins show different distributions in vivo in the layers of the epidermis, i.e., depending on the state of differentiation. Adhesion molecules (dsg-1, 3 and P-cad), which are involved in the patogenesis of pemphigus vulgaris and foliaceus, were investigated. In the light of the fact that the PKC isoenzyme family performs a regulatory function in cell proliferation and differentiation and that this regulatory function is well studied in the human epidermal HaCaT keratinocyte cell line, it seemed logical to investigate the effect of each PKC isoenzyme on the expression of adhesion molecules with the used overexpression, RNA interference technics and the antagonist, rottlerin. Our results suggest that the two Ca-independent 'novel' nPKC isoforms regulate dsg-1, found on more differentiated cells, in the opposite ways, i.e., nPKCδ, which promotes differentiation, increased, while ε, which is responsible for proliferation, decreased dsg-1 expression. In contrast, the expression of dsg-3, expressed on undifferentiated cells, was reduced by nPKCδ, while was increased by nPKC ε; the differentiation-inducing Ca-dependent 'conventional' PKCα behaved similarly to nPKCδ. In the case of P-cad, present on proliferating keratinocytes, all PKC isoforms examined by us seemed to have regulatory role, in an opposite way (cPKCα／nPKCδ inhibited, while cPKCβ／nPKCε increased expression). Thus, we have shown that PKC isoenzymes are involved in the regulation of the expression of adhesion molecules that make up desmosomes and adherent juctions.

Another important element in maintaining integrity is the provision of hydration, which can be aided by two topical polyols, glycerol and xylitol. Previous publications have suggested that in addition to providing hydration, these polyols can also affect signal transduction pathways. Thus, in the other part of our experiments we examined this. We investigated the effect of polyols on the expression of differentiation markers using non-toxic doses (xylitol: 0.0045% - 0.45%; glycerol: 0.0027% -0.27%). We found that the expression of filaggrin, loricrine, involucrin, and occludin was enhanced by both substances. This effect was mediated by the MAPK pathway for both polyols and by the additional activation of nPKCδ for xylitol. In inflammation models, it was also shown that both glycerol and xylitol had an anti-inflammatory effect by influencing the expression of various cytokines. All these results suggest that in addition to their moisturizing effect, glycerol and xylitol i) influence the production of proteins,

which are important for barrier restoration and ii) can also help reduce inflammation by activating different signaling pathways. In addition, based on theoretical considerations, it can also be assumed that their combined application even more effectively improves the skin barrier function.

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Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology

List of publications related to the dissertation

1. **Páyer, E.**, Szabó-Papp, J., Ambrus, L., Szöllősi, A. G., Andrási, M., Dikstein, S., Kemény, L., Juhász, I., Szegedi, A., Bíró, T., Oláh, A.: Beyond the physico-chemical barrier: glycerol and xylitol markedly yet differentially alter gene expression profiles and modify signalling pathways in human epidermal keratinocytes.
Exp. Dermatol. 27 (3), 280-284, 2018.
DOI: http://dx.doi.org/10.1111/exd.13493
IF: 2.868

2. Szegedi, A., **Páyer, E.**, Czifra, G., Tóth, I. B., Schmidt, E., Kovács, L., Blumberg, P. M., Bíró, T.: Protein kinase C isoenzymes differentially regulate the differentiation-dependent expression of adhesion molecules in human epidermal keratinocytes.
Exp. Dermatol. 18 (2), 122-129, 2009.
DOI: http://dx.doi.org/10.1111/j.1600-0625.2008.00771.x
IF: 3.239

List of other publications

3. Szántó, M., Oláh, A., Szöllősi, A. G., Tóth, K. F., **Páyer, E.**, Czakó, N., Pór, Á., Kovács, I., Zouboulis, C. C., Kemény, L. V., Bíró, T., Tóth, I. B.: Activation of TRPV3 inhibits lipogenesis and stimulates production of inflammatory mediators in human sebocytes: a putative contributor to dry skin dermatoses.
J. Invest. Dermatol. 139 (1), 250-253, 2019.
DOI: http://dx.doi.org/10.1016/j.jid.2018.07.015
IF: 7.143

4. Illés, Á., **Páyer, E.**, Simon, Z., Miltényi, Z.: A sokszínű Castleman-betegség.
Hematol. Transzfuziol. 48, 81-85, 2015.

5. **Páyer, E.**, Miltényi, Z., Simon, Z., Magyari, F., Barna, S., Méhes, G., Illés, Á.: Diagnostic and Therapeutic Difficulties in Diffuse Large B-cell Lymphoma Arising From HHV8 Positive Castleman Disease.
J. Hematol. 1 (2-3), 65-69, 2012.

6. Simon, Z., Jóna, Á., Miltényi, Z., **Páyer, E.**, Lieber, A., Szilasi, M., Illés, Á.: Diagnosztikai nehézséget okozó pulmonalis infiltrátum.
Orv. Hetil. 153 (27), 1077-1081, 2012.
DOI: http://dx.doi.org/10.1556/OH.2012.29404

7. Váróczy, L., **Páyer, E.**, Kádár, Z., Gergely, L., Miltényi, Z., Magyari, F., Szodoray, P., Illés, Á.: Malignant lymphomas and autoimmunity - a single center experience from Hungary.
Clin. Rheumatol. 31 (2), 219-224, 2012.
DOI: http://dx.doi.org/10.1007/s10067-011-1807-1
IF: 2.037

8. **Páyer, E.**, Miltényi, Z., Simon, Z., Szabados, L., Dull, K., Méhes, G., Illés, Á.: Uncommon late relapse of angioimmunoblastic T-Cell lymphoma after 16-year remission period.
Pathol. Oncol. Res. 18 (3), 737-741, 2012.
DOI: http://dx.doi.org/10.1007/s12253-011-9475-7
IF: 1.555

9. Magyari, F., **Páyer, E.**, Barna, S., Simon, Z., Miltényi, Z., Udvardy, M., Váróczy, L., Illés, Á.: Az első tapasztalatok rituximab-bendamustin kezeléssel Hodgkin-lymphoma autológ perifériás őssejt-transzplantációt követő progressziójában.
Magyar Belorv. Arch. 2011 (64), 167-169, 2011.

10. Miltényi, Z., Simon, Z., **Páyer, E.**, Váróczy, L., Gergely, L., Jóna, Á., Illés, Á.: Changing patterns in the clinical pathological features of hodgkin lymphoma: a report from Debrecen, Hungary.
ISRN Hematology. 2011, 810708, 2011.
DOI: http://dx.doi.org/10.5402/2011/810708

11. Váróczy, L., Gergely, L., **Páyer, E.**, Miltényi, Z., Simon, Z., Illés, Á.: Myeloid growth factor therapy in malignant lymphomas: a 5-year retrospective study from Hungary.
Support. Care Cancer. 19 (10), 1619-1623, 2011.
DOI: http://dx.doi.org/10.1007/s00520-010-0992-9
IF: 2.597

12. Miltényi, Z., Simon, Z., **Páyer, E.**, Váróczy, L., Gergely, L., Illés, Á.: Refrakter és relabált Hodgkin-lymphomás betegeink kezelésével szerzett tapasztalataink.
Orv. Hetil. 151 (5), 172-178, 2010.
DOI: http://dx.doi.org/10.1556/OH.2010.28799

13. Miltényi, Z., Simon, Z., **Páyer, E.**, Váróczy, L., Gergely, L., Jóna, Á., Illés, Á.: Változnak-e a Hodgkin-lymphoma klinikopatológiai jellemzői?
Orv. Hetil. 151 (49), 2011-2018, 2010.
DOI: http://dx.doi.org/10.1556/OH.2010.28990