UVB- and plasma radiation-induced cellular responses of human keratinocytes

Ph.D. Thesis

Barbara Fazekas

Department of Dermatology and Allergology

University of Szeged

Szeged, Hungary

2015

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Barbara Fazekas

Supervisors:

Dr. Éva Ádám

Dr. Márta Széll

Graduate School of Clinical Medicine

Department of Dermatology and Allergology

University of Szeged, Szeged, Hungary

Institute of Plant Biology

Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary

Szeged

2015

LIST OF PUBLICATIONS

Publications directly related to the subject of the dissertation

I. **Barbara Fazekas**, Hilda Polyánka, Attila Bebes, Gábor Tax, Kornélia Szabó, Katalin Farkas, Ágnes Kinyó, Ferenc Nagy, Lajos Kemény, Márta Széll, Éva Ádám: UVB-dependent changes in the expression of fast-responding early genes is modulated by huCOP1 in keratinocytes.
Journal of Photochemistry and Photobiology B-Biology 140: pp. 215-222. (2014)
IF: 2.803

II. Ihor Korolov, **Barbara Fazekas**, Márta Széll, Lajos Kemény, Kinga Kutasi: The effect of the plasma needle on the human keratinocytes related to the wound healing process.
Journal of Physics D: Applied Physics (Accepted for publication) (2015)
IF: 2.721

Publication not related to the subject of the dissertation

1. Nikoletta Nagy, Katalin Farkas, Ágnes Kinyó, **Barbara Fazekas**, Kornélia Szabó, Edit Kollár, Balázs Sztano, Angéla Meszes, Dóra Beke, Lajos Kemény, László Rovó, Márta Széll: A synonimous polymorphism of APCDD1 affects translation efficacy and is associated with androgenic alopecia.
Journal of Life Sciences (Libertyville) 8:(2) pp. 106-114. (2014)

2. Hilda Polyánka, Kornélia Szabó, Vilmos Tubak, Erzsébet Kusz, Lilla Erdei, Gábor Tax, Beáta Szilvia Bolla, **Barbara Fazekas**, Zsuzsanna Ujfaludi, Róbert Katona, Imre Boros, Zsuzsanna Bata-Csörgő, Lajos Kemény, Márta Széll: A novel immortalized keratinocyte cell line (HPV-KER) is a suitable model for in vitro analysis of UV-B-induced processes of keratinocytes.
(Under review in *Archives of Dermatological Research;* **IF: 1.902,** 2015**)**

INTRODUCTION

1. UVB-induced processes in the skin and in keratinocytes

UVB light with a wavelength range between 290 and 320 nm is one of the most important physical carcinogens in the environment, and the skin is the first and major barrier to protect the body from its harmful effects. The epidermis, our first line of defence from UV light, bears the majority of photo damage, which results in skin thinning, wrinkling, keratosis, and malignancy. The clinical and histological manifestations of UV damage have been well known for some time, but the molecular mechanisms that cause them have only recently become a focus of concerted studies. Although epidermal keratinocytes, the main site of environmental UVB damage, provide a useful model system to study UVB-induced cellular responses, information on the molecular pathways mediating these processes is currently limited.

1.1. COP1, the Constitutive Photomorphogenic Protein I

The COP1 (constitutive photomorphogenic protein 1) protein was first identified as a central negative regulator of light-regulated development in *Arabidopsis thaliana*. *Arabidopsis thaliana* COP1 (AtCOP1) functions as an E3 ubiquitin ligase targeting several transcription factors for proteosomal degradation in plants. In the dark, COP1 is confined to the nucleus, where it directs ubiquitylation and proteasomal degradation of various transcription factors, including Elongated hypocotyl 5 (HY5), HY5-homolog (HYH) and Long hypocotyl in far red (HFR1) and Long after far-red light 1 (LAF1) that turn on light-activated genes. In the light, however, COP1 resides in the cytoplasm, allowing the light-responsive transcription factors to activate their downstream targets. Among its substrates is the HY5 protein, a basic domain/leucine zipper (bZIP) transcription factor, which is one of the key regulators of photomorphogenesis under all light conditions, including UVB.

Sequence analysis of COP1 orthologs from the *Arabidopsis*, human and mouse genome indicated that the COP1 domain structure is highly conserved in higher plants and vertebrates. The mouse (MmCOP1) and human COP1 (huCOP1) are located on chromosome 1 and the high degree of sequence conservation with AtCOP1 suggested functional conservation.

The role of mammalian COP1 (also known as RFWD2) has been revealed from experiments carried out mostly on mouse model. COP1 is an E3 ubiquitin ligase that is ubiquitously expressed, although not at high levels. It mainly resides in the nucleus and a small amount may also be present in the cytosol. COP1 is primarily involved in the ubiquitylation of various protein substrates and itself to trigger their proteasomal degradation. COP1 contributes to UVB-induced signalling in plants and also in human keratinocytes.

1.1.1. The role of COP1 in the UVB response of plant and human cells

1.1.1.1. COP1 in the UVB response of plants

In contrast the negative regulation observed for visible-light responses, AtCOP1 is a critical positive regulator of responses to low levels of UVB. Genome-wide expression changes in response to UVB are blocked to a large extent in the *Arabidopsis* cop1-4 mutant, and, in addition, AtCOP1 is required for HY5 gene activation. According to the latest data, UVB triggers the physical and functional disassociation of the AtCOP1–SPA core complex from CUL4-DDB1 and the formation of a new complex containing the UVB photoreceptor, UV Resistance Locus 8 (UVR8). This UVB-induced machinery is associated with the positive role of AtCOP1 in facilitating HY5 stability and activity.

1.1.1.2. The role of huCOP1 in the UVB response of keratinocytes and its mechanism

The UVB-dependent molecular network in which p53 plays a pivotal role has not been completely revealed yet. TP53 is a transcription factor that functions as a central component of most cellular stress responses. Our research group has previously demonstrated that one of the p53-interacting partners, the E3 ubiquitin ligase, huCOP1 is expressed in keratinocytes in an UVB-regulated manner and is a negative regulator of p53 as a post-translational modifier. The regulation of p53 by huCOP1 in keratinocytes is of particular importance, as this role suggests involvement in cellular UV responses. COP1 also promotes its own ubiquitylation and degradation, and this process is accelerated by DNA damage. Lee et al. discovered that the constitutive photomorphogenesis 9

signalosome (CSN) plays a role in the control of DNA damage and carcinogenesis caused by UV light.

2. Gas discharge plasma, the therapeutic gas mixture

The atmospheric pressure gas discharge plasmas are promising candidates for new therapeutic tools. Plasma medicine is a new emerging area of interdisciplinary research in plasma physics, combining biology, chemistry and physics. The gas discharge plasma is a mixture of ions, electrons, radicals (reactive oxygen species (ROS) and reactive nitrogen species (RNS)), excited species that contribute to the UV radiation, as well as is characterized by an electrical field. Low-temperature plasmas can have a huge potential in dermatological applications based on their unique mixture of reactive components sparking both stimulatory as well as inhibitory processes. Accordingly, the treatment of chronic wounds by plasmas may have selective antimicrobial or antiseptic effect without damaging the surrounding tissue, while stimulating the tissue regeneration. It has been shown that cold atmospheric plasmas (CAP) entail no risk on humans in terms of temperature increase, UV radiation or by free radical formation by the plasma, while can considerably decrease the bacterial load of the skin.

2.1. The effect of the plasma treatment on the wound healing processes

The *plasma needle* is a plasma source with a non-thermal atmospheric glow discharge ignited at the tip of a needle. From its initial application the *plasma needle* went through a series of transformations, which made it more and more suitable for biomedical applications. Several studies have been conducted, which aimed to investigate the effect of the plasma needle on biological samples, such as, reattachment and apoptosis of 3T3 mouse fibroblast after plasma treatment, the proliferation and differentiation of mesenchymal stem cells, as well as its bactericidal effect. Since *plasma needle* insures the sample to be in direct contact with the active discharge plasma - in contrast with the atmospheric plasma jets where only afterglow species reach the sample - makes possible the study of the effect of the active plasma and electric field on the wound healing processes.

AIMS

UVB light is one of the most important physical carcinogens in the environment, and the skin is the first and major barrier to protect the body from its harmful effects. HuCOP1 is involved in the orchestrating of several cellular processes, and contributes to cellular stress response after the DNA damage caused by UVB. Although it is evident from the available data that huCOP1 is a posttranslational regulator of late UV responses in a wide range of organisms, it was not known how huCOP1 acts on early transcriptional responses in human keratinocytes.

Low-temperature gas discharge plasmas can have a huge potential in medical applications by stimulating the living cells and tissues. In the process of wound healing the major contributors are the keratinocytes, which migrate to fill in the gap created by the wound. The active discharge plasma generated by the *plasma needle* gives the possibility to study the effect of plasma on the wound healing processes.

Therefore, we aimed

— to establish and characterize a keratinocyte cell line in which the expression of huCOP1 is silenced (siCOP1)
— to investigate the effect of UVB on huCOP1 protein abundance in the siCOP1 cell line
— to characterize the role of huCOP1 in early UVB-induced signalling processes that lead to transcriptional changes in keratinocytes
— to characterize the effect of the *plasma needle* on the proliferation and migration of human keratinocytes
— to verify the influence of a non-thermal atmospheric pressure plasma on the wound healing process on human keratinocytes

MATERIALS AND METHODS

- Stable transformation of HPV-KER II/15 was performed with either the empty vector (pSuperior puro vector) or a vector harbouring huCOP1 silencing sequences.
- Cell viability of the established keratinocyte cell lines was measured with the impedance measurement-based xCELLigence RTCA System.
- Distinction of dead cells from living ones were observed using propidium iodide staining and afterwards by confocal laser scanning microscopy using a confocal laser scanning microscope.
- Cells were irradiated with a 20 mJ/cm^2 dose of 312 nm UVB from an FS20 lamp (Westinghouse, Pittsburgh, PA).
- For the Western blot analysis, equal amounts of total protein extracts (prepared from 1.0 million cells in a 100 µl solution) were run on SDS-PAGE and then transferred onto a nitrocellulose or PVDF membrane. Membrane were incubated with COP1 or α-Actin primary antibodies and subsequently with secondary antibody (anti-rabbit IgG–HRP). The blots were developed with the Immobilon Western Chemiluminescent HRP substrate (Merck Millipore Corporation, Billerica, MA, USA). Luminescent signals were detected using a liquid-nitrogen-cooled charge-coupled-device camera.
- For Immunocytochemistry, keratinocytes were grown on culture slides and were immunostained 24 hours after UVB irradiation. Samples were incubated with COP1 primary antibody and subsequently with Alexa Fluor 488-labeled secondary antibody. A Zeiss AxioImager fluorescent light microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) fitted with a PixeLINK CCD camera (PixeLINK, Ottawa, ON, Canada) was used for detection.
- For Real-time RT–PCR array, total RNA was isolated from the cells. The cDNA was synthesized from 5 µg total RNA. Gene expression profiling was carried out with the custom-made StellARray™ Gene Expression System (Bar Harbor BioTechnology, Trenton, ME) carrying 30 UVB-regulated genes. The RT-PCR arrays were performed with the ABI Prism 7300 PCR machine. The expression of each gene was normalized to the 18S ribosomal RNA gene. Results are averages of three parallel experiments. The relative mRNA expression levels were calculated by the $^{\Delta\Delta}C_t$ method.

- The validation real-time RT–PCR experiments were carried out with the Universal Probe Library system (F. Hoffmann-La Roche AG, Basel, Switzerland).
- Pathway analysis was performed using the Ingenuity Pathway Analysis (IPA) software. For all analyses, Fisher's exact test was used to calculate a P-value. IPA uses a z-score algorithm to make predictions.
- In order to investigate the effect of the gas discharge plasma on the cells the gas discharge generated at the tip of the needle, the so-called *plasma needle* was used. The needle consisted of a 0.3 mm diameter central electrode made of wolfram. The needle was covered by a slightly larger ceramic tube - except for a 2 mm segment at the tip - and placed in a glass tube of 4 mm inner and 6 mm outer diameter. The tip of the needle was placed in line with the edge of the glass tube. The needle body was made of Teflon. Helium was flowing between the ceramic and the glass tube, the flow rates used are in the 1 - 1.45 slm (standard liters per minute) range. The discharge was operated in the 15-30 W input power range and the dissipated plasma power was estimated to be less than 0.5 W. The optical emission spectra in the 250-800 nm spectral range showed the dominance of the He lines, accompanied by the O atomic line, the OH and low intensity N_2 and N_2^+ bands.
- In the case of the wound-healing model experiments 1×10^5 cells per well were seeded into 24-well plates. Before treatment a scratch wounding was performed with a cell scraper of 4 mm width. The evolution of the scratch in time was observed and recorded using a Nikon Eclipse TS100 inverted routine microscope fitted with a Nikon Coolpix 4500 camera. The width of the scratch was measured using the Gimp2 software. The scratch width reduction was calculated by subtraction of scratch width at t = n hours from scratch width at t = 0 h.
- *Propionibacterium acnes* (ATCC 11828; 1×10^9 cfu/ml) bacterial suspension was transferred into the medium covering the scratched keratinocytes, achieving the multiplicity of infection 50 microorganisms. The general cell performance were observed during 24 h cocultivation with keratinocytes.

RESULTS

1. The role of human Constitutive Photomorphogenic Protein 1 in the UVB light response of human keratinocytes

UVB light is undoubtedly the most important carcinogenic environmental stressor of human skin, and UV-induced changes in keratinocytes have been widely studied.

COP1, the Constitutive Photomorphogenic Protein 1, is well conserved across species. In plants, the function of COP1 is closely tied to the light signalling pathway. COP1 acts as a central repressor in the pathway, where it promotes the ubiquitination and degradation of the positive regulators and is itself regulated by multiple photoreceptors. A recent study revealed that under UVB, however, COP1 acts as a positive regulator in the signalling pathway. Several data suggest that huCOP1 has been implicated in the negative regulation of important cancer-related genes acting in the cellular response to UVB. Nevertheless, the possible role of huCOP1 in keratinocyte UVB response has not yet been investigated in detail. Our aim was therefore to investigate the function of huCOP1 in keratinocytes, the cell type most exposed to UVB irradiation.

To address these issues we produced transgenic cell lines in which the expression of huCOP1 was stably silenced (siCOP1). For this purpose, a novel, human papillomavirus type 16 *E6* oncogene-immortalized keratinocyte cell line (HPV-KER) was used and characterized. Since this cell line exhibited a normal p53 UVB response, we considered it suitable for our purposes.

After establishing the huCOP1 silenced transgenic keratinocyte cell line, we examined the expression of selected UVB-regulated genes with or without UVB irradiation. Our experiments revealed that the silencing of huCOP1 did not affect cell viability before and with a short time after UVB irradiation. However, very importantly, we found that the residual huCOP1 level was further reduced by UVB and the significantly reduced huCOP1 level resulted in more pronounced UVB-induced changes in the expression of genes as compared to non-transgenic keratinocytes. These data demonstrate that this cell line is a particularly suitable tool for studying huCOP1-dependent UVB-induced changes in early gene expression responses.

In order to elucidate the function of huCOP1 in the early UVB-response of human keratinocytes, we carried out array analysis. DNA array and validating real-time RT–PCR

experiments confirmed that transcript levels of the selected genes exhibited changes as early as 2 h after UVB irradiation and that these changes were in good agreement with previously published data. Our experiments revealed that the expression level of the selected genes was not affected by huCOP1 silencing in unirradiated cells. Our results and the data available in the literature indicate that decreased huCOP1 levels sensitize the cells to UVB damage or oxidative stress and modify the UVB-induced stress response of keratinocytes.

To analyse the possible interactions among the examined genes we used the Ingenuity Pathway Analysis software. This software uses published interaction data for composing potential new networks based on novel experimental data. The pathway analysis identified a network in which 13 of the 30 examined genes were organized around three central molecules, *ERK1/2, CREB* and ubiquitin. Functional connections between certain members of the identified network have already been described. All 13 genes were differentially expressed after UVB irradiation, and their expression was increased in siCOP1 cells compared to control. Similar changes have been detected in *ERK1/2* and *CREB* gene expression, affirming their central role in the identified network. Based on results available in literature data, the loss of some members in the identified network increases the sensitivity of the cells to UVB. Presumably, the absence of certain members of the huCOP1-mediated transcriptional cascade leads to an abnormal UVB response.

Some of the components of the identified network have already been implicated in huCOP1-mediated processes. The expression of huCOP1 is altered in non-melanoma skin cancers and several members of the identified regulatory network have been implicated in the pathogenesis of these skin diseases. Thus, huCOP1 emerges as a potential target molecule for the treatment basal cell carcinoma (BCC) and/or squamous cell carcinoma (SCC).

2. The effect of the *plasma needle* on the human keratinocytes related to the wound healing process

The devices based on atmospheric pressure gas discharge plasmas are good candidates for future medical tools. Initial studies have been conducted aiming to model the wound healing process, e.g. the penetration of plasma or plasma generated species into the wound mimicking model surfaces. However, the mechanisms of plasma

interaction with living tissues and cells are very complex, therefore further studies are needed to clarify the physical and biological mechanisms.

The *plasma needle*, which is a plasma source with a non-thermal atmospheric glow discharge ignited at the tip of a needle, is a suitable device for studying biomedical applications. Since *plasma needle* insures the sample to be in direct contact with the active discharge plasma - in contrast with the atmospheric plasma jets, where only afterglow species reach the sample - it is a suitable device to study the effect of the active plasma on the wound healing process.

In the wound healing process the major contributors are the keratinocytes, which migrate to fill in the gap created by the wound. Therefore, we performed the direct treatment of HPV-immortalized human keratinocytes with the glow discharge generated in flowing helium by a *plasma needle*. The cells protected by a layer of phosphate buffered saline (PBS) solution were directly treated with the *plasma needle*. We conducted two types of experiments: (i) cell proliferation and (ii) wound-healing model experiments.

In order to choose the optimal *plasma needle* configuration, the plasma treatment conditions and the thickness of the protecting PBS layer, viability experiments were carried out. We found that the minimum PBS layer necessary to protect the cells during treatment depended on the wells' dimensions, namely, in larger wells thinner layer appeared to be sufficient to protect the cells. This shows the difficulty of scaling the treatment conditions.

The proliferation studies showed that short (5-10 s) and low power (18 W and 20 W input power) treatments could positively influence cell proliferation when keratinocytes were protected by PBS. We also found that the cells treated in cell medium were more affected than those treated in PBS (i.e. cell proliferation decreased), which shows that the plasma induced liquid chemistry - i.e. the created active species, which interact with the cells - in the two liquid mediums were different. Therefore we concluded that the choice of the interaction medium is very important when setting the treatment conditions.

To study the effect of the *plasma needle* on keratinocytes related to the wound healing process, we modelled it with an *in vitro* scratch assay. Monitoring the reduction of the scratch width after plasma treatment for 48 h, we found that there was a maximum in the wound reduction as a function of the input power and treatment time, namely, at 18W and 5 s. Nevertheless, favourable conditions were also found in the case of 18 W

and 20 W input powers and 5-25 s long treatments. We also found that the wound reduction strongly depended on the treated cell – PBS interaction time.

In the case of the proliferation experiments conducted in the 96-well plates the cells were in contact with the full plasma spot, thus the cell culture was exposed to the whole radial electric field distribution of the plasma, in contrast with the scratch assay experiments, where only the wings of the field distribution were in contact with the cells. Therefore, the differences in the two types of experiments may be attributed to the field effect. In what concerns the chemical change of the treated PBS, we did not observe any change in the PH level.

In order to mimic the natural microbial environment of human keratinocytes, they were cocultivated with the most common commensal bacteria of the skin, the *Propionibacterium acnes* (*P.acnes*) Gram-positive bacterium. The plasma treatment of this assay resulted in closing of the scratch, while in the non-treated assay the wound did not close at all.

The results overall suggest, that for the activation of the healing process a minor stress induction of keratinocytes is sufficient, and the treatment medium should be carefully chosen.

SUMMARY

- We found that huCOP1 contributes to the transcriptional regulation of the keratinocyte UVB response by the down-regulation of an UVB inducible network operating through three newly identified central organizers.
- This network is under the control of upstream regulators also showing UVB response. We hypothesize that huCOP1 operates as a negative factor of the identified transcriptional network by modifying the function of the upstream regulators.
- The expression of huCOP1 is altered in non-melanoma skin cancers and several members of the identified regulatory network have been implicated in the pathogenesis of these skin diseases. Thus, huCOP1 emerges as a potential target molecule for the treatment of BCC and/or SCC.
- We could verify the influence of a non-thermal atmospheric pressure plasma on the wound healing process.
- Our results revealed that plasma could positively influence the cell proliferation of keratinocytes protected by PBS. On the other hand, the plasma treatment of cell medium covered keratinocytes resulted in the decrease of proliferation.
- The wound-healing model studies showed that there was a maximum in the wound reduction as a function of the input power and treatment time, namely, at 18 W and 5 s. Furthermore, the wound reduction strongly depended on the treated cell - PBS interaction time.
- To mimic the natural microbial environment of human keratinocytes, they were cocultivated with the most common commensal bacteria of the skin, the *Propionibacterium acnes* (*P.acnes*) Gram-positive bacterium. The plasma treatment of this assay resulted in closing of the scratch, while in the non-treated assay the wound did not close at all.
- The results overall suggest, that for the activation of the healing process a minor stress induction of keratinocytes is sufficient, and the treatment medium should be carefully chosen.

ACKNOWLEDGEMENT

I would like to thank to Prof. Dr Lajos Kemény for the opportunity to participate in their Ph.D. program and to perform these studies in the laboratory of the Department of Dermatology and Allergology, University of Szeged.

I would like to express my deepest and sincere gratitude to my mentors and advisors Prof. Dr Márta Széll at the Department of Medical Genetics, MTA-SZTE Dermatological Research Group, University of Szeged and Dr Éva Ádám at the Institute of Plant Biology, Biological Research Centre of the Hungarian Academy of Sciences for their supervision of this work and for their constant support and advice. I admire their broad knowledge of science, their way of analyzing scientific problems, and their ability to come up with exciting new ideas. I am most grateful for their patient guidance and inspiration during my Ph.D. studies.

It also gives me great pleasure to acknowledge the support and help of Prof. Dr Ferenc Nagy and Dr Michael P Carty for their excellent suggestions and invaluable advice.

I would like to thank Dr Kinga Kutasi and Dr Ihor Korolov for the opportunity to look inside the magic of plasma physics.

I am grateful to my colleagues Gábor Tax, Dr Attila Bebes, Katalin Farkas, Hilda Polyánka and Anikó Göblös for introducing me to all the research techniques and for their caring support during my first steps in the laboratory of Department of Dermatology and Allergology, University of Szeged. I owe warm thanks to all of my colleagues for their help in the preparative work and for having had the opportunity to work with them.

This work was supported by the TÁMOP-4.2.2/B-10/1-2010-0012 and TÁMOP-4.2.2.A11/1/KONV-2012-0035 Grants.

I am also very grateful to all members of my family who helped me with their endless love, support and encouragement.