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„Investigation on the role of circadian system in pathological skin conditions"

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1. The circadian system in psoriasis

Psoriasis is a chronic inflammatory skin disease characterized by abnormal differentiation and proliferation of the keratinocytes. As circadian genes have a regulatory function in these two processes, it is assumed that their altered expression may have a role in the pathomechanism of psoriasis.

1.1. Results of in vitro experiments

During this work, we examined whether the key cytokines (TNFα, IL-22, IL-1β, IL-17A) implicated in the pathogenesis of psoriasis can modulate the circadian oscillation of the clock genes in HaCaT keratinocytes. Compared to the untreated keratinocytes, decreases or moderate increases in mRNA expression of the studied clock genes (*clock, bmal1, per2, cry1, rev-erbα*) were detected after the treatment with IL-22, IL-1β and IL-17A cytokines. In the TNFα-treated keratinocytes, *clock* mRNA showed significantly increased levels of expression during the whole study period. Moderate elevation were found for *per2* and *cry1* transcripts. Moreover, slight downregulation of *per1* and *rev-erbα* was observed in all cytokine-treated cells.

The results of this study demonstrated the perturbation in the expression of the core clock genes by proinflammatory cytokines in vitro.

1.2. Results of human experiments

Since little is known about the involvement of the circadian system in psoriasis, we compared the oscillation and relative mRNA expression of core clock genes between the healthy, non-lesionaland lesional human skin samples. Analysis of skin biopsies shows that *bmal1*, *per1* and *per2* genes are more highly expressed in psoriatic skin, especially in samples from non-lesional areas. Upregulation is observed in psoriatic skin for clock gene expression compared to healthy skin. For *rev-erbα*, we found a consequent mild downregulation in the psoriatic skin samples. The mRNA level of *cry1* was downregulated in symptomatic skin and elevated in asymptomatic skin. The CRY proteins have been described as positive regulators of anti-inflammatory responses, it is hypothesized that those may exert this role in non-lesional skin through their upregulation. Furthermore, we found different expression pattern between non-lesional psoriatic and healthy skin. Asymptomatic psoriatic skin appears clinically similar to normal

skin, although it has been exposed to the systematic effects of the disorder. It is important to further investigate the asymptomatic (nonlesional) areas to identify what processes are different from the symptomatic areas.

In the immunohistochemistry study, different epidermal distribution of the analyzed circadian proteins was observed in healthy and psoriatic skin. Strong CLOCK and PER2 positive stainings were detected in the stratum granulosum of lesional epidermis compared to the healthy and non-lesional psoriatic skin. In the case of the REV-ERBα protein, intense cytoplasmic but no nuclear staining was observed in healthy epidermis, except for the stratum basale.

Our human results indicate that, compared to healthy skin, transcripts of the clock genes are perturbed in nonlesional and lesional psoriatic areas. The alterations in circadian genes (ones with anti-inflammatory role) and protein expression among the different skin samples (healthy vs. nonlesional psoriasis vs. lesional psoriasis skin) may serve as possible therapeutic targets in the future. Modulation of the circadian clock by systemic or local therapies (e.g. agonists or ligands) may be effective in regulating not only psoriasis but also the accompanying inflammatory condition (e.g., REV-ERB agonists or ligands).

2. The circadian system in UVB induced inflammation

Of the solar spectrum, ultraviolet (UV) light posesses a range of skin-damaging effects. The nucleotide excision repair (NER), the primary pathway to remove UV photoproducts, is regulated by clock proteins, and exhibits diurnal pattern. There may be a time-of-day variation in the UV-induced apoptosis, inflammatory cytokine production, and erythema. In this study, we examined in vitro and in human samples the expressional pattern of the clock, clock-controlled genes, and the nucleotide excision repair (NER) gene *xpa* following UVB irradiation.

2.1 Results of in vitro expriments

In this study, we investigated whether exposure to low-dose UVB light could modulate the diurnal oscillations of the core circadian genes in human keratinocytes, and what are the effects of pretreatment with nicotinamide (NAM) on eventual alterations. NAM (vitamin B3) can enhance the DNA repair mechanism, reduce the levels of the fotoproducts in human keratinocytes. Hence regarding the circadian system, no data is available about its role on the diurnal pattern of core clock genes alongside with UV radiation.

The changes in gene expression were monitored for 48 hours. Two experimental setups were performed in which UVB treatment was applied to keratinocytes 12 h apart (baseline and

baseline+12h). In HaCaT cells irradiated12 hours later, as the core circadian gene expressions are in antiphase, different effects of UV radiation are expected, except for the *clock* gene, which has constitutive expression.

The pre-treatment with NAM did not induce significant changes in the expression of circadian genes and *xpa*. In both arrangements, the expression of circadian genes in HaCaT cells was influenced immediately by UVB treatment. At baseline irradiation, UVB rays downregulated the expression of *clock*, *per1-3* and *bmal1* genes, and increased the mRNA level of *rev-erbα*, *cry1-2* genes. A minimum of 24 hours was required for gene expressions to normalise and show similar to untreated control cells. After UVB exposure, a slight difference in mRNA levels was observed in HaCaT cells receiving nicotinamide pretreatment compared to cells irradiated UVB without NAM. In NAM pre-treated cells, significantly increased the *cry1* mRNA expression compared to the untreated control and the UVB-treated cells. For the *bmal1* and *per2* genes, nicotinamide may reduce the expression-enhancing effects of UVB radiation, as transcription is observed at untreated control levels for both genes.

Cells irradiated 12 hours later also showed a decrease in *clock*, *bmal1*, *per1-3* mRNA levels. As opposed to baseline irradiated HaCaT cells, *rev-erbα* expression reduced after irradiation, and for *cry1-2*, after a sudden upregulation, transcript levels declined 12-18 h post UVB exposure.

NER-related gene, xpa expression was higher in the late irradiated keratinocytes, furthermore, NAM significantly enhanced expression compared to cells exposed to UVB at baseline. We did not find robust diurnal changes in xpa expression in untreated HaCaT cells, but significant diurnal changes were observed in UVB-treated cells, which were similar to untreated controls during NAM pre-treatment.

Our results indicate, that exposure to UVB at different times causes a modest difference in expression, which may be explained by the diurnal oscillation. However, this low difference may also have an impact on the downstream processes influenced by the clock genes. Therefore, the role of locally altered expression of clock genes needs further investigation.

2.2. Results of human experiments

Healthy adult volunteers (n=6) with skin phototype II-III were eligible to participate. One square cm area of buttock skin was exposed to narrowband UVB for determination of the

minimal erythema dose (MED). After that, the volunteers received 4-fold MED. The UVB treatment was carried out at 7 am and 3 pm. The 5 mm punch biopsies were sampled 24 hours after the exposure. Four types of skin samples were collected at each time point: (i) untreated control skin, (ii) skin receiving a single MED, (iii) skin receiving a 4-fold MED, (iv) skin receiving a 4-fold MED after treatment with commercial sunscreen (SPF 50+) (8 samples/patient). We use RNA-seq array to quantify the transcriptomes in the skin biopsies. Bioinformatic analysis is ongoing and preliminary results can be reported. Analyzing all samples per time point, 18 mostly circadian genes were found to be significant in the morning samples. Regarding the effect of UVB, metabolism and keratinocyte differentiation were significant in skin receiving light treatment. Opposite regulation of the major skin structural genes, filaggrin vs involucrin and SPRR genes, is seen during treatment. These processes are seen in both 1MED and 4MED treatments, but the difference is more pronounced at higher doses compared to untreated skin. The expression pattern is similar in sunscreen pretreated skin compared to untreated skin. Time of day differences are being investigated.

3. Changes during the research project

Up to date we have not been able to perform the real-time in vivo imaging experiments described in the work plan due to the fact, that the equipment for real-time circadian studies was not available at our project partner, the Institute of Anatomy. From year 5 of the project, we will be able to carry out real-time microscopic analysis, but these processes will only be performed after the end of the project. Consequently, in vitro UVB experiments were performed as detailed above, and the focus was on the diurnal effects of UVB radiation in human studies. There were also external difficulties, which led us to request one-year extension from the NKFI Office. Namely, the coronavirus pandemic significantly affected the progress of the project in the second and third years of the project. The members of the project are health workers who were involved in patient care, diagnostics and vaccination during the pandemic. Furthermore, the laboratory was closed for almost a year. Accordingly, UV experiments were postponed for nearly two years. Regarding the structure of the participants, PhD students were not involved in the project.

From the grant we purchased laboratory equipments: refrigerated microcentrifuge, three notebooks, fluorometer for DNA/RNA/protein concentration determination, homogenizer,

laminar flow cabinet, digital UV radiometer. The last three items were not included in the budget, but their purchase was necessary to carry out the studies.

4. Importance and impact of the findings of the research project

The results associated with the funded project help us to better understand the diurnal changes in two inflammatory skin conditions. A large subset of genes is rhythmically expressed, so various physiological processes are under circadian control. Consequently, disruption of the daily expression of the genes can lead to pathological conditions. We showed that the circadian system is perturbed in psoriatic human skin (both lesional and nonlesional psoriatic skin). This disruption was significant in the non-lesional psoriatic skin, which indicates the systemic effect of the disease. Altered expression of circadian genes and proteins may serve as therapeutic targets in the future. Modulating the circadian clock by systemic or local therapies (e.g. targeting clock gene with anti-inflammatory role) may also be effective in regulating inflammatory conditions. In the other part of the study on the circadian system, the diurnal changes in the impacts of UV radiation were investigated. This would be of practical use in understanding and preventing cancer caused by UV radiation. The results so far have shown us that clock genes depict different expression patterns in response to UV exposure at different time points. These alterations may contribute to the processes that protect against UV damage. Research on the biological clock is increasing, emphasising its importance in the fine-tuning of various regulatory mechanisms.

Research on the circadian system has come to the forefront, but the precise mechanisms between the circadian system and various skin diseases are still the subject of ongoing studies. Our results will contribute to this knowledge.

We published the results from the psoriasis study in peer-reviewed journal with impact factor (Journal of Molecular Sciences, IF: 5.6). Our data from the UV study have been submitted to the Journal of Molecular Sciences. The evaluation of the results of the UV experiment containing human samples is in progress and the manuscript is under preparationto a peer-reviewed journal. Results were presented at national and international conferences as oral presentations and posters, and several abstracts have been published in a peer-reviewed journal (JID).

5. Selected publications, abstracts

Németh V, Horváth S, Kinyó Á, Gyulai R, Lengyel Z: Expression patterns of clock gene mRNAs and clock proteins in human psoriatic skin samples. International Journal of Molecular Sciences (2022) 23(1):121
doi: 10.3390/ijms23010121

Németh V, Kinyó Á, Horváth S, Gyulai R, Lengyel Z: Investigation of the circadian system in psoriasis. Journal of Investigative Dermatology (2021) 141(10) S169
doi: 10.1016/j.jid.2021.08.127

Németh V, Horváth S, Kinyó Á, Gyulai R, Lengyel Z: A cirkadián rendszer vizsgálata psoriasisban. Magyar Dermatológiai Társulat 93. Nagygyűlés, BVSZ 2020.96/6.275-298

Németh V, Gyulai R, Lengyel Z: Effects of UVB radiation on circadian clock genes along nicotinamide treatment in keratinocytes. Journal of Investigative Dermatology (2023) 143 (5) S199doi: 10.1016/j.jid.2023.03.1175

Németh V, Gyulai R, Lengyel Z: Effect of UVB radiation on circadian clock genes in keratinocyte. Magyar Dermatológiai Társulat 96. Nagygyűlés, 2023

Németh V, Gyulai R, Lengyel Z: Effect of UVB radiation on circadian clock genes along nicotinamide treatment in keratinocytes. Submitted to Journal of Molecular Sciences