CHARACTERIZATION OF MOLECULES SHOWING ALTERED EXPRESSION PROFILE IN PSORIASIS

Ph. D. Thesis

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LIST OF PUBLICATIONS

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I. Szegedi K*, **Göblös A***, Bacsa S, Antal M, Németh IB, Bata-Csörgő Zs, Kemény L, Dobozy A, Széll M. Expression and Functional Studies on the Noncoding RNA, PRINS. International Journal of Molecular Sciences, 14(1):205-25, 2012

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* These two authors contributed equally to this work

II. **Göblös A**, Danis J, Krisztina V, Bata-Csörgő Zs, Kemény L, Széll M. Keratinocytes express functional CARD18, a negative regulator of inflammasome activation, and its altered expression in psoriasis may contribute to disease pathogenesis. Molecular Immunology, 73:10-18, 2016

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Publications not related to the subject of the dissertation

I. Gellért L, Fuzik J, **Göblös A**, Sárközi K, Marosi M, Kis Z, Farkas T, Szatmári I, Fülöp F, Vécsei L, Toldi J. Neuroprotection with a new kynurenic acid analog in the four-vessel occlusion model of ischemia. European Journal of Pharmacology 667(1-3): 182-7, 2011

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II. Szabó EZ, Manczinger M, **Göblös A**, Kemény L, Lakatos L. Switching on RNA silencing suppressor activity by restoring Argonaute binding to a viral protein. Journal of Virology 86(15):8324-7, 2012

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INTRODUCTION

1. Psoriasis

Psoriasis is a common, lifelong skin disease, affecting approximately 2% of the population and predominantly presented in the Caucasian population. The prevalence of psoriasis is approximately the same in males and females. Psoriasis has been defined as a chronic inflammatory disease with important immune-mediated features that involves a multifactorial cellular and molecular network. Psoriasis is generally characterized by exacerbation and remission and usually persists for life style. Psoriasis compromises innate and adaptive immune system components and has a well-defined network of chemokines and messengers. The cause of psoriasis is complex, with evidence that the interplay of the multigenic susceptibility and environmental as well as life-style factors lead to the development of the symptoms.

The disease is usually manifests as raised, well-demarcated, erythematosus oval plaques with adherent silvery scales. Psoriasis is associated with a high degree of morbidity; moreover, patients with psoriasis have reduced levels of employment and income as well as a decreased quality of life. In addition to skin plaques, psoriasis can present with various co-morbidities including arthritis, atherosclerosis, inflammatory bowel disease, obesity, type 2 diabetes mellitus and depression. The current existing therapies only relieve symptoms but cannot cure the disease.

The altered skin tissue homeostasis of the normal looking skin of psoriatic patients has key importance in the initiation of the disease. The normal looking skin contains abundant stores of T lymphocytes as well as resident population of DCs, suggesting that skin might be a potential site for the direct triggering of recall immune responses. The keratinocytes of the non-involved psoriatic epidermis are inherently oversensitive to proliferative signals, and this elevated level of sensitivity plays a crucial role in the development of psoriatic lesions. The aim of our workgroup is to identify and characterize abnormal molecular patterns in non-lesional psoriatic keratinocytes contributing to the initiation of the disease phenotype and factors that make these keratinocytes prone to respond with hyperproliferation to cytokines produced by skin infiltrating lymphocytes. For that we have previously performed a differential display- and cDNA microarray experiment. These two studies identified among others a novel

non-coding RNA gene, PRINS (psoriasis susceptibility-related RNA gene induced by stress) and CARD18 (caspase recruitment domain family member 18, Iceberg) molecule.

2. PRINS, a newly identified long non-coding RNA

PRINS (Psoriasis susceptibility-related RNA gene INduced by Stress), identified by our research group, is a 3681 nucleotide-long ncRNA molecule, transcribed by RNA polymerase II. BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the PRINS gene is specific for anthropoid primates; its rodent orthologue could not be identified. Our studies demonstrated a higher PRINS expression in the non-involved psoriatic epidermis than in either the psoriatic involved or healthy epidermis. These results suggest that the overexpression of PRINS in the uninvolved psoriatic epidermis may play a role in psoriasis susceptibility rather than in the precipitation of psoriatic lesions, and are in agreement with the previous observations that the keratinocytes of the uninvolved epidermis differ from healthy keratinocytes in their responses to external stimuli. Real time reverse transcription-PCR analysis showed that stress signals such as ultraviolet-B irradiation, viral infection (herpes simplex virus), and translational inhibition increased the RNA expression level of PRINS. Gene-specific silencing of PRINS by RNA interference revealed that down-regulation of PRINS impairs cell viability after serum starvation but not under normal serum conditions, suggesting that PRINS functions as a noncoding regulatory RNA, playing a protective role in cells exposed to stress. It is described that long mRNA-like ncRNAs form complexes with proteins, and act as regulators of various cellular functions. Therefore we have performed an *in vitro* binding assay which revealed that PRINS interacts physically with the molecular chaperone protein nucleophosmin (NPM, B23) in HaCaT and normal human epidermal keratinocyte (NHEK) lysates and describe the first upregulation of this protein in psoriasis.

NPM, a multifunctional nucleolar phosphoprotein, has a potential role as a positive regulator in cell proliferation, moreover it is implicated in mRNA processing and also in the acute response of mammalian cells to environmental stress, when it stimulates DNA repair and reduces apoptosis. A number of studies have shown that UV irradiation results in a rapid nucleoplasmic translocation of the otherwise predominantly nucleolar protein NPM in fibroblasts, and cancer cells. It has been demonstrated that multiple functions of NPM conferred by the shuttling of protein between the nucleus and cytoplasm.

3. Inflammasome mediated processes in psoriasis

The importance of innate immune dysregulation in psoriasis has long been recognized. Inflammasomes and inflammatory caspases, such caspase-1, are also described as potential inducers and regulators of skin inflammation in contact hypersensitivity and in psoriasis. Inflammasomes and inflammatory caspases are the part of the innate immune system. Inflammasomes are cytoplasmic multiprotein complexes containing pattern recognition receptors that detect invading pathogens or self-derived danger signals and initiate the innate immune response. Inflammasomes control the activation of the proteolytic enzyme caspase-1; caspase-1 in turn regulates maturation of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. IL-1β and IL-18, have been shown to play an important role in the pathogenesis of psoriasis, i.e., initiating and mediating the infiltration of immune cells and stimulating keratinocytes to proliferate. Elevated IL-1β and IL-18 expression has been observed in inflamed skin; moreover, the increased IL-1β is produced mostly by keratinocytes.

Cytosolic DNA is a potent inducer of the innate immune response and has been proposed to be involved in the pathogenesis of psoriasis. The DNA-sensor-containing absence in melanoma 2 (AIM2) inflammasome is triggered by both self-derived and pathogen-released (*Francisella tularensis*, *L. monocytogenes*, vaccinia virus) cytosolic double-stranded DNA. Recently, abundant cytosolic DNA and increased AIM2 expression were found to be present in keratinocytes in psoriatic lesions but not in healthy skin, and suggested to contribute to the abnormal IL-1β secretion in psoriasis.

Our recent large-scale gene expression study has revealed that CARD18 transcript is differentially expressed in psoriatic non-involved epidermis compared to healthy epidermis. CARD18, a member of CARD-only protein (COP) family, consists of only one CARD domain which exhibits sequence similarity to the CARD prodomain of caspase-1. CARD18 serves as a decoy protein which can modify the activity of inflammasome by inhibiting the generation of active IL-1β and IL-18 through direct interaction with pro-caspase-1. CARD18 is detected mainly in placenta and in many human cell lines and its expression is upregulated by proinflammatory stimuli (LPS and TNF-α) in THP-1 monocytes. Moreover, CARD18 inhibited the LPS or INF-ɣ induced secretion of IL-1β in monocytes. These data suggest that CARD18 may be a part of a negative feedback loop.

AIMS

1. To investigate the physical and functional characteristics of PRINS non-coding RNA and its interacting partner, nucleophosmin (NPM)

   • To examine the histological and cellular distribution of PRINS non-coding RNA in different tissue types

   • To study the intracellular localization of NPM under normal conditions and after UV-B irradiation in human keratinocytes

   • To analyze the effect of PRINS gene silencing by RNA interference on NPM UV-B induced shuttling in human keratinocytes

2. To investigate the characteristics and functions of the CARD18 molecule in normal human keratinocytes and in the skin

   • To study the basal expression of CARD18 and its expression changes upon T-cell lymphokine treatment in healthy and psoriatic non-involved epidermis

   • To analyze the distribution of CARD18 expression under normal conditions and after mild injury (tape stripping) in healthy and psoriatic non-involved as well as involved epidermis

   • To study the cellular expression of CARD18 in spontaneously differentiating normal human epidermal keratinocytes

   • To analyze the gene expression of molecules involved in inflammasome signaling in keratinocytes exposed to cytosolic DNA treatment

   • To explore whether the silencing of CARD18 has an effect on keratinocyte innate immune functions

MATERIALS AND METHODS

- Samples for organotypic skin cultures shave biopsy were taken from the non-involved buttock area of five young male psoriasis patients and five age- and gender-matched healthy controls.

- For immunohistochemical staining, patients with moderate-to-severe psoriasis vulgaris (n=2) and healthy (n=2) volunteers were enrolled in the study. In another set of experiments, the skin of psoriatic patients (n=2) and healthy volunteers (n=2) were subjected tape stripping (TS). Punch biopsies were taken from the area once before and twice after (24 and 48 hours) the treatment.
  During the staining, anti-CARD18 goat polyclonal IgG antibody (1:250), biotinylated anti-goat IgG, Extravidin Peroxidase and AEC reagent were applied.

- Normal human epidermal keratinocyte cells (NHEK) and Human immortalized keratinocyte cell line (HPV-KER) were used to analyze the cellular properties of human keratinocytes *in vitro*.

- Upon T-cell lymphokine treatment cells were stimulated with the addition of 0.3 ng/ml IL-3, 1 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF), and 1 ng/ml IFN-ɣ to the medium.

- Upon inflammasome activation induction cells were subjected to 5 ng/ml tumor necrosis factor (TNF) α and 5 ng/ml interferon (IFN) ɣ pretreatment and 1 µg/ml polydeoxyadenylic acid–polydeoxythymidylic acid double-stranded homopolymer (poly(dA:dT)) transfection.

- Cells were irradiated with a 40 mJ/cm2 dose of 312 nm UVB in PBS

- For In Situ Hybridization, Custom Digoxigenin-labeled LNA mRNA detection probes for PRINS and scrambled control sequence were used. Six patients with moderate-to-severe psoriasis vulgaris and ten healthy volunteers were enrolled. Tissue chips (n=2) contained thirteen different human tissues that were placed into a tissue microarray (TMA) block.

- For real-time RT-PCR total RNA was isolated from cells. cDNA was synthesized from 1 µg total RNA. Real-time RT-PCR experiments were carried out with the Universal Probe Library system or with a Cy5-labeled probe. PCR assays were performed with the C1000 Touch Thermal Cycler. The expression of each gene was normalized to the 18S ribosomal RNA gene. Relative mRNA levels were

calculated by the ΔΔCt method. Data from different treatments were compared using one-tailed t test. Differences were considered significant when P ≤ 0.05.

· Upon immunfluorescent staining, for NPM detection anti-NPM monoclonal antibody (1:500) was applied. Next we used anti-mouse Alexa Fluor 546 goat anti-mouse IgG (1:500) as secondary antibody. For CARD18 detection anti-CARD18 goat polyclonal IgG antibody (1:250) was applied. As secondary antibody we used anti-goat Alexa Fluor 546 donkey antibody (1:500). In both cases cell nuclei were counterstained with DAPI (1:100).

For the semiquantitative analysis of the intracellular localization of NPM (nucleolar or nuclear), at least 25 fields of view were counted by observing the localization of NPM immunostaining in the nucleolus or in the nucleoplasm.

· Gene-specific silencing of PRINS was performed with a vector-based method. The transient transfection of HPV-Ker cells with the in vivo pSilencer™ 2.1-U6 hygro vector was carried out. Gene-specific silencing of CARD18 was performed with an siRNA duplex transfection method on NHEK cells. The effectiveness of the silencing was measured by real-time RT-PCR.

· To determine the IL-1β secretion of NHEK cells, the cell supernatants were centrifuged to pellet cell debris and the amount of IL-1 was determined by ELISA.

RESULTS

1. PRINS ncRNA shows variable histological and cellular expression level

For a detailed analysis of cellular and histological distribution of PRINS ncRNA we have applied ISH on paraffin-embedded tissue chips containing thirteen sections from different healthy human organs on one slide. The intracellular distribution of PRINS was also examined in cultured NHEKs. The ISH experiments demonstrated variable levels of expression in different human tissue samples: high levels of PRINS expression were detected in the gut, lungs, lymph nodes, uterus, testicles and skin, whereas no staining was observed in the cerebrum and cerebellum. The breast, kidney, stomach, and gallbladder tissue specimens displayed only moderate PRINS expressions.

ISH demonstrated a relatively high PRINS positivity in the healthy epidermis. The dermal and epidermal expression of PRINS significantly differed while a strong staining intensity in the epidermis was similar in the various layers. In *in vitro* cultured keratinocytes exhibited strong nucleolar and perinuclear PRINS positivity and moderate homogeneous cytoplasmic staining.

Further ISH experiments were performed to compare the expressions of PRINS in normal healthy (n = 10), psoriatic uninvolved (n = 6) and psoriatic involved (n = 6) skin samples. Our results partially confirmed the previous real time RT-PCR findings indicating a moderately elevated level of PRINS expression in the uninvolved and involved epidermis relative to the healthy epidermis.

2. Modifying effect of PRINS ncRNA on the intracellular localization of NPM protein in keratinocytes upon UV exposure

Nucleophosmin was identified as a putative PRINS-binding protein in three independent binding experiments. NPM is predominantly localized in the nucleoli and known to redistributed from the nucleolus in response to cytotoxic drugs and genotoxic stress, for example in fibroblasts and cancer cells, UV irradiation results in a rapid nucleoplasmic translocation of the nucleolar NPM

To investigate whether we could observe a similar intracellular localization of NPM in keratinocytes (NHEKs and HPV-Ker cells) after UV-irradiation, we applied an immunocytochemistry method. UV-B exposure caused a relocalization of NPM from the nucleolus to the nucleoplasm in both cells: 3 h after treatment, slight translocational

changes were observed; the most increased immunofluorescence in the nucleoplasm was detected at 12 and 24 h. Forty-eight hours following UV-B treatment, the protein had mostly returned to the nucleolus. The time course studies showed that the stimulation of NPM shuttling by UV-B irradiation was a rapid and transient process.

To determine whether PRINS had any effect on NPM intracellular trafficking, we silenced the expression of PRINS in HPV-Ker cells with a vector-based method and studied the NPM shuttling under normal conditions and after UV-B exposure. NPM shuttling was compared in HPV-Ker cells transfected with a specific PRINS-silencing construct (AK696) and in HPV-Ker cells transfected with a control construct (SC1313). In untreated samples, the NPM was localized mostly within the nucleolus in both the SC1313 and the AK696-transfected cells. The relocalization of NPM from the nucleolus to the nucleoplasm in the control SC1313-transfected cells at the indicated times after UV-B exposure was similar to our preliminary observations in HPV-Ker cells. As compared with the control cells, the AK696-transfected cells showed a moderate retention of NPM in the nucleolus following UV-B treatment. To validate the immunocytochemical observations, a semiquantitative analysis were performed. The nuclear transition of NPM was inhibited in AK696-transfected cells after UV-B irradiation. Interestingly, we observed that the transfection itself slightly modified the shuttling of NPM even in the UV-B negative group.

3. CARD18 gene expression in keratinocytes and in the skin

We have recently reported the results of cDNA microarray experiments in which 61 annotated genes were differentially expressed in psoriatic non-involved and healthy epidermis upon T-cell lymphokine treatment. Among others, CARD18 has been identified as differentially expressed transcript. CARD18 is a negative regulator of IL-1β maturation, inhibiting inflammasome activation directly by interacting with pro-caspase-1. To validate the cDNA microarray results, real-time RT-PCR analysis was carried out to measure the abundance of CARD18 mRNA. Our results confirmed the cDNA microarray results: the basal expression levels were relatively high in the psoriatic non-involved epidermis (2 fold compared to healthy samples), it was not further induced in response to T-cell lymphokines. This was in contrast to the findings for healthy skin, where lower basal expression levels were induced with a 1.65-fold elevation in response to the same treatment.

To examine the characteristics of CARD18 in NHEKs we examined mRNA and protein changes in spontaneously differentiating third-passage NHEKs. The 0-day samples were taken from subconfluent cultures. The *in vitro* gene-expression study revealed low-level CARD18 mRNA expression in the proliferative state of cells, and this expression continuously increased during differentiation: up to 43.2-fold elevation in the 10-day samples. To examine the expression of CARD18 protein, immunofluorescence staining was applied to keratinocytes, revealing that moderate positive CARD18 protein expression in samples taken on days 0–4 gradually increased in the 6–10-day samples. The immunocytochemistry staining revealed that CARD18 was located in the cytoplasm of NHEKs. Thus, CARD18 protein expression followed a similar pattern observed for the mRNA expression during the 10-day course.

To compare CARD18 protein levels in healthy and diseased skin, immunohistochemical staining was performed on paraffin-embedded samples from healthy, psoriatic non-involved and psoriatic involved skin samples in an independent experiment. In all examined sections, CARD18 appeared exclusively in the epidermis, specifically in the cytoplasm of the epidermal keratinocytes. IHC revealed differential CARD18 expression in different skin samples: elevated levels were observed in the psoriatic non-involved and psoriatic-involved epidermis compared to healthy epidermis. These results confirmed our real-time RT-PCR findings. No differences were detected in the different keratinocyte layers of the epidermis.

It has long been known that baseline proliferative activity of keratinocytes of non-involved skin of psoriatic patients does not differ from keratinocytes in skin of healthy individuals; however, the proliferative response to mechanical stress (tape stripping, TS) is significantly higher in psoriatic non-involved skin compared to healthy skin. CARD18 protein expression was observed in mechanically stimulated skin from healthy and non-involves skin of psoriatic donors. Punch biopsies were taken before the procedure and two times after TS (24 and 48 hours). Compared to untreated sections, CARD18 protein expression level was induced 24 hours after TS in all examined samples, however, the elevation of CARD18 expression in treated and untreated psoriatic non-involved samples was not as pronounced as in healthy or psoriatic involved skin.

4. The modifying role of CARD18 in cellular inflammatory processes

Keratinocytes, important immune-competent cells, are able to sense danger signals and mediate immune response through the activation of pro-inflammatory signaling pathways, resulting in the expression of inflammasome components and secretion of pro-inflammatory cytokines.

To elucidate the cellular processes in which CARD18 participates and to gain further insight into its role in the pathomechanism of psoriasis, we investigated CARD18 expression in response to psoriasis-associated stress signals. Cytosolic DNA has been identified recently as a trigger for AIM2 inflammasome activation and is abundant in psoriatic-involved epidermis. To mimic the effect of cytosolic DNA, a synthetic dsDNA analogue poly(dA:dT) was transfected into cultured keratinocytes.

A significant increase in IL-1β release - up to 50 pg/ml - as well as 3–5 fold increase in caspase-1 expression and a 200-fold increase in AIM2 expression were observed, although changes in CARD18 mRNA were not detected.

To provoke enhanced inflammation, IFN-γ and TNF-α pre-treatment was performed before poly(dA:dT) transfection. IFN-γ is known to induce AIM2 gene expression, whereas TNF-α mediates pro-IL-1β transcription, and both cytokines are consistently elevated in psoriatic skin. The co-treatment resulted in increased IL-1β secretion - up to 100 pg/ml - and increased the expression of caspase-1 and AIM2 mRNAs by 13-fold and 250-fold, respectively. CARD18 mRNA level was also increased by six fold.

These results encouraged us to further characterize the function of CARD18 in keratinocytes in which inflammatory processes had been induced. CARD18 expression was silenced by siRNA 24 hours before IFN-γ and TNF-α treatment and subsequent poly(dA:dT) transfection. The applied siRNA effectively down-regulated CARD18 expression to 50% of the levels observed for the scrambled control cells. The CARD18 silencing resulted in a significant decrease in AIM2 gene expression 12 and 24 hours after treatment and significantly reduced caspase-1 mRNA expression 24 hours after the treatment. For further confirmation, we determined whether the silencing of CARD18 had any effect on IL-1β secretion: our results indicated that downregulation of CARD18 expression affected an elevation of IL-1β production in keratinocytes. These results indicate that CARD18 might indeed contribute to the fine-tuning of inflammatory

processes in keratinocytes and suggest that — similar to the role in professional immune cells — functions as a negative regulator of inflammasome activation in keratinocytes.

SUMMARY

In our work, we have investigated the characteristics and function of two molecules, PRINS and CARD18, which may contribute to the stress responses of epithelial cell and the initiation of psoriasis. Both molecules have been identified previously by our research group as differentially expressed transcripts in healthy and psoriatic epidermis.

We have earlier described a novel non-coding RNA, PRINS that was overexpressed in non-lesional psoriatic epidermis and its expression was induced by various stress factors such as serum starvation, contact inhibition, UV-B irradiation, and microbial agents in HaCaT cells. In this work we analyzed PRINS expression in thirteen different tissue types. *In situ* hybridization experiments demonstrated variable staining patterns in human tissue samples, moreover, we observed tissue specific expression of PRINS in human skin: in the epidermis strong PRINS staining was seen in contrast to the dermis, where a very slight PRINS expression could be detected. Next, we compared the healthy and psoriatic skin: a low PRINS expression elevation was observed in psoriatic involved and non-involved epidermis compared to the healthy epidermis. The previous examination of our workgroup identified nucleophosmin (NPM) as a PRINS-interacting partner. We investigated the subcellular localization changes of NPM protein upon UV-irradiation. We demonstrated an induction of NPM shuttling from the nucleolus to the nucleoplasm in keratinocytes. To explore whether PRINS ncRNA has any functional role in UV-mediated cellular stress responses, we silenced the PRINS gene expression in HPV-Ker cells and monitored NPM intracellular localization. Silencing of the PRINS non-coding RNA expression in the UV-B irradiated keratinocytes resulted in the retention of NPM in the nucleolus, suggesting that PRINS is physically and functionally linked to NPM, thus plays a role in the NPM-mediated cellular stress response.

CARD18 is known as a negative regulatory molecule that inhibits inflammatory events by terminating inflammasome activation due to a direct interaction with pro-caspase-1. During the investigation of molecular mechanisms in keratinocytes that contribute to the pathogenesis of psoriasis, we found that CARD18 expression differs in healthy and psoriatic skin; moreover, CARD18 demonstrated altered response under inflammatory conditions in healthy and psoriatic skin. In healthy skin, low basal CARD18 expression was detected, which showed significant elevation in response to inflammatory stimuli

(lymphokine treatment or mechanical injury). In contrast with this, higher basal expression was observed in psoriatic non-involved skin, but no further CARD18 induction could be detected. The investigation of cellular inflammatory processes revealed that psoriasis-associated danger signals triggered the expression of inflammasome components (AIM2, caspase-1) and CARD18 as well as IL-1β production of keratinocytes. Furthermore, gene-specific silencing of CARD18 in cells treated with cytosolic DNA (poly(dA:dT)) resulted in increased IL-1β secretion, suggesting a negative regulatory role for CARD18 in keratinocyte inflammatory signaling. The differential regulation of CARD18 in healthy and psoriatic uninvolved epidermis may contribute to psoriasis susceptibility. Furthermore, our *in vitro* results indicate that CARD18 may contribute to the fine tuning of keratinocyte innate immune processes.

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