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STUDIES ON THE ROLE OF THE SKIN MICROBIOME IN HEALTHY SKIN AND UNDER PATHOGENIC CONDITIONS

Ph.D. thesis

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Publications related to the subject of the thesis

I. Tax G, Urbán E, Palotás Z, Puskás R, Kónya Z, Bíró T, Kemény L, Szabó K. **Propionic Acid Produced by *Propionibacterium acnes* Strains Contributes to Their Pathogenicity.** Acta Derm Venereol. 2015 Jun 3. doi: 10.2340/00015555-2154. [Epub ahead of print] IF: 3,025 (2014)

II. Szabó K, Tax G, Teodorescu-Brinzeu D, Koreck A, Kemény L. **TNFα gene polymorphisms in the pathogenesis of acne vulgaris.** Arch Dermatol Res. 2011 Jan;303(1):19-27. doi: 10.1007/s00403-010-1050-7. Epub 2010 Apr 13. IF: 2.279, Citation: 27 (24/3)

III. Szabó K, Tax G, Kis K, Szegedi K, Teodorescu-Brinzeu DG, Diószegi C, Koreck A, Széll M, Kemény L. **Interleukin-1A +4845(G> T) polymorphism is a factor predisposing to acne vulgaris.** Tissue Antigens. 2010 Nov;76(5):411-5. doi: 10.1111/j.1399-0039.2010.01530.x. Epub 2010 Aug 19. IF: 3.024, Citation: 15 (14/1)

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VI. Agodi A, Barchitta M, Valenti G, Quattrocchi A, Pettinato M, Tax G, Szabò K, Szell M. **Role of the TNFA -308G > A polymorphism in the genetic susceptibility to acne vulgaris in a Sicilian population.** Ann Ig. 2012 Sep-Oct;24(5):351-7. IF: -

Introduction

The skin is a complex tissue composed of many different cell types, but not all of them of human origin. From our birth we are exposed to a wide range of microorganisms, including bacteria, fungi and viruses. Some of them are capable of inhabiting our skin and together with the various human cells forming a complex ecosystem. According our current view the specialized microbial flora that is located mostly on the surface of the skin and within the pilosebaceous unit (PSU) is an important and integral component, and together they form a complex ecosystem. The exact function of this microbial community is not yet fully understood, but it has been suggested that the balanced interaction of the microbial and the human cells is important for the promotion and maintenance of healthy skin functions.

The composition of skin microflora differs on various body parts, and although *Propionibacterium acnes* (*P. acnes*) is a major constituent, it is especially abundant in the sebum-rich skin regions from the early teenage years. On the other hand, the role of this commensal bacterium has also been suggested in acne pathogenesis for a long time, but it is still not clear how and why an otherwise harmless commensal would turn to pathogenic.

Results from our and other laboratories suggest that the keratinocytes recognize the presence of the *P. acnes* bacterium, which in turn can activate pathogen recognition receptors, such as Toll-like receptor 2 and 4 (TLR 2, TLR4). This activation initiates characteristic signaling events in the cytoplasm of the affected cells and subsequently causes increased expression of different cytokines, chemokines and antimicrobial peptide-coding genes.

According to recent data obtained based on sequence analysis of bacterial *recA* and *tly* genes and the results of multilocus sequence typing (MLST) experiments at least six distinct phylogenetic groups within the *P. acnes* species have been described (IA1, IA2, IB, IC, II, III). It has also been suggested that various *P. acnes* strains may differentially affect the cellular and molecular biological properties of keratinocytes, and such differences may contribute to the determination of the severity of individual acne lesion around puberty.

Apart from the above described bacterial properties, the response of different individuals' to the presence of the skin microbiome may also differ, and inherited factors can modify the outcome and extent of these reactions. Inherited susceptibility to complex diseases is frequently a result of a combination of commonly occurring single nucleotide polymorphisms (SNPs) that may affect the expression, structure and/or function of various genes playing a key role in the regulation of *P. acnes* induced signaling events. Based on all these it is possible that such genetic factors can modify the reactivity of carrier individuals' to the presence of various members of our commensal flora, and subsequently leads to chronic inflammatory events, such as acne vulgaris.

Aims

In the course of our studies we were interested in studying the exact role and properties of the skin microbiome in the healthy skin and during pathogenic conditions, with a special focus on the most common inflammatory skin disease, acne vulgaris.

In the first part of our studies we analyzed the interaction of the skin colonizing *P. acnes* bacterium and the epidermal keratinocytes, to find out if and how this commensal bacterium affects the cellular properties of human cells.

For that, we aimed to investigate:

- whether there is a difference in the effect of selected *P. acnes* strains belonging to different phylogenetic groups within the species (*889*: 1A, *6609*: 1B, *ATCC 11828*: II) on the cellular responses of an *in vitro* cultured immortalized human keratinocyte cell line, HPV-KER,
- whether the keratinocyte responses are depending on the dose of the bacterial treatment,
- the nature of the bacterially-derived factors that are responsible for the induction of cellular responses in keratinocytes.

In the second part we aimed to identify and analyze genetic susceptibility or protective factors that may modify the carrier individuals' response to the presence of the skin microbiome, and thus affect the severity of inflammatory acne symptoms. Thus, in retrospective case-control studies we analyzed the role of different polymorphisms of genes playing an important role in the initiation and/or maintenance of *P. acnes*-induced innate immune and inflammatory events. For that we analyzed the effect of:

- five different promoter polymorphisms of the tumor necrosis factor alpha pro-inflammatory cytokine (TNFα) coding TNFA gene (-238G>A, rs361525; -308G>A, rs1800629; -857C>T, rs1799724; -863C>A, rs1800630; -1031T>C, rs1799964),

- the +4845G>T (rs17561) SNP located at the coding region of the interleukin-1 alpha (IL-1A) gene,
- and the variable number of tandem repeats (VNTR) polymorphism of the anti-inflammatory interleukin-1 receptor antagonist (IL-1RA) coding IL-1RN gene in acne pathogenesis.

Materials and methods

- Human immortalized keratinocyte cell line (HPV-KER) was used to analyze the cellular properties of human keratinocytes *in vitro*.
- For *P. acnes* treatments strains belonging to various phylogenetic groups within the species (*889, 6609, ATCC 11828*) were applied at different doses (multiplicity of infection, MOI=25-300).
- Real-time, label-free analysis of the interaction between HPV-KER or NHEK cells and *P. acnes* was performed using an impedance measurement-based analysis method (xCELLigence system; RTCA SP).
- Cell number changes were followed in the keratinocyte cultures by a Trypan Blue exclusion assay.
- Morphological changes of the *P. acnes* or propionic acid (PA) treated HPV-KER cultures were analyzed by fluorescence microscopy.
- The cytolytic effect of different *P. acnes* strains and PA was investigated with lactate dehydrogenase (LDH) and spectrophotometric hemoglobin assay using HPV-KER cells and washed human erythrocytes, respectively.
- pH sensitive phenol-red dye was used for the visualization of pH changes of the bacterial-treated HPV-KER culture supernatants. Color changes of the culturing media were followed by visual inspection and photo documentation, as well as using a litmus paper.
- Mass spectrometry was used to measure the amount ofshort-chain fatty acids (SCFA) secreted by the different *P. acnes* strains.
- Comparison of the growth properties of different *P. acnes* strains was performed by a spectrophotometric growth curve analysis (OD600).
- Restriction fragment length polymorphism (RFLP) analysis was done to determine the genotype and allele frequencies of the TNFA and IL-1A polymorphisms.
- Luciferase reporter assay was used to measure the effect of different TNFA -857C>T alleles on the TNFA promoter activity.

- VNTR polymorphism of the IL1RN gene was analyzed by PCR and subsequent agarose gel electrophoresis.

Results

1. *In vitro* monitoring of the interaction of the *P. acnes* bacterium and the epidermal keratinocytes

In order to gain a better understanding on the properties of the interaction that exists between the skin colonizing *P. acnes* bacterium and the epidermal keratinocytes, we set up an *in vitro* model system. We investigated how the bacterium affects the different cellular properties (e.g. growth and viability) of HPV-KER cells when they are co-cultured.

To determine if there were any strain specific differences we used three bacterial strains belonging to different phylogenetic groups within the species (*P. acnes 889*: IA, *6609*: IB, *ATCC11828*: II). We applied the bacteria in different doses (MOI=25-300) to find out if the effects depended on the *P. acnes* dose. Cellular changes were monitored in real time using impedance (Z) measurement-based technologies, the xCELLIgence system. We found that, when the *P. acnes 889* strain was applied in a high dose (MOI=300), a rapid elevation of the Z, as well as the derived dimension free normalized cell index (nCi) values were detected. At later time-points, when higher bacterial loads (MOI= 200, 300) of the *889* and *ATCC 11828* strains were applied, a small increase was followed by a sharp decrease of the nCi values. Similar changes were not observed in case of the *P. acnes 6609* strain.

To investigate the cell biological properties leading to the observed nCi changes, we monitored the number of the cells that is present in the control and *P. acnes*-treated cultures using a Trypan Blue dye exclusion assay. High dose (MOI=300) of the *P. acnes 889* strain resulted a transient increase, followed by a decrease of cell numbers compared to untreated and low-dose treated samples. We also noted the presence of abnormally shaped rounded cells exhibiting irregular membrane morphology in case of the *P. acnes 889* and *ATCC 11828* treatments using fluorescent microscopic analysis.

To determine whether *P. acnes*-induced morphological changes and cytotoxicity were due to the damage of the keratinocyte membrane caused by the bacterium or by bacterially derived toxins, we treated HPV-KER cells with different doses of the

P. acnes strains and measured the amount of free LDH enzyme from the supernatant samples. We noted a dose-dependent increased of the free LDH levels when keratinocytes were treated with high dose (MOI= 300) of the *889* and *ATCC 11828* strains compared to the untreated control and *P. acnes 6609*-treated samples.

We repeated the experiment using freshly isolated washed human erythrocytes and observed the presence of increased amount of free hemoglobin (HgB) in the supernatants in response to the same strains; the *P. acnes 889* and *ATCC 11828*. These results suggested that the observed cytotoxic effect may not be a result of cell type-specific interactions.

Apart from these observations we also noted enhanced acidification of the cultures treated with *P. acnes 889* and *ATCC 11828*, which was much less apparent in case of the *P. acnes 6609* strain.

2. Investigation of bacterially-derived factors that may contribute to the induction of cellular responses in keratinocytes

Under anaerobic conditions *Propionibacterium* species can metabolize carbon sources such as glycerol and glucose by anaerobic fermentation and generate short chain fatty acids (SCFA), including acetic and propionic acid (AA and PA, respectively). Based on our previous experiments we hypothesized that strain- and dose-dependent differences in the production of such metabolites may be present in case of the different *P. acnes* strains. To prove this hypothesis, the amount of secreted AA and PA was measured in the supernatant samples of control and bacterial-treated HPV-KER cells by mass spectrometric analysis. We noted that while the amount of AA was relatively comparable, the PA concentrations varied in a strain-, and dose-dependent manner; higher levels were detected when the *889* and *ATCC 11828* strains were applied in high doses (MOI=300).

One of the studied strains, *P. acnes 6609* appeared to be the least effective in our *in vitro* assays, and MS analysis revealed that the PA levels were the lowest in these samples. This appears to be the consequence of the impaired growth properties of this strain, which was apparent under anaerobic as well as aerobic conditions in our microbial growth assays.

To determine the exact role of PA in the *P. acnes* induced cellular changes, we treated HPV-KER cells in different concentrations (1-5 mM). Microscopic analysis of rhodamine-labeled phalloidin-stained cells revealed the appearance of cells exhibiting similar irregular membrane morphologies as we observed earlier in case of high dose *P. acnes 889* and *ATCC 11828* treatments. These results suggest that high concentrations of PA may contribute to the formation of abnormal keratinocyte functions.

In our experiments *P. acnes 6609* showed impaired growth properties and fermented less PA than the other two strains. Thus, we repeated our earlier experiments and treated washed human erythrocytes with the *P. acnes 6609* strain (MOI=300), but now in the presence and absence of PA (1-5 mM), and analyzed the level of free hemoglobin (HgB) that is present in the supernatant sample 72 hours after treatment. We found that PA dose-dependently increased the level of free HgB, and this affect was amplified in the presence of the *P. acnes 6609* strain, which alone did not induce the appearance of elevated free HgB levels in our earlier experiments.

Our results suggest that differences in the growth properties of various *P. acnes* strains, and as a result in the amount of secreted metabolic end-products (including PA) may contribute to the biologic effect of the bacterium. Together with other bacterially-derived molecules PA may be an active contributor of the *P. acnes*-induced cellular changes in human skin cells.

3. Studying the role of different TNFA promoter SNPs in the genetic predisposition to acne

During the course of our studies we aimed to characterize genetic susceptibility or protective factors that may modify the *P. acnes*-induced innate immune and inflammatory events and the severity of acne vulgaris. Through the activation of Toll-like receptors the bacterium is known to induce a transcriptional program and increase the expression of various cytokines, chemokines and antimicrobial peptides. The tumor necrosis factor alpha (TNFα) and interleukin-1 alpha (IL-1α) are important pro-inflammatory cytokines, whose expressions are rapidly increase in response to *P. acnes* treatment and have been shown to play a role in acne pathogenesis.

Single nucleotide polymorphisms (SNPs) located in the promoter region of the TNFA gene encoding the TNFα cytokine have already been associated with an increased risk to develop different chronic inflammatory diseases. In order to investigate the role of these in the pathogenesis of acne, we selected five different SNPs (-238G>A, -308G>A, -857C>T, -863C>A, -1031T>C) and analyzed them in retrospective case-control studies using the PCR-RFLP method. We found no correlation in case of the -238G>A, −863C>A and the −1031T>C SNPs and acne.

In case of the -308G>A polymorphism no association was detected in our overall dataset, but a significant association was discovered between the minor A allele and acne in female patients. We also found an association between the severity of acne and the increasing frequency of the rare allele-containing GA+AA genotypes in the female patient group.

Next, we genotyped our study population for the -857C>T TNFA polymorphism, and found a positive association between the major C allele of this SNP and acne. We also noted that the frequency of the minor T allele-containing genotypes decreased parallel to the severity of inflammatory acne symptoms, suggesting that this allele may have a protective effect.

In order to investigate the effect of the SNP on the regulation of the promoter activity in keratinocytes, we generated luciferase reporter constructs carrying a piece of the proximal TNFA promoter. One construct contained the minor T, whereas another the major C allele at the -857th nucleotide position in the TNFA promoter fragment (pGL4.20-TNFA-857C-3' and pGL4.20-TNFA-857T-3', respectively). The two constructs were transiently transfected to HPV-KER cells and then treated with the *P. acnes 889* strain. In case of the construct containing the rare T allele (pGL4.20-TNFA-857T-3') we measured significantly lower basal luciferase activities. *P. acnes* treatments lead to increased promoter activities in case of both constructs, but the induced luciferase levels were also lower in case of the pGL4.20-TNFA-857T-3'construct.

4. Studying the role of selected polymorphisms of IL1 family members in the genetic predisposition to acne

The IL-1 family plays an important role in immune regulation. Genetic polymorphisms of the IL-1A gene encoding the IL-1α cytokine, as well as and the IL1RN gene that codes for the interleukin-1 receptor antagonist (IL-1RA) have frequently been implicated in the pathogenesis of various chronic inflammatory diseases. We chose to study the role of the IL-1A +4845G>T SNP and the IL1RN VNTR polymorphism in acne pathogenesis, analyzed them in the available control and acne patient samples also in retrospective case-control studies.

In case of the IL-1A +4845G>T polymorphism the percentage of individuals carrying the minor T allele in a homozygote form correlated with the severity of acne symptoms, suggesting that the minor T allele is positively associated with acne. This SNP might influence the calpain-mediated cleavage of the pro-IL-1α during the maturation process of the protein and cause a shift in the ratio of cytoplasmic versus secreted isoforms in keratinocytes.

In contrast to this we did not find any association between the VNTR polymorphism of IL1RN gene and acne.

Summary

- Assorted *P. acnes* strains have different strain- and dose-specific effects on the cell biological properties (proliferation, viability) of human *in vitro* cultured immortalized keratinocytes (HPV-KER cells).
- High dose treatment of certain strains (*P. acnes 889, ATCC 11828*) induced cytotoxicity that may be a result of keratinocyte membrane damage.
- Cytotoxic effect can be a direct result of differences in the growth properties, and parallel to that in the secretion of bacterially derived secreted metabolic end-product (e.g. PA) of the various *P. acnes* strains. PA may be an active contributor to the *P. acnes* induced cytotoxic effect.
- The -238G>A (rs361525), −863C>A (rs1800630), −1031T>C (rs1799964) TNFA SNPs and the IL1RN VNTR polymorphism are not associated with acne vulgaris in our study population.
- The minor T allele of the TNFA -857C>T (rs1799724) is a protective factor in acne pathogenesis. According to our *in vitro* luciferase reporter analysis in the presence of this allele both the basal and the NF-κB-driven signaling events may be compromised in cultured keratinocytes.
- The rare A allele of the -308 (rs1800629) TNFA polymorphism can have a role in the genetic predisposition to acne in the female patients of our study population.
- Positive association was also found between the rare T allele of the +4845(G>T) (rs17561) SNP of the IL-1A gene and acne, as well as a correlation between the severity of acne symptoms and the frequency of the minor allele–containing genotypes. This SNP might influence the calpain-mediated cleavage of the pro-IL-1α during the maturation process of the protein and causing a shift in the ratio of cytoplasmic versus secreted isoforms, and by that can influence the initiation and/or maintenance of *P. acnes*-induced immunological and inflammatory events in keratinocytes.

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