The effect of *Cutibacterium acnes* on the barrier properties of *in vitro* cultured keratinocytes

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Summary of the Ph.D. thesis

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Publications related to the subjects of the thesis

I. **Bolla, Beáta Szilvia; Erdei, Lilla; Urbán, Edit; Burián, Katalin; Kemény, Lajos; Szabó, Kornélia:** *Cutibacterium acnes* regulates the epidermal barrier properties of HPV-KER human immortalized keratinocyte cultures SCIENTIFIC REPORTS 10 : 1 Paper: 12815 , 13 p. (2020) **IF: 3.998**

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IV. **Manczinger, M; Bodnar, V; Papp, BT; Bolla, BS; Szabo, K; Balazs, B; Csanyi, E; Szel, E; Eros, G; Kemeny, L.:** Drug repurposing by simulating flow through protein-protein interaction networks. CLINICAL PHARMACOLOGY & THERAPEUTICS 103 : 3 pp. 511-520. , 10 p. (2018) **IF: 6.336**

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V. **Megyeri, K; Orosz, L; Bolla, S; Erdei, L; Razga, Z; Seprenyi, G; Urban, E; Szabo, K; Kemeny, L.:** *Propionibacterium acnes* induces autophagy in keratinocytes: involvement of multiple mechanisms JOURNAL OF INVESTIGATIVE DERMATOLOGY 138:4 pp. 750-759., 10 p. (2018) **IF: 6.290**

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VI. **Szabo, K ; Erdei, L ; Bolla, BS ; Tax, G ; Biro, T ; Kemeny, L.:** Factors shaping the composition of the cutaneous microbiota BRITISH JOURNAL OF DERMATOLOGY 176:2 pp. 344-351., 8 p. (2017) **IF: 6.129**

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1. Introduction

The human skin provides not only a physical or mechanical barrier but also an immunological and microbial barrier too. The cutaneous microbiota is part of this barrier. It helps to prevent the colonization of pathogenic microbes on the skin surface, aid the differentiation and maturation of a functionally full-fledged tissue, and play important roles in the maintenance of skin homeostasis.

The outermost layer of the skin is the epidermis, which is built up by keratinocytes. It has different layers, depending on the differentiation state of the cells: the *stratum basale*, the *stratum spinosum*, the *stratum granulosum*, and the *stratum corneum.* In the epidermis, the keratinocytes are tightly packed to one another in the different layers, even though their morphological appearances continuously change according to their differentiation state. The cells are also connected by cell junctions. Specialized types are the tight junctions (TJ), which further help to seal the space and aid the establishment of skin barrier functions.

TJs are multiprotein junctional complexes between neighboring cells. They seal the space (paracellular space) between the adjacent cells and control the transport of various chemicals through the tissue. These complexes are located only in the 2nd layer of the *stratum granulosum* as functional sealing complexes, forming intercellular contact sites, so-called ‘kissing points’ between the cells. TJs are consist of at least 40 different proteins. The most well-studied structural components are the claudin (CLDN) protein family members, occludin (OCLN), junctional adhesion molecules (JAMs), and other well-studied and characterized molecules, including zonula occludens 1, 2, and 3 (ZO-1, 2, and 3), cingulin, and afadin.

Based on their structures, TJs generate a tight barrier to prevent the free transportation of molecules and ions through the paracellular space. Their regulated movement is called the paracellular transport pathway, which has two forms: the pore pathway and the leak pathway. The pore pathway is highly conductive and allows the movement of ions and small charged molecules between the two sides of the membrane. TJs can regulate the properties of this transport pathway through their protein composition, for example, through the CLDN content of the TJ complexes. In contrast to that, the leak pathway is responsible for the transportation of large molecules. It is a low-capacity, charge non-selective transport method, depending on the presence of transient breaks within the TJ fibril network. These transport pathways play important roles in the regulation of barrier functions but also contribute to the determination of apical and basolateral polarization in signaling events and also affect cellular behavior and differentiation processes.

In healthy skin, *C. acnes* is a member of the healthy cutaneous microbiota. During its metabolism, short-chain free fatty acids (SCFAs) are produced, which play immunoregulatory functions, help to maintain the acidic pH in the skin, and inhibit the growth of potential pathogens like *Staphylococcus aureus*. *C. acnes* strains also produce a bacteriocin-like substance that regulates the growth of other species. The bacterium also affects keratinocyte functions. It stimulates their proliferation and modifies the expression of genes involved in the keratinocyte differentiation processes, and through this, it may also contribute to the maturation of the fully functional epidermal tissue. *C. acnes* also induces innate immune and inflammatory events in keratinocytes through the activation of Toll-like receptors 2 and 4 (TLR2, TLR4). Extensive activation of these pathways leads to the pathogenesis of *acne vulgaris.*

Acne is the most common, multifactorial, inflammatory skin disease, which affects a large proportion (approximately 85%) of the adolescent population. Its pathogenesis is complex. It starts parallel with the puberty-induced hormonal changes and elevated androgen hormone levels, which leads to increased sebum secretion and abnormal *C. acnes* colonization of the pilosebaceous units (PSUs) or follicles. Keratinocyte hyperproliferation and abnormal differentiation are also prominent features but the exact role the bacterium plays in these processes is still not completely understood. Overall, these events result in the abnormal interaction of skin cells, among others, keratinocytes, sebocytes, and the *C. acnes* bacterium, called bacterial dysbiosis, leading to the formation of the characteristic acne lesions.

2. Aims

Currently, we do not know whether and how the epidermal barrier is affected in acne lesion development. Thus, in our studies, we aimed to investigate the effect of *C. acnes*, which is a prominent member of the cutaneous microbiota, on keratinocyte barriers. We specifically analyzed the following questions:

- How does *C. acnes* affect the barrier properties of confluent *in vitro* keratinocyte monolayer cultures, representing various differentiation states?
- What are the underlying cellular and molecular changes?
- Does *C. acnes* has any effect on the barrier properties of *ex vivo* cultured organotypic skin models?

3. Materials and methods

- *C. acnes* 889 (type IA), 6609 (type IB), and ATCC11828 (type II) strains were cultured and stored as previously described in detail.
- For our experiments, we used *in vitro* cultured, confluent, contact inhibited (Ca-low), and calcium-differentiated (Ca-high) keratinocyte cultures. The latter was established with the addition of 1.7 mM $CaCl_2$. We used a human immortalized keratinocyte cell line (HPV-KER), and normal human epidermal keratinocytes (NHEK).
- To perform studies on *ex vivo* full-thickness skin biopsy samples, organotypic skin (OS) models were also established and analyzed.
- The integrity and the barrier properties of confluent or differentiated HPV-KER and NHEK monolayer cultures were detected in real-time using an impedance-measurement based technology (xCELLigence system) after treatment with different doses of live or heat-killed *C. acnes* strains (889 – type IA, 6609 – type IB, ATCC 11828 – type II,) at various multiplicity of infection (MOI).
- The effect of different concentrations of propionic acid (PA) was determined using HPV-KER (Ca-low) cultures.
- For TJ protein level analysis, western blot assays were carried out. For protein detection actin, ZO1, OCLN, CLDN1, and 4 primary antibodies were used. HRP-conjugated secondary antibodies were applied, and the resulting bands were visualized using the C-DiGit Blot Scanner or the Omega Lum G Chemidoc Imaging System.
- Total RNA was isolated from the monolayer cultures and OS models using TRI-Reagent. cDNA synthesis was performed using EvoScript cDNA Synthesis Kit, and changes in the expression of TJ genes (CLDN1, CLDN4, OCLN, and ZO-1) were analyzed by real-time RT-PCR using the Universal Probe Library system.
- Immunohistochemical (IHC) stainings were used to analyze changes in TJ protein levels after *C. acnes* 889 treatment in the paraffin embedded OS samples with a Bond-Max automated IHC/ISH stainer. Primary antibodies against TJ proteins (CLDN1, CLDN4, OCLN, ZO-1) and matching isotype controls (mouse IgG2a, rabbit poly IgG, and mouse IgG1 κ) were used. Samples were developed with DAB-Chromogen and dyed with hematoxylin. After covering with coverslips, samples were analyzed with an inverted light microscope.

- Transepithelial electrical resistance measurements (TEER) were carried out on confluent (Ca-low) and differentiated (Ca-high) HPV-KER monolayer cultures using an Epithelial Volt/Ohm (TEER) Meter EVOM2.
- Trypan blue exclusion assay was carried out on HPV-KER cultures to follow the cell number changes of the cultures upon *C. acnes* treatment.
- Lucifer yellow (LY) penetration assay was performed on HPV-KER monolayers and OS models. Fluorescence intensities were measured with BMG FLUOstar OPTIMA. In the case of the OS models, LY dye penetration was analyzed on frozen sections using a fluorescence microscope.
- The effect of antibiotic treatment of *C. acnes* co-cultured HPV-KER monolayers was observed in real-time using the xCELLIgence system.
- For statistical analysis, data were compared using paired Student's t-test and one-way ANOVA with post-hoc Tukey-test. A probability value of less than 0.05 was considered significant.

4. Results

4. 1. Ca^{2+}-induced differentiation leads to marked nCi and corresponding barrier changes of *in vitro* keratinocyte cultures

Ohmic resistance and impedance measurements across a wide spectrum of frequencies are considered as good indicators of the integrity of cellular barriers. Thus, we investigated how different culturing conditions affect the measurable impedance (Z), and the calculated Cell index (Ci) values of confluent NHEK and HPV-KER cultures using real-time cellular analysis and interpreted the changes as alterations in barrier properties of the *in vitro* monolayer cultures. We allowed the cells to form confluent monolayers using basal KSFM and then raised the concentration of extracellular Ca^{2+}, which is known to induce keratinocyte differentiation. We found that, after an initial growth phase, Ci values reached a plateau as the cultures became confluent and contact-inhibited. Replacing the media with fresh media containing high Ca^{2+} concentrations (Ca-high) induced a marked and immediate increase in Ci compared to samples that were only contact inhibited and maintained in low Ca^{2+} media (Ca-low). NHEK and HPV-KER cultures behaved similarly, suggesting that the latter cells may be used as a model to analyze keratinocyte barrier functions in monolayer cultures. These findings also agree with the available literature data and suggest that high extracellular Ca^{2+} concentration leads to the stabilization of keratinocyte barriers. In addition, the capability of the xCELLigence RTCA system for real-time monitoring of these properties was confirmed.

4. 2. Analysis of the effect of *C. acnes* on the integrity of *in vitro* keratinocyte cultures

We used the same experimental setup to analyze how the addition of *C. acnes* 889 strain to the Ca-low and Ca-high NHEK and HPV-KER cultures affected keratinocyte barriers. In Ca-low NHEK and HPV-KER cultures, we first observed a rapid and transient increase of nCi values, suggesting improved barrier properties of the cultures. The peak in nCi was followed by a drop in the Ca-low cultures, suggesting that after reaching a threshold, continuously growing bacterial cells may have deleterious effects on the barrier properties of keratinocyte monolayers. We also repeated the experiments on differentiated, Ca-high NHEK, and HPV-KER monolayers. In these cell cultures, only the dose-dependent decrease of nCi values was detected, independent of the used cell type and *C. acnes* MOI.

For determining possible strain-specific effects, we repeated the bacterium treatments of HPV-KER and NHEK monolayers using different *C. acnes* strains (6609 and ATCC11828). Our results showed that the measured nCi values changed in a similar manner that we detected

with the *C. acnes* 889 strain both in the Ca-low HPV-KER and NHEK cultures. Our results also showed that the exact timing of the nCi changes depended on the used *C. acnes* strain, the treatment dose, and the cell type.

Next, we also investigated whether the observed cellular changes were reproducible by using heat-killed *C. acnes* 889, 6609, and ATCC11828 strains. Our results showed no significant changes in the measured nCi values, suggesting that the impedance changes observed in previous experiments depended on the presence of live bacteria.

As Ci changes may reflect differences in the cell numbers, we monitored the effect of the *C. acnes* 889 strain on the number of cells both in the Ca-low and Ca-high HPV-KER cell cultures. In our experimental setup no significant change in cell number was detected using a trypan blue exclusion assay, suggesting that keratinocyte proliferation and viability changes were not causing the Ci differences.

Our data raised the possibility, that the nCi changes we detected in our real-time cellular analysis were due to barrier changes. To test this hypothesis, we also performed TEER measurements and found that the TEER resistance values first increased in the Ca-low cultures, followed by a decrease in both models in the presence of the bacterium, similarly to what we found in the xCELLigence analysis. Altogether, these results strongly suggested that *C. acnes* can influence the state of the *in vitro* cultured keratinocyte barrier.

4. 3. Analysis of the effect of propionic acid (PA) on the integrity of *in vitro* keratinocyte cultures

We also analyzed the effect of PA, which is an important molecule produced during the anaerobic fermentation processes of *C. acnes*, on the barrier properties of HPV-KER monolayer cultures. Our results showed that PA treatment alone had similar effects on the measured nCi values than that of live *C. acnes*. Indeed, treatments with high concentrations of PA resulted in a transient increase in nCi, followed by marked decreases suggesting that metabolic compounds produced by the *C. acnes* also affected the barrier properties of the *in vitro* cultured keratinocytes.

4. 4. Expression changes of selected TJ proteins in HPV-KER monolayers and OS models upon co-culturing with *C. acnes* 889 strain

To study the cellular changes leading to *C. acnes*-induced nCi alterations, we treated Ca-low and Ca-high HPV-KER cultures with *C. acnes* 889 strain (MOI=100, 300) and analyzed the expression of selected genes encoding important TJ components: claudin 1 and 4 (CLDN1,

CLDN4), occludin (OCLN) and zonula occludens 1 (ZO-1). ZO-1 mRNA levels did not markedly change, and OCLN expression increased upon bacterial treatment in both models. The expression of the two claudins exhibited opposing changes: CLDN1 mRNA levels decreased in both models, while CLDN4 mRNA increased in the Ca-low cultures. We found that, upon the addition of the bacterium, ZO-1 protein levels increased markedly in the Ca-high cultures, and a small but consistent elevation was detected in the Ca-low HPV-KER cultures. OCLN protein expression increased slightly, whereas CLDN1 protein levels decreased in both cultures. CLDN4 levels also increased but only in the Ca-low cultures 72 hours after treatment. Our findings suggest that *C. acnes* induces the expression changes of selected TJ structural proteins.

We also investigated whether the *C. acnes*-induced cellular changes were specific to monolayer cultures by studying the expression of selected TJ structural components in *ex vivo* OS cultures upon *C. acnes* treatment. We found that ZO-1 and OCLN protein levels increased in all epidermal layers in the presence of *C. acnes* 889 strain. CLDN1 expression, which was strongly expressed throughout the epidermis in the untreated control samples, decreased everywhere except the basal layer. In contrast, CLDN4, which was restricted to the *stratum granulosum* in control samples, also appeared in the lower layers of the epidermis in the presence of the bacterium. These findings suggest that the abundance, as well as the localization of selected TJ proteins, change in the presence of the bacterium.

To analyze the functional consequences of the observed nCi changes, an LY penetration assay was performed on the Ca-high HPV-KER monolayers and OS models. We found that fluorescence intensities increased in treated samples compared to controls, indicating LY dye penetration by 72 hours after treatment. No differences were detected in the 24-hour samples. We also repeated the dye penetration experiments in the OS models to confirm that the observed barrier changes were not specific to monolayer cultures. As observed for the monolayer cultures, LY dye penetration increased in the presence of high-MOI *C. acnes* 889 treatment, as indicated by the appearance of increased, diffuse fluorescent signal in the subepidermal skin regions.

4. 5. Antibiotic treatment of HPV-KER monolayers co-cultured with *C. acnes* 889 strain leads to a partial reversal of bacterium-induced nCi decreases

As many of the acne treatments currently available exhibit bacteriostatic and/or antibacterial properties, we were interested to see if decreasing the *C. acnes* activity and/or viability in co-cultures with the addition of AB/AM treatment reverses the bacterium induced

nCi decreases we noted in previous experiments. Decreasing the load of live bacteria in the co-cultures by the addition of AB/AM solution partially reversed the deleterious effect of *C. acnes* 889 strain and led to a marked increase of nCi values, characteristic of improved barrier functions of *in vitro* keratinocyte cultures.

5. Summary

- We showed that Ca^{2+} treatment of keratinocyte cultures helps the establishment and stabilization of the keratinocyte barrier functions, and the xCELLIgence system is able to detect these changes.
- In less differentiated, Ca-low HPV-KER and NHEK cultures, co-culturing with live *C. acnes* bacterium induced rapid, transient nCi increases, which are characteristic of improved barrier properties. These changes were strain-specific and depended on the applied bacterial dose.
- Continuously growing *C. acnes* strains gradually exhibited deleterious effects on the integrity and barrier properties of keratinocyte monolayer cultures, as indicated by the marked decrease in nCi values. This effect was dependent on the dose of live bacteria applied, but independent of cell differentiation.
- We proved that nCi changes reflected altered barrier properties of keratinocyte monolayer cultures using TEER measurements.
- Barrier modifying effect of *C. acnes* was only apparent in the presence of live bacteria.
- We analyzed the effect of PA, a special metabolic product of this bacterium that is linked to bacterial growth and metabolism. We found that PA may be an important factor: Ca-low cultures treated with high doses of PA exhibited clear, transient Ci changes that were comparable to what we observed with live *C. acnes* strains.
- *C. acnes* treatment induced expression and localization changes in the TJ proteins (CLDN1,4; OCLN; ZO-1) in monolayer cultures and OS models as well.
- Treatment with LY, a large molecular weight, fluorescently labelled compound that cannot freely permeate lipophilic barriers, clearly indicated that the nCi decrease we observed in Ca-low HPV-KER keratinocytes could be the consequence of bacterium-induced deterioration of the integrity of the cultures. This effect was not specific to monolayer cultures: similar results were found when the experiments were repeated in OS models, suggesting that *C. acnes* may also regulate cutaneous barrier properties, tightness and paracellular transport properties of the epidermis, even in a tissue environment.
- We tested whether reducing the presence of viable *C. acnes* using antibiotics in the HPV-KER co-cultures restores the barrier properties and found clear increases in the measured nCi values.

Based on our results, *C. acnes* can modulate the expression and localization of the TJ proteins and through this it can play important roles in the regulation and maintenance of healthy skin homeostasis.

6. New findings

1. *C.* acnes, a prominent member of the skin microbiota, have a complex, strain-specific, and dose-dependent effect on keratinocyte barriers. It improves barrier properties when present in low doses but may also exhibit deleterious effects due to its excess growth. The latter effect may play roles during lesion development in acne vulgaris pathogenesis.
2. Keratinocyte barrier changes are accompanied by altered expression and protein distribution of important TJ structural components, CLDN1, 4, OCLN, and ZO-1, leading to modified paracellular transport properties.
3. The barrier modifying effect of *C. acnes* is partly mediated by the short-chain fatty acids of anaerobic fermentation.
4. Antibiotics are widely used therapeutic modalities for acne vulgaris treatment. Their therapeutic effect may include the restoration of epidermal barrier functions by decreasing the number of live bacteria in the diseased follicles.
5. Our results highlight the complex effect of *C. acnes* on the cutaneous barrier functions in healthy skin
6. We propose that acne pathogenesis may be an even more complex event than previously suspected, and apart from the role of *C. acnes* induced immune and inflammatory events, changes in cutaneous barrier properties can play important roles.

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