

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigation of the Role of Endocannabinoid Tone in the
Biology of Selected Non-Neuronal Skin Cells

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University of Debrecen
DOCTORAL SCHOOL OF KÁLMÁN LAKI
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Éva Margittai, MD, PhD

The Examination takes place at the Department of Laboratory Medicine,
University of Debrecen Clinical Center
December 19, 2018, 11:00

Head of the **Defense Committee:** János István Kappelmayer, MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Building A,
Department of Internal Medicine, Faculty of Medicine, University of Debrecen.
December 19, 2018, 13:00

INTRODUCTION AND OVERVIEW OF LITERATURE

Skin as a barrier

Human skin is one of the largest organs of the body with its 1.8 m² (average adult). It serves not only as a well-structured protection shield against various physicochemical and biological hazards, but it also plays a crucial role in thermoregulation and water balance. It produces several hormones (e.g., vitamin D), stores a variety of substances (e.g., different fats), and also allows to detect temperature, pressure, touch, vibration, and pain.

The skin implements its multiple tasks by acting as a physicochemical, immunological, and microbiological barrier. The major part of the skin's physicochemical barrier resides in the epidermis, more specifically in the *stratum corneum*. This layer consists of terminally differentiated keratinocytes (KCs) that are tightly surrounded by extracellular lipid matrix. This protective lipid layer is complete with the oily, waxy product of sebaceous glands (SGs), the so-called sebum that is brought up to the skin surface.

Those advantages microbes that live on the skin surface provide with a microbiological barrier. These microorganisms are able to take actions against pathogens that represent a major threat for the human body.

The immunological protection is created by different antimicrobial peptides (AMPs) and lipids (AMLs) that are produced by the cells of the skin. This function is implemented in close collaboration with the members of the innate and adaptive immune system. The members of the in-born system consists of numerous epidermal (e.g., KCs and Langerhans cells) and dermal (e.g., sebocytes, mast cells [MCs] and dendritic cells) cells.

Biology of human sebaceous glands (SGs) and related diseases

Besides the lipid production, SGs are also important in stem cell biology, cellular differentiation, skin aging, and inflammatory skin processes. Just like the composition of sebum, these functions of the SGs are also well-controlled among

others by sex steroids, growth factors, neuropeptides, and by a variety of other receptors and modulators (e.g., by peroxisome proliferator-activated receptors [PPARs], histamine, etc.).

The immunological role of SGs is also significant. Sebocytes are armed with pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and cluster of CD14 that can recognize several dangerous pathogens.

Diseases affecting SGs are common dermatological disorders such as acne and seborrhea that have very high occurrence within the general population. Acne affects approximately 85% of young adults aged 12–25 years. The following multiple factors result in its development: increased sebum production, androgen activity, follicular hyperkeratinization, the action of *Cutibacterium* (formerly called *Propioibacterium*) *acnes* within the sebaceous follicle.

Unfortunately, the present therapeutic treatments of illnesses associated with SG disorders face various obstacles: beyond the disruption of the homeostatic function of SGs, most of them can cause serious systematic side effects (e.g., skin dryness, itch, hepatic impairment, and anemia). Therefore clinical practice has a great demand for therapies that can supply better solutions to these problems.

The model of human SGs: SZ95 sebocyte cell line

To better understand the pathophysiology of SG-associated disorders and their multiple factors, the development of experimental SG models is needed. Unfortunately, animal models that would provide a way to examine the functions of intact SGs within their natural tissue environment are not available. Even though the primary cell lines show natural sebocyte characteristics, they also face some major limitations, such as short sustainability (3-6 passages) and a need for large number of donors.

One solution for overcoming these challenges recently was to create immortalized human sebocyte cell lines. Among them, the first and the best characterized is the SZ95 cell line. Compared to normal human sebocytes, these

cells show not only similar phenotypic, but functional characteristics, and as cell lines in general they are proven to be stable even at high passages.

Based on above mentioned reasons, in this thesis we have performed our experiments using the SZ95 cell line.

Biology of human mast cells (MCs) and related diseases

MCs are one of the most important effector cells of the cutaneous immune system. Within the skin, they play a pivotal role in many physiological functions such as host defense, wound healing, tissue remodeling, and hair growth.

After leaving the bone marrow, immature MCs circulate through the vascular system. Due to chemotactic signals they predominantly migrate into connective or mucosal tissues, where due to the specific local tissue milieu and to the presence of certain factors they complete their maturation process.

Undifferentiated MC progenitors can also be deposited in tissues, namely the stroma of rodent and human hair follicles (HFs), more specifically within their connective tissue sheath (CTS). From these precursors, mature MCs can also be generated *in situ* in the absence of bone marrow.

Thus, we hypothesized here such potent regulatory mechanisms that can effectively regulate MCs functions.

MCs participate in the development of several diseases that are mostly associated with allergic and inflammatory actions. The incidences of these illnesses are increasing to epidemic proportions, and constitute a considerable burden to affected patients and to health-care providers. For example, worldwide 15-20% of children and 1-3% of adults affected by atopic dermatitis. Also because of these reasons we clearly need a better understanding of how healthy human MC-rich tissues avoid excessive activities and number of MCs under physiological circumstances.

In situ model of skin MCs: human hair follicle (HF) organ culture

The isolation and maintenance of MCs have been challenging for several reasons. Firstly, there are only a low number of MCs in their resident tissues and

it is hence relatively difficult to obtain them in reasonably large quantities. Secondly, methods with multiple purification processes can act as serious stress resulting in a poor yield and sometimes altered properties of the cells, which can lead to misleading results during their investigation. Finally, we should also keep in mind that the maturation process of MCs is strictly dependent on their local tissue milieu. Under cell culture conditions these microenvironmental factors are missing. Therefore, human MCs are best analyzed under *in situ* conditions. In human system, MC-rich human skin can particularly be suitable for such investigations and moreover it has recently become a relatively easily accessible organ, especially as the “by-product” of routine plastic surgeries (e.g., facelift operations).

As already mentioned, mature MCs that are already presented can be differentiated from MC precursors, notably in the CTS of murine and human HFs, without the bone marrow being present. Thus, we hypothesized potent regulatory mechanisms that help to prevent the excessive increases in the number of MCs by controlling their intracutaneous maturation from the resident progenitors within the human skin, more specifically within the HFs’ CTS.

Thus, in the line of all these, for the investigation of MCs, HF organ culture was used.

Endocannabinoid system (ECS)

Cannabinoids ligands, together with their specific receptors and enzymes, form the so-called ECS. Over the past couple of decades, this complex lipid signaling network has emerged as one of the most remarkable regulatory systems involved in establishing and maintaining a variety of physiological processes, including appetite, pain sensation, and memory.

The cannabinoid ligands are arranged in three different categories: plant originated phytocannabinoids, endocannabinoids (eCBs), and their synthetic analogues.

The psychotropic (-)-trans- Δ^9 -tetrahydrocannabinol (THC) is the most studied phytocannabinoid that has a greater influence on key functions like motor- and cognitive skills, as well as memory, emotions, appetite, and analgesia. Among eCBs, the best-known are N-arachidonylethanolamine (AEA, also called anandamide) and 2-arachidonoylglycerol (2-AG). eCBs, in addition, also include eCB-like ligands, namely N-acylethanolamines (such as oleoylethanolamide [OEA] and palmitoylethanolamide [PEA]) and monoacylglycerols (e.g., 2-palmitoylglycerol). In recent years several synthetic cannabinoids have been created for therapeutical purposes. These compounds typically show functional similarity with e.g. THC, but are more effective in stimulating different cannabinoid sensitive receptors.

Cannabinoids can explicate their effects on target tissues throughout the whole body via specific receptors. To date two G protein-coupled receptors (GPCRs) have been identified as classical targets of cannabinoid ligands, namely cannabinoid receptor type 1 (CB₁) and cannabinoid receptor type 2 (CB₂). The effects of THC are mostly the outcome of its engagement with CB₁ that is mainly, but not exclusively, expressed in the central nervous system. In contrast, CB₂ rather appears within the cells of the immune system, and affects biological processes such as immune responses.

Cannabinoid compounds can evoke a number of cannabinoid-like effects without activating the above mentioned classical cannabinoid pathways, through other metabotropic (e.g., GPR18, GPR55, and GPR119), ionotropic (transient receptor potential [TRP] channels, e.g. previously known as vanilloid receptor 1 [TRPV1]), and intranuclear receptors (PPARs).

The metabolism of eCB ligands and its therapeutic modulation

eCB metabolism is based on the biological necessity of arising from their lipid moiety. In general, these ligands act near the site of production (typically paracrine and autocrine ways) and their degradation takes place within a relative short period of time.

The best way to outline the general metabolism of the ECS is probably an overview of the two best studied eCBs' (AEA and 2-AG) synthesis and degradation processes. N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) is mainly responsible for catalyzing the release of AEA, while fatty acid amide hydrolase (FAAH) for its degradation. 2-AG is mostly degraded by monoacylglycerol lipases (MAGLs) and synthesized by diacyl-glycerols lipase (DAGL).

Before being degraded, cannabinoids are removed from their activation site and diffuse back (most probably via facilitated diffusion) into the appropriate cells. It is assumed that a yet uncharacterized specific eCB membrane transporter (EMT) might be responsible for the reuptake of these lipid molecules. However, to date, the details of these processes have not been fully clarified.

Setting the appropriate levels, or tone of the eCB compounds can offer novel therapeutic perspectives in various diseases. Therefore, ECS research is currently focusing on the modification of its main metabolic enzymes.

The c(ut)annabinoid system

Functional members of the ECS are widely represented in the skin and play an important role in various regulatory mechanisms, both under physiological and pathological conditions. Both resident and non-resident skin cells have also been shown to express eCB receptors, enzymes and many of them were shown to be able to produce eCB ligands. In the light of all these findings, the effects of the ECS on epidermal homeostasis are highly significant.

Role of the ECS in SGs biology

We have previously demonstrated that important eCBs such as AEA and 2-AG are continuously produced in SGs. In addition, we have observed that these locally generated substances were able to increase the cellular differentiation through the activation of CB₂, and in this way the induction of the lipid production of human immortalized SZ95 sebocytes.

Even so, we still do not know much about the expression of the enzyme apparatus involved in the synthesis and degradation of the eCBs, nor about the role of the local eCB tone created by these enzymes in human SGs. Thus, current doctoral thesis explores the expression of the major members of the ECS *in vitro* in human sebocytes, as well as *in situ* in human skin. We also investigate whether the pharmacological modulation of eCB homeostasis is able to regulate SLP.

Role of the ECS in MCs biology

The regulatory effects exerted by ECS in skin mostly affect the viability, proliferation, maturation, phagocytic actions, cytokine and chemokine release of cutaneous immune cells such as Langerhans cells, intraepithelial lymphocytes, dermal dendritic cells, cutaneous memory T cells and MCs.

Recently turned out that ECS (without knowing the details of the mechanism) has a high potential as a neuroendocrine control in the regulation of MCs. *In vitro* results that have been published so far on how CB stimulation affects rodent and human MC lines are sometimes contradictory. Based on this data, the role of CB₁ and CB₂ stimulation in the control of MCs activation is not completely understood. Moreover, it remains unclear how the ECS affects primary human MCs and under clinically relevant conditions human skin MCs *in situ*. Whether the maturation of MCs from human MC progenitors *in situ* are affected by the ECS is yet to be studied.

Taking all these into account, this doctoral thesis also investigates how eCB stimulation/inhibition affects normal, experimentally un-manipulated human skin MCs *in situ*. In particular, we study whether resident CTS-MCs of human HFs express functional CB₁ and how the local ECS regulates the activation and/or maturation of these cells.

GOALS

Main questions regarding the possible role of the ECS as a neuroendocrine regulator of MCs:

- 1.) Is the human HF organ culture a clinically relevant model to study the role of the ECS in skin MCs under physiological conditions?
- 2.) Do the resident MCs that are situated in the CTS of human HFs express CB₁?
- 3.) If so, how does the stimulation/inhibition of CB₁ affect the behavior of these MCs?

To further investigate the role of eCBs in connection with the biology of SGs, we primarily aimed to answer the following questions:

- 1.) Do human SZ95 sebocytes express the major eCB synthesizing and degrading enzymes?
- 2.) How does the modulation of eCB tone affect the biology of human SZ95 sebocytes?

MATERIALS AND METHODS

Human HF organ culture and cell cultures

Normal micro-dissected anagen VI HFs were isolated from occipital and temporal scalp skin, obtained from 8 healthy individuals (aged: 49-72 years) undergoing routine facelift surgery. Human tissue collection and handling were performed according to the Helsinki guidelines, following Institutional Research Ethics approval (University of Lübeck, Germany, n. 06-109, 18-07-06) after patients' informed consent. In total, 414 micro-dissected HFs were used and cultured for specified times in 24-multiwell plates at 37°C in 5% CO₂ and 95% air in Williams E Medium. The latter's supplements were the following: insulin 10 µg/ml, hydrocortisone 10 ng/ml, 2 mmol L-glutamine and 1% antibiotic mixture. To adapt to the culture conditions, HFs were incubated overnight (with the exception of gene silencing experiments). Medium has been changed every second day, while treatments were done daily.

Human outer root sheath (ORS) KCs were obtained and cultured as following. Plucked HFs went through a 1 hour enzymatic digestion (0.2% trypsin, 0.1% ethylenediaminetetraacetic acid in calcium and magnesium free phosphate buffered saline (CMF-PBS) at 37°C. ORS-KCs were then collected by centrifugation (1000 rpm, 10 min) and then seeded in culture medium, which contained 3:1 mixture of Dulbecco's modified Eagle medium (supplemented with L- glutamine, Na-pyruvate, 4.5 g/L glucose) and Ham's F12 (supplemented with 10% Fetal Clone II (Hyclone), 5 µg/ml insulin, 0.4 µg/ml hydrocortisone, 2.43 µg/ml adenin, 2 nM triiodothyronine, 0.1 nM cholera toxin, 10 ng/ml human epidermal growth factor [EGF], 1 mM ascorbyl-2-phosphate, 100 U/ml penicillin G, and 25 µg/ml gentamycin). During the maintenance mitomycin treated human dermal fibroblasts were used as feeder-layer. Primary human material was collected after obtaining written informed consent, adhering to the Helsinki Declaration, and after obtaining permission from the Institutional Research Ethics Committee and Government Office for Hajdú-Bihar County (document

identifications: IX-R-052/01396-2/2012, IF-12817/2015, IF-1647/2016, and IF-778-5/2017).

Human immortalized SZ95 sebocytes were grown in Sebomed® Basal Medium supplemented with 10 (V/V)% foetal bovine serum, 1 mM CaCl₂, 5 ng/ml human EGF, and MycoZap™ Plus-CL. The medium was changed every second day, and cells were passaged once they reached 60-70% confluence.

Preparation of cryo and paraffin sections

After the adequate treatment time, cryo samples were embedded into cryo-matrix and were frozen in liquid nitrogen. From these specimens, 6 µm thick cryosections were cut. Sections were then placed onto SuperFrost slides and were kept at -80°C, until they were used for different stainings.

Samples for paraffin embedding were fixed after the adequate treatment time for 24 hrs in 4% phosphate buffered formalin solution. Fixed samples then went through a dehydration process in an embedding machine. Before the staining procedures, 4 µm sections were cut. Right before each staining procedure, sections were deparaffinized.

MC histochemistry

Human skin MCs were detected with 2 sensitive histochemical staining methods, namely Leder's esterase and alkaline-Giemsa staining.

c-Kit (CD117), CB₁, tryptase, chymase, and FcεRIα immunostaining

For the detection of c-Kit (CD117), CB₁, tryptase, chymase, and FcεRIα, Tyramide Signal Amplification (TSA™) technology was applied according to the manufacturer's protocol. After fixation, primary antibodies (rabbit-anti human CD117, rat anti-mouse CD117, rabbit anti-human CB₁, mouse anti-human FcεRIα, mouse anti-human chymase, and mouse anti-human tryptase) were applied during an overnight incubation. The following day, biotinylated secondary antibodies were used. After horseradish peroxidase was administered, the reaction was amplified by fluorescein isothiocyanate (FITC) tyramide

amplification reagent. Finally slides were counterstained with 4',6-diamidino-2-phenylindole-dihydrochlorid (DAPI). Negative controls were obtained by not adding the primary antibodies to the samples.

c-Kit/CB₁ double immunostaining

TSA method was applied according to the previous paragraph. c-Kit was visualized by FITC, while CB₁ by tetramer rhodamine–conjugated tyramide. Negative controls were obtained by not adding the primary antibodies to the samples.

c-Kit/Tocrifluor double immunostaining

After the incubation with Tocrifluor and fixation, the endogenous peroxidase activity was saturated using 3% H₂O₂. In the next step, the endogenous avidin-biotin were blocked and sections were incubated with the primary antibody (rabbit-anti human CD117) overnight. Slides were then stained with secondary antibody and incubated with streptavidin-conjugated horseradish peroxidase. Finally, FITC-conjugated tyramide was applied. Primary antibodies were omitted as negative control for the unspecific binding of the secondary antibody. Finally slides were counterstained with DAPI. Negative controls were obtained by not adding the primary antibodies to the samples.

c-Kit/Ki67 double immunostaining

After the c-Kit TSA staining, mouse anti-human Ki67 or rat anti-mouse Ki67 antibodies were applied. As secondary antibodies, rhodamine goat anti-mouse or goat-anti-rat IgGs were used. Finally slides were counterstained with DAPI. Negative controls were obtained by not adding the primary antibodies to the samples.

Ki67/TUNEL double immunostaining

Double Ki67/TUNEL (“terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling”) immunostaining was performed to evaluate the number of proliferating and apoptotic cells within the HFs. After fixation, cryosections were

labeled with digoxigenin-deoxyUTP in the presence of TdT enzyme. This was followed by an overnight incubation with mouse anti-human Ki67 antigen. TUNEL cells were visualized by an antidigoxigenin FITC-conjugated antibody, whereas Ki67 was detected by a rhodamine-labeled goat anti-mouse antibody. Finally, sections were counterstained by DAPI. The number of Ki67 and TUNEL positive cells were counted in defined areas in the CTS of HFs and were normalized to the number of total (DAPI) cells. Primary antibody and TdT enzyme were omitted as negative control for the unspecific binding of the secondary antibody.

c-Kit/TUNEL double immunostaining

This staining was applied to check the ratio of the apoptotic c-Kit⁺ MCs. Above described c-Kit TSA method was followed by a TUNEL reaction. Primary antibody and TdT enzyme were omitted as negative control for the unspecific binding of the secondary antibody.

SCF immunostaining

To identify the SCF immunoreactivity of the organ-cultured human HFs and isolated human ORS-KCs, an indirect immunofluorescence method was used. Sections were incubated with anti-human SCF during night. As a secondary antibody, FITC conjugated (rhodamine for ORS-KCs) goat anti-mouse IgG was applied. As a positive control, an intact human scalp skin sample was used. Primary antibodies were omitted as negative control for the unspecific binding of the secondary antibody.

SG immunohistochemistry

For the immunohistochemical investigation of NAPE-PLD, FAAH, MAGL and DAGL β formalin fixed paraffin embedded skin samples (rich in SGs) from three donors were used, all diagnosed as trichilemmal cyst in Gyula Kenézy County Hospital, Hungary. To examine the expression pattern of DAGL α in SGs, frozen skin samples obtained from three donors (vertex) were used.

Cryostat samples were then fixed in acetone, whereas formalin fixed cuts were deparaffinised and prepared for heat-induced antigen retrieval. FAAH, MAGL, and DAGL β epitopes were retrieved in 11 mM citrate buffer (pH 6) using pressure cooker on full pressure (2 min). NAPE-PLD was retrieved in EnVision FLEX Target Retrieval Solution High pH in water bath (95 °C, 30 min). After blocking the endogenous peroxidase activity (3% H₂O₂) in both type of sections, primary antibodies diluted in 1% Bovine Serum Albumin were applied. Following this step, EnVision FLEX Labeled polymer-HRP System were used with 3,3'-diaminobenzidine (DAB) visualization techniques. Lastly, slides were counterstained with haematoxylin. Negative controls were obtained by not adding the primary antibodies to the samples.

Microscopy

For fluorescence and light microscopy the following microscopes were used in case of experiments with MCs: Keyence Biozero-8000 and Keyence Biozero-8100 microscopes in combination with Nikon lenses. Images were analysed with Biozero Image Analyzer Software, Version 2.5. For the immunohistochemistry of SGs and their positive controls Nikon Eclipse E600 microscope was applied.

After adequate sample preparation, sections were analyzed by transmission electron microscope JEM-1200EXII, JEOL.

High magnification images of c-Kit and CB₁ double positive cells were taken by using laser scanning confocal microscopy (Fluoview 300, Olympus) running Fluoview 2.1 software (Olympus).

Determination of the number of MCs

MCs were counted as degranulated, when five or more extracellularly situated metachromatic granules could be histochemically detected at 400x magnification, using light microscopy (visual field). The number of degranulated and total CTS-MCs were counted along the HFs per visual field, which

individually means at least 13 fields per one HF (a minimum of 2 donors and 6 HFs/treatment group). MCs within human and mouse skin samples were counted per visual field, which individually means at least 13 fields per sample (a minimum of 2 donors).

Signal intensity measurements

Signal intensity of stainings were evaluated by semiquantitative analysis, using NIH image J software.

RNA isolation, reverse transcription and quantitative “real-time” polymerase chain reaction (Q-PCR)

Q-PCR was performed on an ABI Prism 7000 sequence detection system using the 5' nuclease assay, TaqMan primers and probes. As internal control, transcripts of internal housekeeping genes for glyceraldehyde 3-phosphate dehydrogenase, for β -actin, for 18S RNA, and for cyclophilin A were determined.

CB₁ knockdown in situ

Freshly isolated HFs were placed into cold William's E medium and were kept there right before the knockdown procedure. During transfection, HFs were incubated in 500 ml transfection medium mixed with CB₁ specific siRNA (2.5 μ l), siRNA transfection reagent (2.5 μ l), or control siRNA (2.5 μ l) for 6 hours. Mediums were then changed back to normal supplemented William's E medium. Cryo-embedding of samples was made 24 hrs after the transfection.

CB₁ KO mice

CB₁ gene was inactivated with its targeted disruption within MPI2 embryonic stem cells, using a non-receptor sequence during homologous recombination. The resulting mice were mated more than 13 times with C57BL/GJ animals, and therefore considered to be congenic. CB₁^{+/-} types were generated with the crossing of wild (CB₁^{+/+}) and KO mice (CB₁^{-/-}).

Tryptase immunoassay

The level of tryptase was measured by a fluorescent enzyme immunoassay in the supernatants of CB₁ gene knockdown HF_s by using a commercial assay from Phadia (ImmunoCap Tryptase).

Determination of intracellular lipids

For quantitative measurement of sebaceous (neutral) lipid content fluorescent Nile Red staining was performed. Intensity was measured on FlexStation 3 multi-mode microplate reader.

Determination of cellular viability

To determine the viability of the SZ95 cells MTT assay was used. Cells were seeded in 96-well plates (20,000 cells/well) in quadruplicates, and were treated as indicated for 48 hrs. This step was followed by a 2 hrs of incubation with MTT solution. Finally, the concentration of formazan crystals (as an indicator of the number of viable cells) was measured colorimetrically at 565 nm by using FlexStation 3 multi-mode microplate reader.

Determination of apoptosis and necrosis

To determine the mitochondrial membrane potential of SZ95 sebocytes 1,1',3,3',3',3'-hexamethylindodicarbo-cyanine iodide containing MitoProbe™ DiIC₁(5) Assay Kit was used. To check the unprogrammed death of cells (necrosis) SYTOX Green staining was used. Selective decrease of DiIC₁(5) intensity indicated mitochondrial depolarization (i.e., the onset of early apoptotic processes), whereas an increase of SYTOX Green staining intensity revealed necrotic cell death.

Determination of cytokine release (ELISA)

Cells were seeded in standardized way. Treatments were done as indicated. The released amounts of IL-1 α , IL-1 β , IL-6, IL-8, and TNF α were determined, using the collected supernatants, according to the manufacturers' protocols.

To measure the SCF level in the supernatants of the human ORS-KCs Quantikine Human SCF ELISA Kit was used, according to the manufacturer's protocol.

Western blotting

To measure the sample's protein content, a modified BCA protein assay was used. The proteins of the samples were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Samples were loaded with equal amount of protein per lane into a 10% Mini Protean TGX gel. They were then moved onto a membrane made of nitrocellulose. The membrane was then incubated overnight at 4°C with dilute solution (5g/100 ml milk containing PBS) of specific primary antibodies. After this step, the membrane was exposed to horseradish peroxidase-conjugated rabbit IgG Fc segment-specific antibodies. To detect the probes that were labeled and bound to the protein of interest, a SuperSignal® West Pico Chemiluminescent Substrate enhanced chemiluminescence kit and KODAK Gel Logic 1500 Imaging System were used.

[³H]-AEA uptake assay in SZ95 sebocytes

The cellular uptake of AEA in SZ95 sebocytes was measured by using an established multi-phase assay protocol. Samples were incubated with either vehicle control, or the reference AEA uptake inhibitor, UCM707 (10 µM) in 500 µl RPMI medium at 37°C in AquaSil™ silanized screw-cap Eppendorf tubes. Afterwards a mixture of [ethanolamine-1-³H]AEA (0.5 nM) and unlabelled AEA (final 100 nM) was added to them and they were further incubated for 15 min. To stop the uptake process, the samples were placed on ice following a rapid centrifugation at 800xg for 5 min at 4°C. The supernatants were collected separately and the cell pellets were washed and resuspended using 500 µl ice-cold PBS containing 1 % BSA (fatty acid-free). The washing solution was also kept, and after phase separation (1:1 CHCl₃:MeOH, ice cold, 1 ml, 10000xg), was mixed with the organic phase, extracted from the primary supernatant resulting as the extracellular phase. The aqueous phase was collected apart. The cell pellet was

resuspended in 250 μ l PBS. All phases were transferred into scintillation tubes and mixed with Ultima Gold scintillation cocktail after liquid scintillation counting (LCS) using a Packard Tri-Carb 2100 TR beta counter.

Determination of eCB levels

In order to determine eCB levels, samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-APCI-MS). AEA, 2-AG, PEA, and OEA contents (pmol) were normalized per mg of extracted lipids.

Assessment of FAAH-activity

The assessment of FAAH activity in SZ95 cells was performed by our collaboration partners, by measuring the hydrolysis of radioactively labeled AEA.

Statistical analysis

MC data was analyzed using Prism 5.0 Software (GraphPad Prism Program), and the following tests were applied: Student's t-test for unpaired samples or Mann-Whitney U-test. p values <0.05 were regarded as significant.

Sebocytes data was analyzed by Origin Pro Plus 6.0 software, using Student's two tailed, two samples t-test and p<0.05 values were regarded as significant differences. Graphs were plotted using Origin Pro Plus 6.0 software.

RESULTS

1. eCBs limit excessive MC maturation and activation in human skin

Human CTS-MCs express CB₁

First, we aimed to investigate whether CB₁ is expressed on MCs that are directly located in the HFs' CTS. We found that around 75.5% of the CTS-MCs that showed c-Kit (MC growth factor [SCF] receptor) positivity, also expressed CB₁ (c-Kit/CB₁ double immunofluorescence staining). This was the case not only in organ-cultured human HFs, but also in human isolated scalp skin. In the next step, we incubated HFs with the derivate of CB₁ invers agonist AM251 ("Tocrifluor T1117") for 24 hrs. Despite its large size, this compound was able to directly bind to those CTS-MCs, which also showed co-expression of c-Kit marker, showing that Tocrifluor is appropriate for their functional examination.

CB₁ inhibition induces the number and degranulation of CTS-MCs

In the next step, we wished to examine how eCBs through CB₁ stimulation affect the biology of MCs. We showed that specified doses of AEA (non-selective CB₁ agonist, 30 μM) and ACEA (CB₁ selective agonist, 30 μM) had no significant impact on the detectable CTS-MCs' number and activity (Leder's esterase immunohistochemistry). By contrast, AM251 (CB₁ antagonist/inverse agonist, 1 μM) significantly increased both the number and degranulation rate of the cells. These effects, with the co-administration of AEA and/or ACEA, were completely abolished.

CB₁ inhibition induces the maturation, but didn't alert the proliferation of CTS-MCs

We next examined whether CB₁ inhibition also affected the number of the c-Kit⁺ CTS-MCs (c-Kit immunofluorescence staining). We found that in case of CB₁ inverse agonism, the number of c-Kit⁺ CTS-MCs was significantly elevated *in situ*. In addition, we have observed that the number of Ki67⁺/c-Kit⁺

(proliferating MCs) and TUNEL⁺/c-Kit⁺ (apoptotic MCs) cells did not significantly change after AM251 treatment (Ki67/c-Kit and TUNEL/c-Kit immunofluorescence staining).

From these results we assumed that the abrogation of homeostatic CB₁ signaling first may stimulate the differentiation of resident, immature c-Kit⁻ MC progenitors into c-Kit⁺ cells, rather than acting on the proliferation/apoptosis of mature ones.

We next investigated the expression of different antigens, which were characteristically identified on matured MCs. Using the inverse agonist AM251, just as in the aforementioned experiments, the number of tryptase⁺, chymase⁺ and FcεRIα⁺ MCs were significantly increased within the HFs. These results suggest that those MC progenitors, which first become c-Kit⁺, later differentiate into fully mature cells.

CB₁ gene knockdown is possible in organ-cultured human HFs

To further assess the effects observed previously, we attempted to abrogate the eCB signaling through CB₁ (standard siRNA technology). Based on the performed CB₁ immunohistochemistry, the gene silencing in human HFs was successful, since the receptor immunoreactivity was significantly reduced in the CB₁ siRNA treated HFs.

CB₁ gene silencing increased the number and activation of human CTS-MCs

With the help of the successful knockdown experiments, we were able to identify that the lower CB₁ gene level caused a significant increase in the number (Leder's esterase, c-Kit, and tryptase staining) and degranulation (Leder's esterase and tryptase staining) of the CTS-MCs, just like AM251. Similarly to the results of pharmacological studies, the number of the Ki67⁺ cells did not significantly change.

These results suggest that locally produced eCBs in the CTS of human HFs might continuously stimulate CB₁, through which they keep maturation and activation of MCs at a relatively low baseline level.

The excessive activation of MCs is controlled by CB₁

We next investigated whether CB₁ stimulation is able to counteract the MC activator effects of two classical MC secretagogues. We applied the endogen activator, substance P (100 pM) and the exogen activator, compound 48/80 (10 µg/ml), with the combination of AEA (30 µM) or ACEA (30 µM). We found that both eCBs were able to inhibit the degranulation, hence abrogating the effects of the two MC secretagogues (Leder's esterase quantitative immune histomorphometry). These results again drew the attention to the possible importance of ECS as an endogen, clinically relevant, negative regulator of MC activity.

The effects of CB₁ inverse agonism on MCs are partly SCF dependent

CB₁ is expressed along the epithelium of the human HFs, which area has also been shown to be an important source of SCF that has an impact on the maturation of MCs. Given that the functional type of CB₁ is also expressed within this HF area, we next investigated whether CB₁ stimulation/inhibition would be able to effect the intrafollicular expression of SCF.

We showed that AM251 (1 µM) significantly enhanced SCF not only *in situ* (SCF immunostaining), but also at the mRNA (Q-PCR) level in organ cultured human HFs. When this CB₁ inverse agonist was applied in combination with ACEA (30 µM), the previously observed SCF upregulation was abrogated.

Additionally, by analyzing primary ORS-KCs after AM251 treatment, we showed that the SCF concentration was also increased not only in the membrane of the cells (SCF immunstaining), but also in their culture medium (ELISA).

These results indicate that the abrogation of CB₁-mediated signaling might lead to the upregulation of SCF within the epithelium of human HFs. This suggests that ECS might be able to adjust the production of this important growth factor.

As a last step, we applied AM251 (1 µM) together with an SCF-neutralizing antibody (1 µg/ml) within the culture medium of HFs (24 hrs). When comparing

the number of c-Kit⁺ CTS-MCs between control and treated groups, we saw that the CB₁ inhibition caused - previously has already seen cell growth effect - was partially, although significantly reduced.

Degranulation and maturation of CTS-MCs are increased in CB₁ KO mice

To examine the role of CB₁ in an even more explicit model, we checked the *in vivo* relevance of our results by using CB₁ KO mice. In line with our organ cultured human HF data, within the skin of the CB₁^{-/-} animals, the number of c-Kit⁺ CTS-MCs showed CB₁ negativity. Comparing the subcutaneous CTS of the test animals, we found that not only the total number of MCs, but also their degranulation activity was significantly enhanced after CB₁ silencing (Leder's esterase immunohistochemistry).

The total number of MCs was also significantly increased within the CB₁ KO mice. Furthermore, just like in the case of HFs, there was no significant difference in the proliferation activity of MCs when comparing the number of the Ki67⁺/c-Kit⁺ (proliferating) CTS-MCs in the control and KO mice (Ki67/c-Kit immunofluorescence staining). This indicates that (just like *in situ*), CB₁ stimulation also *in vivo* primarily acts on the maturation and activation of murine MCs, rather than on their proliferation. Thus, to control unwanted maturation and activation of MCs, continuous CB₁ stimulation also *in vivo* is needed.

2. The eCB tone regulates the biology of human sebocytes

Major elements of ECS' enzyme apparatus are present in cultured human sebocytes, as well as in human SGs

We began our investigations by checking whether the most significant enzymes involved in the metabolism of AEA (NAPE-PLD and FAAH) and 2-AG (DAGL α and $-\beta$, as well as MAGL) are expressed in human, immortalized SZ95 sebocytes *in vitro*. Our results showed that this was the case for all aforementioned enzymes both at the mRNA (Q-PCR) and protein (Western blot) level, regardless of the cells confluency. To further strengthen these data, we also checked the expression of the above enzymes *in situ* in human skin. In line with our *in vitro* data, the expression of all above enzymes was also confirmed in human SGs *in situ*, except for DAGL α , whose expression was questionable, when comparing it with the neighboring endogenous positive control sweat glands.

eCB uptake of sebocytes can pharmacologically be inhibited

According to the above results, SGs might be able to produce and degrade eCBs. Since EMTs that can be involved in such processes, therefore we checked whether the uptake of *de novo* synthesized and released AEA by the putative EMT can pharmacologically be inhibited in human sebocytes. To do so, we have measured the uptake of radiolabeled [^3H]AEA into SZ95 cells. By using a selective EMT-inhibitor UCM707 (10 μM), our collaboration partners observed that the amount of intracellular [^3H]AEA significantly decreased, while its extracellular level increased, when comparing it to the vehicle control. These results indicate that in human sebocytes EMT is functionally active and can pharmacologically be inhibited.

It is important to mention that the non-specific inhibition of FAAH could also cause a decreased driving force for [^3H]AEA uptake. To exclude this possibility, we examined how the activity of FAAH changes in SZ95 sebocytes when using two different EMT-inhibitors (UCM707 and VDM11) that are widely

used to revoke cellular uptake of eCBs, and a reference FAAH-inhibitor, URB597.

Our results showed that the FAAH activity expressed by SZ95 sebocytes is constitutive, although its level is relatively low. The aforementioned inhibitors were not able to inhibit the function of FAAH in a relevant way.

Overall, our results convincingly suggest that VDM11 can block the eCB uptake in sebocytes and that this inhibition is likely to occur without influencing FAAH activity.

EMT of human sebocytes facilitates the transport of eCB ligands to their site of degradation

Next, we aimed to assess how the inhibition of eCB transport affect the concentration of eCB ligands. To do so, our collaboration partners treated SZ95 cells with vehicle or VDM11 for 24 hrs, and analyzed their eCB content by LC-APCI-MS. The results showed that the concentration of AEA and OEA were significantly increased in the VDM11-treated samples, while the enhancement of 2-AG and PEA levels was not significant.

Given that the inhibition of EMT reduces the degradation of eCBs, our findings suggest that the EMT inhibitor VDM11 might be able to enhance the local eCB tone of sebocytes.

EMT-inhibitors mimic lipogenic actions of direct eCB-treatment, whereas selective FAAH-inhibition does not affect SLP

Previously, we have shown that SLP of eCB-treated sebocytes dramatically elevated. Taking this into account, in the next step, we aimed to investigate whether and how the EMT-controlled eCB-tone plays a role in regulating above mentioned function of SGs. Our results showed that non-cytotoxic concentrations of EMT inhibitors, VDM11 (5-10 μM) and AM404 (10 μM) significantly increased SLP, although in a moderate way (Nile Red assay). Even though both above inhibitors could mimic the lipogenic actions of AEA, they could not

compete with the efficiency of direct AEA-treatment in elevating the SLP of the sebocytes.

Co-administration of VDM11 does not further increase lipogenic action of direct AEA treatment

Next, we investigated the combined effects of AEA and VDM11 on the SLP of human sebocytes. This experiment revealed that VDM11 was unable to further potentiate the AEA-induced SLP of human sebocytes, even at the highest investigated concentration (10 μ M). This suggests that the pro-lipogenic cannabinoid signaling by AEA (30 μ M) was most probably activated to the maximum extent possible.

Up to 10 μ M, VDM11 does not evoke apoptosis in human sebocytes

Right after the level of SLP increases, the sebocytes start differentiating that leads to their apoptotic cell death. We have demonstrated that the inhibition of EMT is able to mildly enhance the lipid synthesis of the sebocytes, therefore, we aimed to check whether it also affects the apoptotic/necrotic processes. We found that the most effective lipogenic concentration of VDM11 (10 μ M) slightly, although not significantly reduced the mitochondrial membrane potential of the cells. This shows that during the investigated period of time (48 hrs) obvious pro-apoptotic effects have not developed.

EMT inhibitor, VDM11 exhibits significant anti-inflammatory activity

A slight increase in the level of homeostatic SLP would be highly desirable when treating e.g. diseases associated with pathologically dry skin. Since skin dryness and cutaneous inflammation are often occur together, we wanted to examine whether and how VDM11 affects the immune properties of human sebocytes. With the application of VDM11 (10 μ M), the LPS-induced expression of IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α of human sebocytes was effectively suppressed at mRNA level (Q-PCR, 24 hrs). In addition to this, VDM11 was showed to significantly decrease the LPS-induced release of IL-6, and tended to

reduce the amount of IL-8 (ELISA, 24 hrs). The concentration of TNF α , IL-1 α , and IL-1 β did not or barely reached the detection limit of the respective assays.

DISCUSSION

The ECS is not only meaningful in the maintenance of skin homeostasis, but also in the development of different pathological skin conditions. Our research group has studied in detail the function and regulatory role of this “*c(ut)annabinoid*” system, with a special focus on the non-neuronal cells of skin. Within the frame of this thesis, we have investigated the effects of the ECS on human skin MCs, and further examined its role within the biology of sebocytes.

ECS is involved in the regulation of cutaneous MC biology

Overall, our results show for the first time that the activation/degranulation, as well as the maturation of normal human skin type of MCs from their resident progenitor cells is limited via CB₁-mediated signaling *in situ*. Based on our findings the homeostatic CB₁ signaling, presumably through both direct (primarily affecting MCs) and indirect (due to the regulation of SCF expression produced in the epithelium of HFs, secondary effect) ways, is able to inhibit the maturation and degranulation of MCs.

Indeed, when neutralizing SCF by a blocking antibody, the stimulatory effect of CB₁ inverse agonism on the maturation of MCs from their progeny was significantly decreased, although it wasn't completely blocked.

Overall, this data suggests that the eCB tone, through the homeostatic stimulation of CB₁ plays role in the adjustment of epithelium-produced SCF within the HF. With the inhibition of CB₁ signaling, this SCF-control might be discontinued. In this way, the increased level of this cytokine might induce then the intracutaneous maturation process of MCs from immature, resident progenitor cells within the CTS.

It is important to note that (although in the current study we did not specifically examined) our findings raise the possibility that CB₁ signaling not only in HFs, but also within the interfollicular epidermis might be able to regulate the expression of SCF. In addition to this, our findings highlight how significant

are the epithelial-mesenchymal interactions in the biology of human skin MCs and how relevant is the CTS of rodent vibrissae and human HFs for immature MC precursors. Furthermore, the results also suggest that the MC progenitors, whose differentiation is (also) under a continuous homeostatic eCB control, can mature "spontaneously" without any other impact.

CB₁ mediated signaling has already been shown to be able to regulate the proliferation and/or cell death of fast-proliferating hair matrix KCs and certain non-neuronal cell populations. In the present case however, it principally seems to influence the CTS-MCs' maturation and activation processes.

In line with our *in situ* HF data, the corresponding *in vivo* murine skin results further testify not only the importance of CB₁ signaling regarding the regulation of skin MCs, but also how substantial and relevant the use of human HF organ-culture is. It provides an unconventional, and also a physiologically and clinically relevant model system for investigating the biology of primary skin MCs *in situ*. In addition, it is also relatively cheap and easily accessible. Besides the possibility to evaluate the seen effects in both qualitative and quantitative manner, HF organ culture also allowed us to perform mechanistically informative methods (*in situ* gene silencing, blocking antibodies), which significantly widened the range of *in situ* experimental methods. Based on our results, this model system well complements the already available methods for studying MCs, e.g. in allergic and chronic inflammatory skin disorders.

In order to better understand the possible link between ECS and MCs, further questions beyond the ones addressed in the scope of this dissertation need to be answered. For example, why high number of MCs are located in organs/tissues of the body (e.g., skin, mucous membranes of respiratory tract), which are in direct contact with the external environment? What are their exact functions under physiological (and not inflammatory) conditions? However, it is certain that the above described CB₁ mediated regulation can be very well a valuable therapeutic tool for disorders associated with excessive MC number and

activation (e.g., allergic diseases, atopic dermatitis, mastocytosis, and MC associated tumors).

Effects of the modulation of eCB tone in human sebocytes

To the best of our knowledge, our results show for the first time that major enzymes of the ECS (i.e., NAPE-PLD, DAGL α and $-\beta$, MAGL, as well as FAAH) are expressed not only in human sebocyte cell culture, but also in SGs of the human skin; however, the *in situ* expression of DAGL α showed dubious immunostaining pattern (especially when comparing its staining expression with the neighboring endogenous positive control sweat glands).

Moreover, we also demonstrated that eCB transport is functionally active and can pharmacologically be inhibited in human sebocytes. Using VDM11, we showed that the EMT plays a role in the degradation of eCB and eCB-like molecules within these cells.

We subsequently investigated the effects of different EMT inhibitors on the viability, lipid synthesis, and immune responses of human sebocytes. We determined that non-cytotoxic concentrations of VDM11 and AM404 significantly increased the SLP of the cells, although this elevation was significantly lower compared to the effect of direct-AEA treatment.

Finally, VDM11 also suppressed the LPS-induced pro-inflammatory cytokine expression of the cells. This result is remarkable in particular in the light of recent data according to which cytokines released by SGs (e.g., IL-6) can help the differentiation of CD4⁺/CD45RA⁺ naïve T cells into T helper (Th) 17 cells. Thus a change in the cytokine production caused by abnormal sebocyte functions can play an important role in the development of diseases associated with Th17-driven inflammation, e.g., psoriasis.

All these results (especially in the light of the above described results on MCs) suggest that the increase of eCB tone caused by the pharmacological inhibition of eCB degradation might be a possible therapeutic strategy in order to treat skin disorders associated with skin dryness and inflammation.

SUMMARY

The endocannabinoid system (ECS) has been shown to be important in the regulation of numerous physiological and pathological processes in the human skin. In this thesis we have investigated the role of endocannabinoid (eCB) tone in the biology of selected non-neuronal human skin cells, more specifically in skin mast cells (MCs) and sebocytes.

We found that human MCs express cannabinoid receptor type 1 (CB₁). Under *in situ* condition, by blocking the homeostatic CB₁ signaling, the inverse agonist AM251 was able to increase not only the activation of human skin MCs, but also their maturation from resident progenitor cells. These effects (at least partially) have also emerged in an indirect way by affecting the expression of stem cell factor in the epithelium of the human hair follicles.

Taken together, CB₁ is an important, homeostatic regulator of MC maturation and activation, thus the stimulation of this receptor might be a promising strategy in the future management of allergic and inflammatory skin diseases associated with excessive MC number and activation.

Based on the results of our experiments, we shown that the most important eCB metabolic enzymes (NAPE-PLD, DAGL β , FAAH, and MAGL) are present both in SZ95 cells and in sebaceous glands. Furthermore, we have also seen that the anandamide-uptake inhibitor VDM11 moderately elevated the concentration of different eCB ligands and also the sebaceous lipid production of SZ95 cells. In addition, VDM11 could also prevent certain actions of lipopolysaccharide-induced pro-inflammation.

Collectively, our results suggest that eCB transport inhibitors might possible be advantageous in treating cutaneous diseases associated with inflammation and skin dryness.

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LIST OF PUBLICATIONS



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