

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

Role of adipokines in sebaceous glands biology

by Dóra Kovács

Supervisor: Dániel Törőcsik MD, PhD



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By **Dóra Kovács**, biologist MSc

Supervisor: Dániel Törőcsik, MD, PhD

Doctoral School of Health Sciences, University of Debrecen

Head of the **Examination Committee**: Margit Balázs, PhD, DSc

Members of the Examination Committee: Péter Holló, MD, PhD

Béla Nagy, MD, PhD

The examination takes place at Department of Dermatology, Faculty of Medicine, University of Debrecen, 9th of May, 2016 at 11:00 a.m

Head of the **Defense Committee**: Margit Balázs, PhD, DSc

Reviewers:

Eszter Baltás, MD, PhD

Bálint László Bálint, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 9th of May, 2016 13:00 p.m

INTRODUCTION

Sebaceous glands (SGs) form the pilosebaceous unit together with hair follicles. Their most apparent function is to secrete sebum which is a mixture of neutral lipids, mostly synthesized *de novo* by SGs. The primary function of sebum is to coat the hair as a hydrophobic protection and to insulate heat in wildlife. Moreover, sebum also transports fat-soluble antioxidants to and from the skin surface and displays a natural photo-protective activity. Recently, several other functions have been suggested for sebaceous glands via their ubiquitous production of antibacterial peptides (human- β -defensins), pro-inflammatory cytokines (interleukin [IL] -1 and 6, tumor necrosis factor- α [TNF- α]), chemokines (IL-8), periglandular peptides and neuropeptides which can all be further induced in the presence of various stimuli such as bacterial compounds. Another interesting feature of sebocytes is that they link lipid metabolism and inflammation at a cellular level, giving rise to many intriguing hypothesis on their role in several (patho)physiological conditions. This dual feature largely resembles adipocytes that are the prime examples for lipid metabolizing cells which in response to various stimuli produce inflammatory mediators (adipokines), such as adiponectin, IL-6, resistin, leptin, serpin E1, visfatin, apelin, chemerin, retinol binding protein 4 (RBP4) and monocyte chemoattractant protein-1 (MCP-1).

Lipids produced by the sebaceous glands

Sebocytes synthesise and accumulate lipids in cytoplasmic droplets that are later secreted via a holocrine mechanism with a complete disintegration of the glandular cells into the follicular duct of the pilosebaceous unit. Sebaceous lipids are well known to contribute to the integrity of the skin barrier, but also have pro- and anti-inflammatory properties, as well as exerting antibacterial effects. The major components are cholesterol, cholesteryl esters, squalene, fatty acids, di- and triglycerides, and wax esters.

Cholesterol, a component of cellular membranes, makes up ~2% of sebaceous gland lipids, while squalene, the linear intermediate in cholesterol biosynthesis, not found in the internal organs or among other epidermal surface lipids accounts for 12% of the lipid composition of sebum. Wax esters are also unique to the sebum accounting for ~25% of sebaceous gland lipids, being important for the survival of the sebaceous gland. The most abundant fatty acids in human sebum are sapienic and sebaleic acids and are exclusively present in humans but not in the sebum of animals.

Sebaceous glands contribute to inflammation

Sebaceous glands have several pattern recognition receptors (e.g. Toll-like receptor [TLR] 2, 4 and 6) whose activation results in the increased production of pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α), chemokines (IL-8), antimicrobial lipids and peptides as well as periglandular peptides and neuropeptides. Moreover, sebaceous glands utilize also their intensive lipid production to establish an inflammatory response via various inflammatory mediators such as leukotrienes (LTs), prostaglandins (PGs) and 15-hydroxyeicosatetraenoic acid.

Pathophysiology of sebaceous glands

Acne vulgaris

Acne vulgaris is one of the most common cutaneous disorders, manifested by comedones, papules, pustules, and cysts in sebaceous gland rich areas of the body such as the face, neck and back. Several hypothesis put *Propionibacterium acnes* (*P. acnes*) in the focus by suggesting that it drives hyperkeratinisation, and thus the accumulated keratinocytes plug the opening of the pilosebaceous unit resulting in the “explosion” of the obstructed pilosebaceous unit as the initiating step of acne development. Whether *P. acnes* has also a direct effect or not

on the SGs in this cascade still needs to be elucidated, however different bacterial compounds definitely possess the ability to activate sebocytes and thus integrate such pathways into the pathogenesis of acne.

Regarding the involvement of sebocytes, another interesting start point is provided with the findings that both the amount and the composition of the sebum changed in acne patients. Increased levels of squalene peroxides, decreased levels of linoleic acid and vitamin E and the significant differences in the ratio of saturated and unsaturated fatty acids all support that sebocytes are not just “innocent bystanders” but are also active players in acne.

Similarities between the sebaceous gland and adipose tissue

One of the central and most interesting questions in sebocyte biology is to compare their function and the related molecular programs behind with that of adipocytes, which also connect lipid metabolism with inflammation at a cellular level. The analogy has been previously suggested on the level of transcriptional factors, such as the CCAAT/enhancer binding proteins (c/EBPs) and the peroxisome proliferator-activated receptors (PPARs) that are pivotal in adipocyte differentiation and function and were found also in the sebaceous gland. *In vivo* and *in vitro* research have proven that c/EBP- α and - β localizes to the basal layer of the sebaceous gland which suggests that they have an important role in the initial phase of differentiation. While of the PPARs (α , β/δ , γ), the γ isoform showed the highest levels in the terminal differentiation of sebocytes. Further investigations also identified several other transcription factors such as liver X receptor (LXR), galectin-12 and sterol regulatory element-binding protein-1c (SREBP-1c) with functions yet to be verified how they could be involved in inducing further similarities between adipocytes and sebocytes.

Adipokines

White adipose tissue is the main site of energy and nutrient storage in mammals. Being a highly dynamic endocrine organ, it is involved in a wide range of (pato)physiological and metabolic processes, in which producing and releasing diverse secretory proteins, the so called adipokines into the microenvironment as well as into the circulation. Adipokines are small molecular weight, bioactive proteins, produced and secreted primarily by the adipocytes. Members include classical cytokines, growth factors, and various proteins that are involved in a wide range of biological processes from the regulation of blood pressure, lipid metabolism, vascular and glucose homeostasis to angiogenesis. The most widely used classification however puts their inflammatory properties in the centre as discussed in the following part.

Cellular and systemic effects of pro-inflammatory adipokines

IL-6

IL-6 is the most well characterized pro-inflammatory cytokine/adipokine, produced by a wide variety of cells besides adipocytes such as monocytes, macrophages, endothelial cells, fibroblasts and sebocytes. It acts in an endocrine, paracrine and autocrine manner on different target cells and is considered to be pivotal in the regulation of immune response during infectious, chronic inflammatory and autoimmune disorders. Clinically, increased plasma IL-6 levels were observed in obese subjects and weight loss leads to a reduction in IL-6 levels.

Leptin

Although via signalling in the hypothalamus, leptin regulates appetite and body weight, this adipokine is more than a sensor of metabolic homeostasis: leptin activates signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa-B (NF- κ B) transcriptional factors in a wide repertoire of cell types, and also increases the production of pro-inflammatory cytokines (e.g. IL-6, IL-12 and TNF- α). Moreover, its levels in the blood

positively correlate with BMI and the size of the adipose tissue also making it to be an important player in obesity related systemic inflammation

Resistin

Although identified in the adipose tissue, recent data suggest that monocytes and macrophages are also important sources of resistin. The regulatory mechanisms behind its production, namely that it's expression can be induced by several pro-inflammatory cytokines (e.g. IL-6 and TNF- α) suggest that it might be involved in complex inflammatory cascades. Moreover, resistin deficient *ob/ob* mice are characterized by massive obesity and represent improved glucose tolerance and insulin sensitivity.

Plasminogen activator inhibitor-1 (PAI-1; serpin E1)

Serpin E1 plays an important role in the maintenance of vascular haemostasis, inhibiting the activation of plasminogen. Interestingly, elevated serpin E1 levels can be observed also in wound healing and melanoma.

Visfatin

Visfatin plays an important role not only in the nicotinamide adenine dinucleotide (NAD) biosynthetic pathway but also in insulin secretion by pancreatic β -cells. It is one of the most important inflammatory mediators in foam cell macrophages within atherosclerotic lesions.

Apelin

Apelin is the endogenous ligand of orphan G protein-coupled receptor APJ, and plays an important role in the regulation of blood pressure, angiogenesis and fluid homeostasis. Its secretion is in strong connection with inflammatory processes mediated by TNF- α .

Chemerin (tazarotone-induced gene 2; retinoic acid receptor responder 2 [RARRES2])

Chemerin mediates inflammation with a strong chemoattractant property that induces infiltration of macrophages, immature dendritic cells (DCs) and natural killer cells in

inflammatory diseases. It's levels positively correlate with BMI, fasting glucose and inflammatory cytokines.

Retinol binding protein 4 (RBP4)

RBP4, with a primary role in the retinol (vitamin A) circulation throughout the body is secreted not only by hepatocytes but also adipocytes and macrophages. Moreover, RBP4 is also an important factor in the regulation of glucose homeostasis since increased serum RBP4 levels were found to be associated with metabolic syndrome and increased BMI.

Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 plays an important role in the infiltration of immune cells, thereby it is essential for the development of an inflammatory microenvironment widely observed in the adipose tissue. However, not only adipocytes are capable of producing MCP-1, but several other immune cells, connective tissue and malignant tumours, altogether making MCP-1 one of the primary chemoattractant proteins in various pathological conditions.

Cellular and systemic effects of anti-inflammatory adiponectin

Adiponectin is the most widely studied adipokine with anti-inflammatory properties, as it can initiate the secretion of anti-inflammatory cytokines (e.g. IL-10 and IL-1 receptor antagonist) in various cell types of the immune system. Decreased levels of adiponectin were observed in obesity, type 2 diabetes and metabolic syndrome further suggesting a potent anti-inflammatory role for adiponectin also in a system wide context.

Role of adipokines in skin biology

In analogy to the established role of adipokines as primary mediators of the malicious consequences of adipose tissue associated chronic low-grade inflammation, recent findings have shown that they may as well be equally important regulatory factors in inflammatory

conditions of the skin. This hypothesis is largely based on the marked correlation between circulating adipokine levels and the onset and severity of numerous dermatological diseases. Patients suffering from psoriasis exhibit elevated levels of TNF- α , IL-6, leptin and resistin, whereas the level of circulating adiponectin decreases with the worsening of the symptoms. Atopic dermatitis, on the other hand, is characterized by elevated levels of serum leptin, resistin and apelin and decreased levels of visfatin. Adipokines also have been intensively investigated in acne, however the results are vastly contradicting. While some studies found increased levels of leptin in acne, others suggested a decrease or no change at all.

Despite the clear indication that adipokine research has a place in dermatology, still very limited information is available regarding their role in skin (patho)physiology, with their possible source and target cells. We, therefore, set out to profile the adipokines produced in the sebaceous glands, asking whether these cells are capable of initiating and regulating skin inflammatory processes through the production of adipokines.

OBJECTIVES

Based on the already characterized similarities between adipocytes and sebocytes, it was reasonable to suppose that adipokines might also be produced and secreted by sebocytes and these proteins could be integrated into sebaceous gland biology.

Our work aims to identify sebaceous glands as possible sources of adipokines within the human skin. The intention of this work is to understand the role of adipokines in sebocyte cells biology in particular for the leptin. For that we investigated the role of leptin signalling in sebaceous lipid metabolism and in the induction of inflammatory enzymes and cytokines.

In particular the objectives of this work are:

1. to identify adipokines in sebaceous glands of healthy and various disease-affected skin samples (acne, rosacea, melanoma and psoriasis);
2. to confirm the presence of the detected adipokines in the human SZ95 sebaceous gland cell line;
3. to characterize their expression and secretion patterns under different stimuli by using TLR2 and 4 activators, or by 13-*cis* retinoic acid (13CRA);
4. to provide evidence that the functionally active full-length form of the leptin receptor (Ob-Rb) is expressed in human sebaceous glands and in cultured human SZ95 sebocytes;
5. to investigate the effect of leptin on SZ95 sebocytes regarding lipogenesis and inflammation.

MATERIALS AND METHODS

Histological samples

Anonymized frozen or formalin-fixed and paraffin embedded (FFPE) sections of human skin from the tissue archive of the Department of Dermatology, University of Debrecen were acquired after the approval of the Regional and Institutional Ethics Committee, University of Debrecen. At least 3 different FFPE samples of each condition (healthy, acne vulgaris, rosacea, melanoma, psoriasis skin samples) were evaluated. Appropriate positive controls were used according to the manufacturer's instructions.

Cell culture and treatments

The SZ95 immortalized human sebaceous gland cell line was cultured at 37°C in a humidified atmosphere containing 5% (v/v) CO₂ in Sebomed Basal Medium® (Biochrom), supplemented with 10% fetal bovine serum ([FBS], Biowest), 1 mM CaCl₂, 1% penicillin/streptomycin (Sigma-Aldrich) and 5 ng/ml epidermal growth factor ([EGF], Sigma-Aldrich). 1 µg/ml LPS (TLR4-activator; derived from *Escherichia coli*; Sigma-Aldrich), 1 µg/ml PAM3CSK4 (TLR1/2-activator; InvivoGen), 1µM 13CRA (kind gift from BASF AG, Ludwigshafen, Germany) and 20 nM human recombinant leptin (R&D Systems) were used for treatments.

Proliferation assay

SZ95 sebocyte proliferation was measured by the 4-methylumbelliferyl heptanoate (MUH) fluorescence assay. Cells were plated and their proliferation was assessed on day 6 after 1µM 13CRA or equivalent amount of DMSO treatment. On the day of evaluation cells were washed and 100 µl of 100 µg/ml MUH was added to each well. After incubation, fluorescence

was detected by using 355 nm excitation and 480 nm emission filters (Synergy H1 microplate reader, BioTek).

Immunohistochemistry

Frozen sections were fixed in ice-cold acetone and dried at room temperature. Paraffin sections were deparaffinised, rehydrated and endogen peroxidases were blocked with 3% H₂O₂. For antigen retrieving, slides were treated with Tris-EDTA buffer and nonspecific binding was blocked with 5% bovine serum albumin (BSA). For immunohistochemical staining, the following antibodies were used: full length form leptin receptor (Ob-Rb; Abbiotec), adiponectin, chemerin, resistin, visfatin (Santa Cruz Biotechnology), leptin, IL-6 (LifeSpan BioSciences), apelin, MCP-1 (Abcam), RBP4 and serpin E1 (Thermo Fisher Scientific). HRP-conjugated secondary antibodies were used in accordance with the manufacturer's instructions (SuperSensitive One-step Polymer-HRP Detection System). Immunoreaction was visualized by Vector VIP Kit (Vector Labs). Sections were counterstained with methylene green. Images were acquired with a Leica DM2000 LED microscope (Leica Microsystems).

Immunocytochemistry

SZ95 sebocytes were grown on Superfrost Ultra plus adhesion slides (Thermo Fisher Scientific) and fixed in 1% acetic acid in 96% ethanol. Nonspecific binding was blocked with 5% normal human serum. For immunocytochemistry, the following antibodies were used: full length form leptin receptor (Ob-Rb; Abbiotec), adiponectin, chemerin, resistin, visfatin (Santa Cruz Biotechnology), leptin, IL-6 (LifeSpan BioSciences), apelin, MCP-1 (Abcam), RBP4 and serpin E1 (Thermo Fisher Scientific). Goat anti-rabbit IgG Alexa Fluor 555 (Molecular Probes), Dylight 549 goat anti-rabbit IgG and Dylight 549 horse anti-mouse IgG (Vector

Labs.) secondary antibodies were used in accordance with the manufacturer's instructions. Slides were mounted with Vectashield mounting medium with DAPI (Vector Labs). Images were acquired by LSM 700 confocal laser scanning microscope (Zeiss Oberkochen) equipped with Plan-Apochromat 63X/1.40 oil objective and solid-state lasers, connected to a CCD IMAC camera (Sony).

Lipid analyses

Briefly, cell samples were collected after leptin treatment and compared to controls using various techniques such as high performance thin layer chromatography (HPTLC), gas-chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography coupled with flight mass spectrometer mass detector (HPLC-ToF/MS).

HPTLC silica gel 60 plates (Merck) were used as the stationary phase. 20 μ L of the concentrated lipid extract from each sample were loaded onto the TLC plate. The separation was achieved by running the HPTLC with two consecutive eluent systems. The plates were charred at 120° C for 60 minutes to develop grey-to-black spots. Measurements of densitometry were performed to assess spot intensities. GC-MS was used for the analyses of Vitamin E, cholesterol and squalene. Briefly, following derivatization of Vitamin E and cholesterol with BSTFA to obtain their volatile trimethylsilyl derivatives, the compounds were separated in the gaseous phase with a Rtx-5MS Crossbond® 5% diphenyl/95% dimethyl polysiloxane column (Restek Corporation). Detection was in single ion monitoring of base and qualifier mass peaks of the respective target compound. The analytes were quantified against calibration curves of the reference standard compounds with the d6-CH internal standard. Quantitative determination of free fatty acids was performed with HPLC-ToF/MS (Agilent Technologies). Elution was performed with the binary system composed by A: water-0.1% formic acid-3% acetonitrile and B: acetonitrile-3% isopropyl alcohol. Compounds

ionized in the electrospray ion source (ESI) were acquired in the negative ion mode. Scan detection was performed in the 80 - 400 mass range. Extracted ion chromatograms were obtained by extracting the exact mass of the [M-H]⁻ ion of the target compounds and the internal standard (d4-LA). The analytes were quantified against calibration curves of the reference standard compounds in the range of concentrations 0.625-20 μM with the d4-LA internal standard.

Lipid body detection and enumeration

Sebocytes were cultured on LabTek chamber slides (Thermo Scientific) and were treated with human recombinant leptin. Cells were fixed in 4% paraformaldehyde. After washing, slides were incubated with 10 μg/mL 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene 493/503 fluorescence lipid stain (BODIPY; Life Technologies). After incubation, images were acquired by Axioplan microscope (Carl Zeiss) equipped with selective filters and connected to a CCD IMAC camera (Sony).

Western blotting

Cells were treated with human recombinant leptin, harvested and lysed in RIPA buffer containing a phosphatase and protease inhibitor mix (aprotinin, leupeptin, pepstatin, bestatin) (Sigma Aldrich). Proteins (20 μg) were separated by electrophoresis using the appropriate polyacrylamide gel and transferred to nitrocellulose membrane (Bio-Rad). After blocking, membranes were probed with anti-OB (R&D Systems), anti-5-LOX (Cell Signalling), anti-COX-2 (Cell Signalling), anti-phospho-NF-κBp65 Ser536 (Cell Signalling), anti-phospho-STAT3 Tyr705 (R&D Systems) and anti-β-actin (Cell Signalling). The Ag-Ab complexes were labelled with appropriate HRP-conjugated secondary Abs (Bio-Rad) and visualized by Immobilon Western HRP Substrate kit (Millipore, Bedford, MA).

Determination of mRNA levels

SZ95 sebocytes were cultured in the presence of human recombinant leptin, TLR1/2 and 4 ligands (PAM3CSK4, LPS), 13CRA or vehicle control. Total RNA was isolated using TRI Reagent (MRC) according to the manufacturer's protocol and quantified by using NanoDrop 2000 (Thermo Fisher Scientific). Reverse transcription was performed using Superscript II reverse transcriptase and random primers (Invitrogen, Life Technologies), according to the manufacturer's protocol. Quantitative PCR was performed using real-time PCR (ABI PRISM 7900; Applied Biosystems) using specific primers and TaqMan assays (Applied Biosystems). Comparative Ct method was used to quantify transcripts and to normalize for Peptidylprolyl Isomerase A ([PPIA] Cyclophilin A).

For RNA sequencing (RNA-Seq) and library preparation cDNA was generated from 1 µg total RNA using TruSeq RNA Sample Preparation Kit (Illumina) according to the manufacturer's protocol. Briefly, poly-A tailed RNAs were purified by oligodT-conjugated magnetic beads and fragmented, then 1st strand cDNA was transcribed using random primers and SuperScript II reverse transcriptase (Life Technologies). Second strand cDNA was synthesized, double stranded cDNA end was repaired and 3' ends were adenylated. After adapter ligation enrichment PCR was performed to amplify adapter ligated cDNA fragments. Fragment size distribution and molarity of libraries were checked on Agilent BioAnalyzer DNA1000 chip (Agilent Technologies). Concentration of RNA-Seq libraries were diluted to 10 nM and 5 libraries were pooled together before sequencing. Single read 50 bp sequencing run was performed on Illumina HiScan SQ instrument (Illumina). Each library pool was sequenced in one lane of sequencing flow cell, 16-18 million reads per sample was obtained.

Analysis of RNA-Seq data

CASAVA software (Illumina) was used for pass filtering and demultiplexing process. Sequenced reads were aligned to Human Genome v19 using TopHat algorithm and bam files were generated. Further statistical analyses were executed using NGS module of GeneSpring 12.6 software (Agilent Technologies). Relative mRNA expression levels were calculated with DESeq algorithm.

ELISA measurements

SZ95 supernatants and cell lysates were collected after human recombinant leptin, LPS, PAM3CSK4, 13CRA or DMSO treatment. Cells were lysed in distilled water containing 0.1% Triton-X. Levels of adiponectin were probed with both ELISA Development Kit and Quantikine ELISA Kit (R&D Systems). For IL-6, IL-8, leptin, serpin E1 and resistin ELISA Development Kit were used while visfatin levels were measured with ELISA Assay Kit (Biovision) according to the manufacturers' instructions.

Statistical analyses

All data are presented as mean \pm SD. In qRT-PCR experiments, the mean and SD were calculated for both the normalized and the normalizer values. To incorporate the random errors of the measurements, we used the propagation of errors to determine the SD of the normalized values. We made at least three biologic replicates for all experiments. Unpaired t-test, one-way ANOVA statistic test and Tukey post-hoc test were used in the analysis of ELISA data. For qRT-PCR experiments and proliferation assay paired t-test was used, furthermore, results for RNA-sequencing were analysed by unpaired t-test. Differences by $p < 0.05$ values were considered statistically significant.

RESULTS

Human sebaceous glands differentially express adipokines

First, we investigated the expression of the major adipokines in human sebaceous glands of healthy skin samples as well as different pathological conditions (acne, rosacea, melanoma, psoriasis). By using immunohistochemical detection with specific antibodies, the pro-inflammatory adipokines IL-6, resistin, leptin, serpin E1 and visfatin, and the anti-inflammatory adiponectin were found to be expressed in all of the tested samples, while apelin, chemerin, RBP4 and MCP-1 could not be detected.

These data show that SGs express a characteristic set of adipokines regardless of the disease background, suggesting that functional differences with a pathological relevance might be related to their secretion.

Human SZ95 sebocytes express multiple adipokines

To further characterize the adipokine expression and secretion, and to define possible mechanisms regulating these processes we used the most accepted *in vitro* human sebocyte model, the SZ95 sebocyte cell line. Both the immunostaining and the ELISA data clearly showed that SZ95 cells expressed IL-6, resistin, leptin, serpin E1 and visfatin. Interestingly, the fluorescent image analysis also revealed that IL-6, resistin, serpine E1 and visfatin were detected in both the nucleus as well as in the cytoplasm while leptin localized mostly if not exclusively only to the cytoplasm.

SZ95 sebocytes differentially secrete adipokines

To see if SZ95 sebocytes not only produce but also secrete the detected adipokines, protein levels were measured by ELISA in the supernatants of untreated SZ95 sebocytes. IL-6, leptin, serpin E1, and visfatin were present in the supernatants, whereas resistin and adiponectin could not be detected. Furthermore, the amounts and ratios of the various adipokines in the supernatants were comparable to the measured levels in the cell lysates, except for leptin that despite showing relatively high protein levels in cell lysates, barely reached the detection limit in the supernatant of the SZ95 sebocytes.

Expression and secretion of adipokines is differentially regulated by various stimuli

To address whether expression and secretion of the measured adipokines can be induced and/or regulated by stimuli leading to inflammation or differentiation, the effects of TLR1/2 (PAM3CSK4) and TLR4 (LPS) activators as well as 13CRA (an anti-acne agent) were tested. The changes in the mRNA expression levels of the SZ95 sebocytes were assessed by RNA sequencing and by ELISA measurements from their supernatants. Based on our mRNA data, the TLR activators led to an induction in the mRNA levels of pro-inflammatory adipokines (leptin, serpin E1 and visfatin), showing the same expression pattern as observed in the case of IL-6, a cytokine known to be induced by TLR1/2 and 4 activation. In contrast, the mRNA expression of the anti-inflammatory adiponectin was down-regulated via the TLR1/2 pathway and also by 13CRA.

For further addressing the changes in the secretion of these adipokines, supernatants from the treated SZ95 sebocytes were used for ELISA measurements. Resistin secretion was not affected by any of the applied stimuli. On the other hand, the levels of leptin, serpin E1 and visfatin increased similarly to IL-6 upon treatment with the applied TLR activators. Interestingly, SZ95 sebocytes only responded to the potent anti-acne agent 13CRA by

enhancement of the expression and secretion of leptin and with a reduction in the adiponectin mRNA levels. In line with the previous ELISA findings, however, the protein levels of adiponectin could not be assessed.

These data altogether show that sebocytes differentially express and secrete adipokines in response to various stimuli.

Ob-Rb is expressed in human sebaceous glands

Since the production of leptin was the most sensitive in the applied conditions, we diverted our attention to the in depth analysis of leptin. To address if leptin has also an affect of SGs and therefore could form a signalling loop, we first performed immunohistochemical analysis using a specific antibody for the Ob-Rb receptor subtype, which is responsible for active signal transduction. We found that Ob-Rb was expressed in the sebaceous glands of both the healthy, as well as the acne-involved skin.

Next, we turned our attention to human SZ95 sebocytes and confirmed the expression of Ob-Rb mRNA and protein levels using qRT-PCR, Western blotting and immunocytochemical analyses. These findings confirmed SZ95 sebocytes to be a valuable model for further investigations on addressing the role of leptin in sebocyte biology.

Leptin alters lipid metabolism in SZ95 sebocytes

The primary function of sebocytes is their potential to synthesize lipids, therefore we investigated the early and late effects of leptin on the lipid profile of leptin-treated SZ95 sebocytes, assessing the synthesis of major sebaceous lipids such as squalene (SQ), cholesterol (CH), triglycerides (TG), free fatty acids (FFA) and Vitamin E (VE). Firstly, HPTLC with densitometric analyses showed that TG levels were significantly elevated in response to leptin at 36h; in contrast, levels of SQ, FFAs and CH did not change significantly.

For a higher sensitivity and specificity, SQ and CH levels were simultaneously quantified by GC-MS. The extended analysis showed that while levels of SQ were not modified over time in control sebocytes, its concentration tended to increase in the leptin-treated cells. In contrast, the concentration of CH increased in a time-dependent manner in both the control and leptin-treated cells, and leptin treatment did not result in any significant change compared with the controls.

In order to investigate further the representative components of the human sebum such as different FFAs and TG lipid classes, samples were analysed by HPLC-ToF/MS and GC-MS. The evaluation of the summarized levels and calculated ratios of monounsaturated fatty acids (MUFAs) to saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs) to SFAs showed significant differences in the PUFA/SFA ratios and was close to significance regarding the MUFA/SFA ratios.

The change of the cell redox status consequent to activation of lipid synthesis has rarely been investigated in sebocytes. Thus, VE, which accounts for the major lipid scavenger of free radicals in the cell membrane, was also analysed. The concentration of VE was significantly lower in leptin-treated sebocytes at 36 h.

These data indicate that desaturation of FFAs is an early effect of leptin treatment, whereas appreciable induction of TG synthesis occurs at later time points. Moreover, a significant decrease in the VE levels indicated that leptin and its lipogenic effects could be associated with consumption of lipophilic antioxidants.

Leptin treatment leads to lipid body enlargement in SZ95 sebocytes

Sebocytes store and metabolize intracellular lipids in hydrophobic organelles called lipid bodies. In order to detect changes in the lipid body formation we used fluorescence microscopy to visualize the lipid droplets stained with a boron-dipyrromethene (BODIPY)

dye. We found that in the leptin-treated samples the number of cells with enlarged lipid droplets was increased when compared with the untreated controls. As BODIPY staining can selectively stain lipid droplets it also allows flow cytometry measurements for quantitative analyses. We confirmed that leptin treated SZ95 sebocytes exhibited an increased fluorescence intensity compared with control cells. Altogether, these results indicate that leptin-treated SZ95 sebocytes display an altered lipid droplet formation.

COX-2 and 5-LOX expression and inflammatory cytokine expression of SZ95 sebocytes is increased by leptin

To investigate the potential role of leptin in inflammation, we measured the protein levels of COX-2 and 5-LOX, the key enzymes in the production of inflammatory mediators in SZ95 sebocytes. Using western blot analysis we showed that the expression levels of COX-2 and 5-LOX were increased on leptin treatment compared with untreated cells.

Next, we determined the effect of leptin on the expression of IL-6 and IL-8, shown to be important in the inflammatory signalling of human sebocytes. In the presence of leptin the mRNA and protein levels of both IL-6 and IL-8 showed a marked upregulation compared with untreated cells. Similar to this, the secreted IL-6 and IL-8 proteins were also upregulated, as detected by ELISA measurements.

These data together show that SZ95 sebocytes respond to leptin stimulus with an enhanced expression of inflammatory enzymes and an increased production and secretion of pro-inflammatory cytokines.

Leptin activates the STAT3 and NF- κ B pathways in SZ95 sebocytes

The prime consequence of leptin binding to its receptor is the activation of the STAT3 pathway leading to STAT3 phosphorylation, while on the other hand NF- κ B also serves as a

downstream signalling mediator of leptin. To explore the mechanisms by which leptin could increase the inflammatory response of SZ95 sebocytes, we examined its effect on STAT3 and NF- κ B activation. We measured the phosphorylation of STAT3 and the NF- κ B p65 subunit in the control and leptin-treated SZ95 sebocytes. Upon leptin treatment, both STAT3 and p65 phosphorylation increased, showing that this treatment resulted in increased STAT3 and NF- κ B activity.

These data suggest that leptin contributes to inflammation through the induction of STAT3 and NF- κ B signalling in SZ95 sebocytes.

DISCUSSION

Intensive research in the past decade demonstrated that sebocytes link lipid metabolism and inflammation at a cellular level, giving rise to many intriguing hypothesis on their role in several (patho)physiological conditions. This dual feature largely resembles adipocytes that are the prime examples for lipid metabolizing cells which in response to various stimuli produce inflammatory mediators, the so called adipokines such as adiponectin, IL-6, resistin, leptin, serpin E1, visfatin, apelin, chemerin, RBP4 and MCP-1. Probably the most extensively studied role of these proteins are their contribution to chronic systemic inflammation observed in obese patients where the elevated serum levels correlate with increased amounts of adipose tissue. Based on the fact that sebocytes with an active lipid metabolism, inflammatory activity and the expression of various lipogenic factors resemble adipocytes in great detail, in our work we aimed to identify sebocytes as a possible source for adipokines within the skin and to define possible regulatory mechanisms behind their production and secretion. Histochemical staining of skin samples originating from healthy skin or from various dermatological diseases revealed that SGs expressed adiponectin, IL-6, resistin , leptin, serpin E1 and visfatin, while apelin, chemerin, RBP4 and MCP-1 could not be detected in SGs in any of the examined samples. Considering the fact that the presence of adipokines in the SGs is independent of the examined disease, their (patho)physiological relevance, is most likely associated with the ability of sebocytes to secrete these proteins in a stimulus dependent manner. Extending our studies with *in vitro* experiments using the SZ95 sebaceous gland cell line we confirmed that sebocytes indeed are capable to exert a stimulus specific regulation in the expression and secretion of these proteins. In response to pro-inflammatory stimuli such as the TLR2 and 4 activators, representing prominent initiators of inflammation during acne pathogenesis, only IL-6 was shown to be induced in sebocytes so far. The observed similar expression patterns in the induction and secretion of leptin, serpin E1 and visfatin with that of

IL-6 defines, therefore, additional important targets in the pathogenesis of acne. We also tested 13CRA, the most widely used anti-acne agent, on sebocytes. Our results showing increased leptin and decreased adiponectin expression and no influence on other inflammatory adipokines, let us propose that 13CRA may affect the differentiation/metabolism of sebocytes via adipokines, as also observed in rat adipose tissue. Moreover, based on the fluorescent image analysis, their accumulation not just in the cytoplasm but also within the nucleus suggested that adipokines might even play a role in so far undefined intranuclear functions.

The high levels of visfatin in sebocytes that was further increased upon different TLR stimuli was an interesting finding in our study. Besides its inflammatory role in diabetes and metabolic syndrome, visfatin was reported to be strongly expressed within lipid-loaded macrophages of atherosclerotic lesions. Regarding skin biology, visfatin was suggested to potentiate the development of psoriasis via stimulating human keratinocytes to an enhanced production of the antimicrobial peptides. The detected high levels of visfatin fully support the pro-inflammatory role of SGs and their possible regulatory effect on the dermal milieu that should be further investigated in disease settings.

Serpin E1, which is a possible link between obesity and the increased risk for thrombosis, was found to be involved also in wound healing and in the skin fibrosis. Moreover, serpin E1 was detected in various cancers including melanoma, where it was considered to be a marker of poor prognosis that may impact invasion and metastatic spread due to its involvement in tissue matrix remodelling. Therefore, the detection of serpin E1 in SGs highlights another interesting aspect of the SGs related adipokines: a possible contribution to extracellular matrix remodelling.

Adiponectin is the prime adipokine identified as an anti-inflammatory protein. Regarding its role in skin biology, adiponectin suppressed the secretion of pro-inflammatory cytokines like TNF- α , IL-17, IL-22, and IFN- γ in keratinocytes and was involved in cutaneous wound

healing. Importantly, adiponectin also promoted hair shaft elongation in cultured human hair follicles, suggesting that altered levels of adipokine derived from the cutaneous adipose tissue and also based on our *in vitro* data, could even affect hair growth. Our findings, that adiponectin could be also detected and regulated in human SGs, suggested that sebaceous glands could possess a unique ability within the human dermis to modify inflammation both in a pro- and an anti-inflammatory manner. Moreover could fulfil roles in various biological processes that were so far linked to the subcutaneous adipose tissue.

Leptin is an adipokine, secreted predominantly by adipocytes in response to increased lipid uptake. Besides its metabolic effect, leptin modulates immune responses in many different cell types: adipocytes, macrophages, DCs, T cells, B cells, NK cells, chondrocytes, fibroblasts and keratinocytes all seem to respond to leptin. Regarding its role in skin physiology, leptin was found to be an important player in skin regeneration and aging, as well as in hair growth. Our new findings that sebocytes not only respond to but also self-secrete leptin, encourage further studies to address how sebocyte derived leptin could contribute to the adipokine-mediated regulation of inflammatory and metabolic pathways within the skin. In our work evidence is provided, that besides the human epidermis and human follicular papilla cells, leptin receptor is also expressed in the human sebaceous glands. The expression of the Ob-Rb receptor was detectable both in control and in acne samples, suggesting that these cells can readily respond to leptin under different conditions. In order to investigate how leptin could alter lipogenesis and inflammation in human sebocytes, initially we confirmed the presence of Ob-Rb in SZ95 sebocytes, followed by a comprehensive analysis of the outcome of leptin stimulus, by measuring the changes in the lipid body formation, lipid profile and the pro-inflammatory responses with the possible signalling pathways behind it. Lipid body formation is a phenomenon widely observed in lipid-metabolizing cells. Using BODIPY fluorescent staining, we found that leptin treatment was associated with an enlargement of the lipid

droplets and increased fluorescence intensity within SZ95 sebocytes. Interestingly, the same phenomenon could be observed also in macrophages upon leptin treatment, pointing out that leptin could affect intracellular lipid storage and metabolism. In order to detect the changes in the levels of lipids that are representative human sebum components, we exploited additional lipidomic methods and provided evidence that leptin treatment altered lipogenesis in SZ95 sebocytes. Detecting a significant increase in the levels of TGs and the relative accumulation of different MUFAs and PUFAs supported our findings on lipid body enlargement as they were shown to promote triglyceride-enriched lipid body formation. Giving biological relevance to these findings, the importance of the unsaturated/saturated lipid ratio in the sebum was addressed in a previous publication, showing that a decrease in unsaturated FFA vs. saturated FFA levels in sebum, was predictive of the clinical improvement in acne, suggesting that the desaturation of sebaceous fatty acids may be involved in acne development. Interestingly, the authors also found that subjects on a low glycaemic load (LGL) diet showed a greater improvement, which correlated with the change in body mass and with the decrease in levels of unsaturated FFAs compared with the increase seen in the control group of patients not on an LGL diet. Concluding these findings, it was hypothesized that an LGL diet may affect sebum composition via undefined metabolic effects, in which leptin may be a key candidate according to our understanding. In our system, we provide evidence that leptin might be implicated into the pathophysiology of acne by inducing an ‘acne-like’ change in the sebaceous lipid profile via increasing the amounts of unsaturated FFAs, which largely resembles the changes in the composition of sebum found in patients with acne. Moreover, the decrease in the levels of the major sebum antioxidant vitamin E on leptin treatment, which is also a hallmark in patients with acne, further supports a possible role for leptin in acne. In addition, with further research aiming to integrate primarily adipogenic signalling cascades, such as the mammalian target of rapamycin (mTOR)-

mediated pathway, into the sebocyte-leptin – and other adipokine – axis, we might also be able to provide explanation for the metabolic changes frequently observed in acne patients.

Multiple lines of evidence indicate that the role of sebocytes is not limited to lipogenesis, but also cover the modulation of inflammation. Previous reports have shown that SZ95 sebocytes express inflammatory enzymes (COX-2 and 5-LOX), cytokines (IL-6) and chemokines (IL-8) at a basal level. We showed that the expression of all these molecules were significantly increased upon leptin treatment supporting that leptin might also be a potential player in inducing inflammatory signalling in human sebocytes. Furthermore, measuring the phosphorylation of STAT3 and NF- κ B p65 after leptin stimulus we found evidence that in leptin-treated SZ95 sebocytes STAT3 and NF- κ B pathways are likely candidates for the mechanisms behind the enhanced inflammatory cytokine production. This is also the first report showing that STAT3 signalling could be induced in SZ95 cells, adding important details on the complexity of inflammatory pathways that are active in human sebocytes.

In summary, our results demonstrate that similarly to adipocytes, sebocytes differentially express and secrete adipokines and that these topically synthesized proteins could influence the homeostasis of the dermis and contribute to cutaneous inflammatory processes. Moreover, our work also opens new avenues for further investigations that may integrate leptin into the currently modified concepts of (patho)physiological signalling in sebaceous gland-associated diseases, such as acne. However, one of the most intriguing questions based on our results, whether SG-derived adipokines could contribute not only to a local but also to systemic inflammation and metabolic effects through affecting serum adipokine levels (similarly to that of seen in the case of the adipose tissue), is yet to be investigated.

SUMMARY

Sebaceous glands and hair follicles form together the pilosebaceous units, whose primary function is the production of sebum. While an altered lipid composition associated with an increased excretion of sebum is one of the most important factors in the development of acne, on the other hand, its impaired production has been proposed to be a key feature in atopic dermatitis. Sebaceous glands also play a central role in skin inflammation. Pattern recognition receptors such as TLR2 and TLR4 are expressed in sebocytes, and their activation increases the secretion of both pro- and anti-inflammatory cytokines (IL-6 and IL-10 respectively), chemokines (IL-8), antimicrobial lipids, peptides, periglandular peptides and neuropeptides. Altogether, besides being important players in the regulation of skin homeostasis, sebocytes represent an interesting cell type that links lipid metabolism with inflammation at a cellular level, a key feature that is also a hallmark of adipocytes.

Adipokines, i.e. proteins that are synthesized and secreted primarily by adipocytes in response to various stimuli, include IL-6 and other small molecular weight bioactive proteins such as adiponectin, resistin, leptin, serpin E1, visfatin, apelin, chemerin, RBP4, and MCP-1. Due to their different biological properties and diverse cellular targets adipokines are involved in a wide array of (patho)physiological processes and are responsible for mediating the inflammatory effects of the adipose tissue in the local tissue environment as well as to different organs via circulation. Based on the already characterized similarities between adipocytes and sebocytes, it was reasonable to suppose that adipokines might also be produced and secreted by sebocytes and to design studies to elucidate if these proteins, as important inflammatory mediators of adipocyte biology, could be integrated into SG biology. Therefore, in this work we aimed to identify sebocytes as possible sources of adipokines within the human skin by showing the presence of different inflammatory adipokines in SGs of various histological specimens. Sebaceous glands in all examined samples expressed

adiponectin, IL-6, resistin, leptin, serpin E1 and visfatin, but not apelin, chemerin, RBP4 and MCP-1. Confirming the presence of the detected adipokines in the human SZ95 sebaceous gland cell line we further characterized their expression and secretion patterns under different stimuli by using TLR1/2 and 4 activators, or by 13CRA, a key anti-acne agent. With the exception of resistin, the expression of all of the detected adipokines (adiponectin, IL-6, leptin, serpin E1 and visfatin) could be further regulated at the level of gene expression, showing a close correlation with the secreted protein levels.

In our work, with the detection of the active, full-length form of leptin receptor in human sebaceous glands and in cultured human SZ95 sebocytes we also provided evidence that SGs are not just the sources but are also possible targets for adipokines. The treatment of SZ95 sebocytes with leptin led to an enlargement of intracellular lipid bodies, increased the ratios of unsaturated/saturated fatty acids and decreased vitamin E levels. Further supporting a pro-inflammatory role, leptin induced COX-2 and 5-LOX expression in SZ95 sebocytes and augmented the production of IL-6 and IL-8 cytokines. Upon leptin treatment, STAT3 and NF- κ B pathways were activated indicating that these known leptin signalling pathways are active in human sebocytes.

Our results suggest that sebocytes are not simply targets of inflammation but may exhibit initiatory and modulatory roles in the inflammatory processes of the skin through the expression and secretion of adipokines.

LIST OF PUBLICATIONS



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Candidate: Dóra Kovács
Neptun ID: TVRI4N
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List of publications related to the dissertation

1. **Kovács, D.**, Lovászi, M., Pólska, S., Oláh, A., Bíró, T., Veres, I., Zouboulis, C.C., Stähle, M., Rühl, R., Remenyik, É., Törőcsik, D.: Sebocytes differentially express and secrete adipokines. *Exp. Dermatol. Epub ahead of print (2015)*
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2. Törőcsik, D., **Kovács, D.**, Camera, E., Lovászi, M., Cseri, K., Nagy, G.G., Molinaro, R., Rühl, R., Tax, G., Szabó, K., Picardo, M., Kemény, L., Zouboulis, C.C., Remenyik, É.: Leptin promotes proinflammatory lipid profile and induces inflammatory pathways in human SZ95 sebocytes. *Br. J. Dermatol. 171(6)/2014*, 1326-35, 2014.
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Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu Web: www.lib.unideb.hu

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