Sebocytes differentially express and secrete adipokines

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Abstract

In addition to producing sebum, sebocytes link lipid metabolism with inflammation at a cellular level and hence, greatly resemble adipocytes. However, so far no analysis was performed to identify and characterize the adipocyte-associated inflammatory proteins, the members of the adipokine family in sebocytes. Therefore, we determined the expression profile of adipokines (adiponectin, interleukin [IL] 6, resistin, leptin, serpin E, visfatin, apelin, chemerin, retinol-binding protein 4 [RBP4] and monocyte chemoattractant protein 1 [MCP1]) in sebaceous glands of healthy and various disease-affected (acne, rosacea, melanoma and psoriasis) skin samples. Sebaceous glands in all examined samples expressed adiponectin, IL6, resistin, leptin, serpin E1 and visfatin, but not apelin, chemerin, RBP4 and MCP1. 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 mimicking bacterial invasion (by using Toll-like receptor [TLR] 2 and 4 activators), or by 13-*cis* retinoic acid (13CRA; also known as isotretinoin), a key anti-acne agent. With the exception of resistin, the expression of all of the detected adipokines (adiponectin, IL6, leptin, serpin E1 and visfatin) could be further regulated at the level of gene expression, showing a close correlation with the secreted protein levels. Besides providing further evidence on similarities between adipocytes and sebocytes, our results strongly 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.

Key words: sebocyte, adipokine, inflammation

Introduction

Sebaceous glands (SGs) together with the hair follicles form the pilosebaceous with a primary role to produce sebum (1). Changes in their lipid metabolism resulting in an altered amount and composition of sebum is associated with skin diseases such as acne vulgaris and atopic dermatitis (2). Moreover, sebocytes are also capable of exerting inflammatory responses via the secretion of pro-inflammatory cytokines, chemokines and antimicrobial peptides upon activation by pathogens (e.g. different *Propionibacterium acnes* strains) and pathogen-associated molecular pattern recognition receptor (like Toll-like receptor [TLR] 2 and TLR4) ligands (3, 4). These data show that sebocytes besides being important players in the regulation of skin homeostasis, also 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. The resemblance between the two cell types was further supported by the detection of lipogenic factors such as resistin, galectin-12, sterol regulatory element-binding protein 1 (SREBP1), peroxisome proliferator-activated receptor gamma (PPARg) and liver X receptor (LXR) in sebaceous glands of histological specimens as well as in SZ95 sebocytes. These factors are all pivotal players in adipocyte differentiation and lipid metabolism, (5-8), and their presence in sebocytes suggest that pathways and signalling mediators in adipocytes might play similar roles in sebocyte biology.

Adipokines, i.e. proteins that are synthesized and secreted primarily by adipocytes in response to various stimuli, include interleukin (IL6) and other small molecular weight bioactive proteins such as adiponectin, resistin, leptin, serpin E1 (also known as plasminogen activator inhibitor 1 [PAI1] and endothelial plasminogen activator inhibitor), visfatin (also known as nicotinamide phosphoribosyltransferase), apelin, chemerin, retinol binding protein 4 (RBP4), and monocyte chemoattractant protein 1 (MCP1) (9). 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. By detecting alterations in their serum levels also in dermatological diseases such as acne vulgaris and psoriasis, extensive studies have also started in dermatological research to identify the possible targets and the cutaneous sources of these proteins (10-12).

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/regulators 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. Using the SZ95 human sebaceous gland cell line we further extended our studies with a systematic analysis to address also their expression and secretion and searched for mechanisms that could regulate these processes.

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, psoriasis, melanoma skin samples) were evaluated. Placenta (for apelin), liver (for chemerin and RBP4) and ductal breast cancer (for MCP1) sections were used as positive controls according to the manufacturer's instructions (Supplemental Figure S1).

Cell culture and treatments

Human immortalized sebaceous gland cell line, SZ95 sebocytes were used for experiments (13). Lipopolysaccharide ([LPS], derived from *Escherichia coli*), PAM3CSK4 or 13-*cis* retinoic acid (13CRA) were used for treatments. SZ95 sebocyte proliferation was measured by the 4-methylumbelliferyl heptanoate (MUH) fluorescence assay as described by Zouboulis *et al.* (14). Detailed protocol is available in supplementary information online.

Immunohistochemistry

Anonymised frozen or FFPE sections of human skin samples were stained with adiponectin, resistin, visfatin, chemerin (Santa Cruz Biotechnology, Heidelberg, Germany), IL6, leptin (LifeSpan BioSciences, Nottingham, UK), apelin, MCP1 (Abcam, Cambridge, UK), serpin E1

and RBP4 (Thermo Fischer Scientific, Wilmington, USA). Staining protocols are detailed in supplementary information online.

Immunocytochemistry

SZ95 sebocytes were stained with anti-adiponectin, anti-IL6, anti-resistin, anti-leptin, anti-serpine E1 and anti-visfatin antibodies (as used for immunohistochemistry). Staining protocols are detailed in supplementary information online.

Determination of mRNA levels

SZ95 sebocytes were cultured in the presence of TLR1/2 and 4 ligands (PAM3CSK4, LPS), 13CRA or vehicle control as described previously. Total RNA was isolated using TRI Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's protocol and quantified by using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, USA). *Quantitative real-time PCR* (qRT-PCR) and *RNA sequencing* (RNA-Seq) protocols are detailed in supplementary information online.

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

Levels of adiponectin were probed with both ELISA Development Kit and Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). For IL6, resistin, leptin and serpin E1 ELISA Development Kit were used while visfatin levels were measured with ELISA Assay Kit (Biovision, Milpitas, CA, USA) according to the manufacturers' instructions. Detailed protocol is available in supplementary information online.

Statistical analyses

All data are presented as mean ± 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. By using immunohistochemical detection with specific antibodies (Supplemental Table S1), the pro-inflammatory adipokines IL6, resistin, leptin, serpin E1and visfatin, as well as the anti-inflammatory adiponectin were found to be expressed, while apelin, chemerin, RBP4 and MCP1 could not be detected (Figure 1a).

In order to assess if the expression pattern is altered in different pathological conditions we have extended our studies with samples of acne and rosacea and of diseases where SGs are not thought to be involved such as melanoma and psoriasis. Based on our results the expressed adipokines were detected in SGs of all of the tested diseases, while the ones that were absent in the healthy skin samples were also absent in the disease involved ones (Figure 1b and Supplemental Figure S1).

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 (13).

By immunofluorescent detection (Figure 2a) and using cell lysates for ELISA measurements (Figure 2b), the expression of adipokines found in the SGs of our histological samples was also confirmed in SZ95 sebocytes. Both the immunostaining and the ELISA data clearly

showed that SZ95 cells expressed IL6, resistin, leptin, serpin E1 and visfatin. Interestingly the fluorescent image analysis also revealed that IL6, resistin, serpine E1 and visfatin were detected in both the nucleus as well as in the cytoplasm of the SZ95 sebocytes while leptin localized mostly if not exclusively only to the cytoplasm. On the basis of the results obtained by ELISA measurements the levels of visfatin were found significantly higher ($p<0.0001$) compared to that of other adipokines (Figure 2b). The levels of adiponectin, that was previously identified in the supernatants of cultured mouse sebocytes (15), and also by immunocytochemistry in SZ95 sebocytes, could not be detected by ELISA.

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. IL6, leptin, serpin E1, and visfatin were present in the supernatants, whereas resistin and adiponectin could not be detected. As expected, 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 (Figure 2c).

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 activating the retinoic acid receptor (RAR) (16) were tested (Supplemental Figure S2). The changes in the mRNA expression levels of the SZ95 sebocytes were assessed by RNA sequencing and RT-Q-PCR measurements 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 *IL6*, a cytokine known to be induced by TLR1/2 and 4 activation (17). 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 E1and visfatin increased similarly to IL6 upon treatment with the applied TLR activators, correlating with the mRNA data. 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 (Figure 3). These data altogether show that sebocytes differentially express and secrete adipokines in response to various stimuli (Figure 4).

Discussion

In the process of skin inflammation the sebum producing SGs were considered until recently as impotent “innocent” bystanders under the control of other cell types, but without giving any feedback to them (18, 19). Intensive research in the past decade however 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 (13, 20). 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, IL6, resistin, leptin, serpin E1, visfatin, apelin, chemerin, RBP4 and MCP1 (9). 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 (21, 22). Interestingly, such correlation was also found for various dermatological diseases pointing on a possible communication between the adipose tissue and the skin and more importantly suggesting that besides adipocytes other cell types in the skin might also be the source of adipokines. However, so far research has only addressed keratinocytes and fibroblasts (23-25). 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 this work we identified sebocytes as another possible source for adipokines within the skin. To the best of our knowledge, only expression of IL6 and resistin have been previously reported in human sebocytes (4, 5). Therefore we aimed to define whether other adipokines are also present in SGs and evaluate their expression profile and 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, IL6,

resistin , leptin, serpin E1 and visfatin, while despite their expression in the adipocytes, apelin, chemerin, RBP4 and MCP1 could not be detected in SGs in any of the examined samples. In order to exclude false negative results due to the histochemical procedure in paraffin embedded tissue and the storage duration, we also performed staining on frozen tissue sections of available normal skin samples and found identical expression profiles.

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. To support this hypothesis we performed *in vitro* experiments with the best characterized human sebocyte cell line, the SZ95 sebaceous gland cell line, with unique biologically and clinically relevant behaviour (13) and confirmed that sebocytes exert a stimulus specific regulation in the expression and secretion of these proteins. Moreover, based on the fluorescent image analysis of the expressed adipokines in SZ95 sebocytes, it is also reasonable to suppose possible intracellular functions for them as well. 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.

In response to pro-inflammatory stimuli such as the TLR2 and -4 activators, representing prominent initiators of inflammation during acne pathogenesis, only IL6 was shown to be induced in sebocytes. The observed similar expression patterns in the induction and secretion of leptin, serpin E1 and visfatin with that of IL6 defines, therefore, additional important targets in the pathogenesis of acne. Moreover, we suggest that besides their complex biological functions, which are not yet fully explored, these proteins and their gene expression profiles could serve as markers for *in vitro* testing of agents with possible pathological as well as therapeutic relevance. As a consequence, 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 affects the differentiation/metabolism of sebocytes via adipokines, as also observed in rat adipose tissue (26).

Among the multiple biological functions of leptin, such as linking nutritional status with neuroendocrine and immune functions (27-29), in our previous studies we showed that sebocytes are also possible targets for leptin altering both lipid metabolism and inflammation via producing a pro-inflammatory lipid profile in association with the enlargement of intracellular lipid droplets (30). Our new findings that sebocytes not just 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. Considering the results of *Frank et al.* that keratinocytes from both leptin-deficient *ob/ob* mice as well as from wild-type mice responded to leptin treatment (both systemic and topical) with improved wound re-epithelisation (31) therefore we add another aspect of sebocyte-derived leptin that could be related to skin regeneration and wound healing processes. Moreover, with further research aiming to integrate primarily adipogenic signalling cascades, such as the 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 (32-34).

The high levels of visfatin in sebocytes that was further increased upon different TLR stimuli was another interesting finding in our study. Besides its possible inflammatory role in diabetes and metabolic syndrome (35), visfatin was reported to be strongly expressed within lipid-loaded macrophages of atherosclerotic lesions with a possible role in matrix degradation and inflammation (36). Regarding skin biology, visfatin was suggested to potentiate the development of psoriasis via stimulating human keratinocytes to an enhanced production of the antimicrobial peptides (37). However, speculations on the contribution of SGs in the

development and/or progression of psoriasis just on the basis of visfatin expression and secretion seem farfetched at this point. Nevertheless, 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 besides the adipose tissue is also produced in high amounts by the endothelium, thus suggesting a link between obesity and the increased risk for thrombosis (38), was also found to be involved in wound healing and in the pathogenesis of skin fibrosis (39, 40). 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 (41). Therefore, the detection of serpin E1 in SGs highlights another interesting aspect of the SGs related adipokines: a possible contribution to extracellular matrix remodelling. Assessing the presence and density of serpin E1-rich SGs in wound healing as well as in melanoma and studies on their correlation with the disease progression and metastasis is definitely another interesting and provocative field to challenge SGs for.

Adiponectin is the prime adipokine identified as an anti-inflammatory protein based on its inhibitory effect on the production of IL6 and on its capability to induce expression of anti-inflammatory cytokines, such IL10 and IL1 receptor antagonists (42). Regarding its role in skin biology, adiponectin suppressed the secretion of further pro-inflammatory cytokines like TNFa, IL17, IL22, and IFNg in keratinocytes (43) and was involved in cutaneous wound healing (44). 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 based on our data also from sebocytes, could even affect hair growth (45). 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 and that sebocytes might be potential candidates to test for functions that are linked to the cutaneous adipose tissue.

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. Furthermore, considering also their high numbers, SGs might even assume their status as "belly of the skin" to reflect their complex role as major metabolic and inflammatory regulators of the skin. 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 whether metabolic effects through affecting serum adipokine levels (similarly to that of seen in the case of the adipose tissue), definitely calls for further studies. Moreover, our findings may also open new avenues for investigations, with both diagnostic and therapeutic relevance, to regulate SG-derived adipokines for the benefit of various inflammatory skin diseases.

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Author contributions

DK performed cell work, immunostaining, ELISA measurements, ML did the RNA work, SZP did the RNA-sequencing and analysed the data, AO performed cell work and edited the manuscript, TB contributed to the research design, IV preformed histological verification of the used samples, CCZ provided the cell line and contributed to research design, MS edited the manuscript, RR analysed data, ER provided samples, DT designed the study, wrote the manuscript and supervised the research group.

Conflict of interests

The authors have declared no conflicting interests.

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Figure 1: Immunohistochemical detection of the expressed adipokines in human sebaceous glands.

(A) Immunostaining of adipokines in frozen tissue sections of human skin samples of healthy individuals as described in the Materials and Methods. Sections were counterstained with methylene green. Scale bars 100 µm.

(B) Immunohistochemical detection of the expressed adipokines (adiponectin, IL6, resistin, leptin, serpin E1 and visfatin) in human sebaceous glands in control skin as well as in rosacea, acne, melanoma and psoriasis affected FFPE tissue samples as described in the Materials and Methods. Images are representative of at least 3 samples from each disease and each staining. Sections were counterstained with methylene green. Scale bars 100 µm.

Figure 2.: Expression and secretion of adipokines in/by SZ95 sebocytes.

(A) Immunofluorescent image analysis using anti-adiponectin, anti-IL6, anti-resistin, anti-leptin, anti-serpin E1 and anti-visfatin antibodies as described in the Materials and Methods confirms the expression of various adipokines in cultured human SZ95 sebocytes. Note that while adiponectin, IL6, resistin, serpin E1 and visfatin was accumulated both in the cytoplasm as well as in the nucleus, leptin showed a prominent cytoplasmic localization. Scale bars 5µm.

(B) ELISA measurements of adiponectin, IL6, resistin, leptin, serpin E1 and visfatin in SZ95 sebocyte lysates as described in the Materials and Methods. Mean ± SD of samples assayed in duplicate is depicted (n=4). Presence of adiponectin is based on the results of immunocytochemical staining in SZ95 sebocytes (marked with white column). One-way ANOVA statistic test and Tukey post-hoc test were used in the analysis of ELISA data.

(C) ELISA measurements of adiponectin, IL6, resistin, leptin, serpin E1 and visfatin in SZ95 supernatants collected after 24 h of culturing as described in Materials and Methods. Mean ±

SD of samples assayed in duplicate is depicted (n=4). One-way ANOVA statistic test and Tukey post-hoc test were used in the analysis of ELISA data.

Figure 3: mRNA expression and protein secretion of adipokines by SZ95 sebocytes upon various stimuli.

Adiponectin, *IL6*, *resistin*, *leptin*, *serpin E1* and *visfatin* mRNA expression in SZ95 sebocytes was determined by RNA sequencing and qRT-PCR (black bars), protein secretion (grey bars) was determined by ELISA measurements from the supernatants of SZ95 sebocytes as described in the Materials and Methods. Cells were treated with vehicle, 1/2 and 4 activators (1 µg/ml PAM3CSK4 and LPS, respectively) and 10^{-6} M 13CRA for 24 h. The expression levels are the average of at least 3 independent experiments, measured in triplicates. Error bars indicate the SD of the relative mRNA and protein expression levels. For analysis of RNA sequencing and ELISA data, unpaired t-test was used.

Figure 4.: Overview on the expression and possible secretion of adipokines by sebocytes.

The pro-inflammatory adipokines IL6, resistin, leptin, serpin E1 and visfatin, and the anti-inflammatory adiponectin were all expressed in sebaceous glands of normal skin, rosacea, acne, melanoma and psoriasis samples regardless of the pathological conditions, as well as in SZ95 sebocytes. With the exception of resistin, all of the expressed adipokines (*adiponectin*, *IL6*, *leptin*, *visfatin* and *serpin E1*) could be regulated at mRNA levels upon treatment of SZ95 sebocytes with various stimuli (PAM3CSK4 for TLR1/2, LPS for TLR4 and 13CRA for Retinoic acid receptor - RAR). The changes in the protein levels of IL6, leptin, visfatin and serpin E1 from the supernatants correlated with the mRNA data. In the case of adiponectin the light green circles refer to a possible secretion supported with previous findings of Akazawa *et al*. (15).