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Systemic Circulating Leptin – Aiding New Dimension of Immune-Related Skin Carcinogenesis and Lipid Metabolism

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Abstract

AIM: The link between the development of melanoma skin cancer and obesity is gaining recently an increased attention. A plethora of immune-related factors sustain a chronic inflammatory state modulated by the metabolic imbalance, the main examples being tumor necrosis factor alpha, interleukin 6, monocyte chemoattractant protein (MCP1), and several adipokines. Among the adipokines leptin (LEP) is steadily increasing interests as it involved in modulating different signaling pathways (e.g., JAK2/STAT3 and MAPK cascade/FOS) and immune cells fate by promoting the switch of memory cells to T helper-1 cell response, increases $CD4^+CD25^-$ T-cell proliferation, and reduces autophagy during T-cell receptor stimulation. Our work investigates in a pilot study, using protein microarray technology, the LEP circulatory levels in melanoma patients in comparison to other cytokines and chemokines already reported as significantly involved in melanoma initiation and progression.

METHODS: Serum from melanoma cases and pool of serum from healthy volunteers were analyzed by an antibody array platform for a 42-cytokine profile.

RESULTS: The investigation of cytokines, chemokines, and growth factors has shown that all the proinflammatory circulating molecules are elevated in the melanoma patient's serum. Inflammatory cytokines were found elevated and were correlated to the more advanced stage of the investigated patient (M2). The highest circulatory level was registered for LEP in both melanoma cases; moreover, in M1 patient, although the LEP level was completely comparable with the one found in M2, its IL-1 serum level was normal. In addition, there are ten analytes that show clear abnormal values and in patient M2 high LEP concentration is correlated with high IL-1, MCP-2, MCP-3, MCSF, and TARC serum concentrations.

CONCLUSION: Leptin is pleiotropic adipocytokine involved in various diseases, including skin cancer and melanoma. As it is involved in favoring inflammation and further tumorigenesis, the development of effective leptin-based therapeutic approaches opens new possibilities of adjuvant therapy in skin cancers.

Introduction

The significant increase in the incidence of obesity embodies a new global health crisis. Obesity affects the entire organism, and as skin is the largest human organ that it affects its functions. Besides various diseases, obesity is involved also in cancer initiation and progression. Recently, the link between the development of melanoma skin cancer and obesity is gaining increased attention. Increased production of cytokines and growth factors is sustained by adipose stromal/stem cells aiding tumorigenesis and metastasis. These immune-related factors sustain a chronic inflammatory state, further modulated by the metabolic imbalance. Therefore, this entire deregulated homeostasis would favor skin malignancies, melanoma included [1]. Melanoma has intrinsic and extrinsic risk factors, sun exposure and lifestyle are the main environmental factors inducing this skin cancer [2]. A recent relationship between the melanocortin pathway that is involved to both melanin synthesis and obesity was established [3]. The biology of adipokines, molecules secreted by the adipose tissue, is linked to inflammation, and their molecular pathways can be involved in angiogenesis, migration, invasion, and proliferation of melanoma cells. Adipose tissue dysfunction triggers chronic inflammation, this process further induces hyperglycemia and insulin resistance, contributing to tumor growth. Moreover, adipocyte secreted proinflammatory factors, including leptin and up-regulate the expression of tumor progression genes associated with cancer (e.g., cellular adhesion, invasion, angiogenesis, and apoptosis resistance) [4]. An increased rate of lipid synthesis is one of the melanoma cells characteristics sustaining the metabolic reprogramming of the tumor cell [5]. In this view, lipid – mediators regulate classical oncogenic pathways, therefore affecting cutaneous melanoma progression. Various lipid-associated alterations have been reported to be linked to melanoma cell aggressiveness, for example, lipogenic fatty acid synthase, fatty acid β-oxidation, glycerophospholipids,

sphingolipids, sterols, and eicosanoids. Chronic inflammation is a condition to be found in the majority of disease states including obesity and cancer [6], [7]. There is a *pleiada* of proinflammatory molecules in the adipose tissue that is related to the inflammatory status of metabolic diseases, the main examples being tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), monocyte chemoattractant protein 1 (MCP1), and several adipokines [8]. Among the adipokines leptin (LEP) is steadily increasing interests as it involved in a network of interactions. LEP is an important player in the regulation of energy balance and body weight control. After entering in circulation, LEP will bind to its receptor (LEPR) inducing both central and peripheral effects. As LEPR is expressed by many tissues, LEP binding will activate different signaling pathways (e.g., JAK2/STAT3 and MAPK cascade/FOS) [9]. STAT3 (Signal transducer and activator of transcription 3) mediates cellular functions as an immune response to IL and to various other factors. Once activated, it can recruit coactivators (e.g., NCOA1 and MED1) to the promoter region of the target gene. In this molecular network, STAT3 can mediate cellular responses to various skin-related receptors (e.g., activated FGFR1, FGFR2, FGFR3, and FGFR4). Moreover, it can bind to the IL-6-responsive elements from the promoters of various acute-phase protein genes. Although not studied in melanoma, recently, it was shown that the inhibition of STAT3 activation can reduce suppressor of cytokine signaling levels, as a negative feedback molecule that regulates cytokine signal transduction through the JAK/STAT pathway [10]. JAK2 (Tyrosine-protein kinase JAK2) is also regulated by LEP. This tyrosine kinase is involved in many processes, cell growth, development, differentiation, or histone modifications and it is mediating essential signaling networks in innate and adaptive immunity. Through the JAK2, PI3K, MAP2K1/MEK1, and MAPK14/p38 pathways, LEP develops its pro-inflammatory role, synergizing with IL1B. By inducing NOS2 LEP promotes IL6, IL8, and prostaglandin E2 production [11], [12]. In T lymphocytes, it promotes the switch of memory cells to T helper-1 cell response, increases $CD4^+CD25^-$ T-cell proliferation, and reduces autophagy during T-cell receptor stimulation [13].

In the past 20 years, there are only 40 papers that focus on leptin assessed in the systemic circulation of melanoma patients and relevant mice models. Acknowledging the increasing role of LEP, our work investigates in a pilot study, using protein microarray technology, the LEP circulatory levels in melanoma patients in comparison to other cytokines, and chemokines already reported as significantly involved in melanoma initiation and progression.

Materials and Methods

Subjects

Two melanoma cases (abbreviated M1, M2) from the Diagnostic Centre of "Victor Babeș" National Institute of Pathology, Bucharest, Romania for which informed consent was obtained, were selected for a 42-cytokines profile in serum. Patients were followed up for standard S100 antigen detection and clinical evolution of the disease. A normal pool serum from healthy volunteers matching gender and age were run alongside with melanomas patients' serum as control. The main clinical parameters of melanoma cases are depicted in Table 1.

Table 1: Characteristics of melanoma samples assessed for serum 42-cytokine profile

Case	Melanoma stage	s100 serum level pg/mL	Age	Gender
M1	I	144.48	52	M
M2	II	754	28	F

Antibody array analysis

Simultaneous detection of 42 human cytokines in each serum sample was assessed using an antibody array platform (*RayBio® Human Cytokine Antibody Array G-Series 3*, RayBiotech, Inc. GA, USA, http://www.raybiotech.com). In addition, a pool of serum from normal volunteers was run in a separate sub-array on the same slide; to normalize signal intensity data, this sub-array was defined as "reference." The chosen array platform semi-quantitatively detects the following analytes: ENA-78, G-CSF, GM-CSF, GRO, GRO α, I-309, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40/p70, IL-13, IL-15, IFN-Y, MCP-1, MCP-2, MCP-3, M-CSF, MDC, MIG, MIP-1δ, RANTES, SCF, SDF-1, TARC, TGF-β1, TNF-α, TNF-β, EGF, IGF-1, Angiogenin, Oncostatin M, Thrombopoietin (TPO), VEGF-A, PDGF-β, and leptin. The slide was scanned for fluorescence using a laser scanner (Innoscan 1100 AL, Innopsys, http://www.innopsys.com/en/) and data acquisition and analysis were performed using MAPIX software (version 8.2.5, Innopsys). The spots intensities were automatically analyzed by software, and further, the values of intensities were normalized according to the formula provided by manufacturer. Increments of above 1.5-fold or decrements below 0.65-fold in signal values were defined as assessable and biologically significant for each analyte.

Results are presented as indexes of normalized expression using as reference the values obtained for normal pool of sera.

Results

Using protein microarray technology [14], 42 analytes from melanoma patients' serum (M1 and M2) were evaluated against a pool of healthy donors' serum. The investigation of cytokines, chemokines, and growth factors has shown that all the proinflammatory circulating molecules are elevated in the patient's serum (Table 2 and Figure 1). Moreover, the circulatory pattern of the investigated patients is different and it is corelated with a high S100 serum level (M2) and a normal S100 serum level (M1).

Table 2: Expression index for tested analytes in melanoma cases assessed by protein microarray platform; index normalized to normal values of serum pool from healthy subjects

Analyte	Normalized expression index		Analyte	Normalized expression index	
	Patient M1	Patient M2		Patient M1	Patient M2
ENA-78	2.14	1.09	MCP-2	1.27	1.54
GCSF	0.89	1.20	MCP-3	2.87	3.13
GM-CSF	0.90	1.23	MCSF	1.52	5.02
GRO	0.73	0.46	MDC	1.83	1.15
GRO-α	1.11	1.30	MIG	1.11	0.98
I-309	1.11	2.29	MIP-1δ	0.37	0.89
IL-1α	0.90	1.13	RANTES	0.99	1.01
IL-1β	0.74	8.21	SCF	1.69	3.34
IL-2	0.79	1.07	SDF-1	1.13	3.25
IL-3	0.97	1.1	TARC	2.60	2.19
IL-4	0.90	1.15	TGF-β	0.98	0.99
IL-5	1.12	1.10	TNF-α	1.12	1.23
IL-6	0.84	0.94	TNF-β	6.37	6.35
IL-7	1.18	1.24	EGF	0.20	0.51
IL-8	0.85	0.88	IGF-I	1.30	1.61
IL-10	0.84	0.91	Angiogenin	1.01	1.03
IL-12 p70	1.67	1.35	Oncostatin M	1.10	1.04
IL-13	0.99	0.85	Thrombopoietin	0.43	0.70
IL-15	1.02	1.20	VEGF	0.91	1.01
IFN-γ	1.12	1.40	PDGF BB	1.02	1.00
MCP-1	1.63	0.82	Leptin	47.65	11.38

MCP: Monocyte chemoattractant protein.

Inflammatory cytokines were found elevated as previously published by us [15] and were correlated to the more advanced stage of the investigated patient (M2). In addition, immune molecules from the CC chemokines family (e.g., MCP-1, 2, 3) were found increased in both cases (exception MCP-1 in M2 patient) and are related to the melanoma development as shown by us in humans [15] and in melanoma mouse models [16].

Cytokines such as IL-3, 4, 7, 12, and IFN were found elevated especially in M2 patient. The highest circulatory level was registered in M2 patient, almost as the high of LEP levels. In M1 patient, although the LEP level was completely comparable with the one found in M2, its IL-1 serum level was normal (Figures 1 and 2).

We have grouped all the factors that were found deregulated high or low serum concentrations in both patients (Figure 2). There are ten analytes that show clear abnormal values. Thus, in patient, M2 high LEP concentration is correlated with high IL-1, MCP-2, MCP-3, MCSF, and TARC serum concentrations. As MCP-2 and 3 are chemokines involved in immune cell activation, MCSF is a growth factor and it was found elevated only in case M2. MCSF is known to be involved in the proliferation, differentiation, and survival of innate immune cells, for example, monocytes and macrophages. Another inflammatory chemokine, CCL17, or TARC is elevated in patients' sera in comparison to normal values and, although reports in melanoma lack, a link with elevated level in other cancers were reported [17].

TPO was found under normal levels in both cases. TPO is produced in the liver, up-regulated by IL-6 [18]. The low levels of TPO detected in both patients were found correlated with the low levels of IL-6. Moreover, TPO shares a family of receptors with leptin, with structural similarities in their extracellular and intracellular domains lacking an intrinsic tyrosine kinase [19].

Discussion

In search of new circulatory biomarkers that could hint on the clinical outcome of the melanoma patients, we have used various identification technologies, including protein microarray [14], [20]. This technology offered us multiple analyte identification

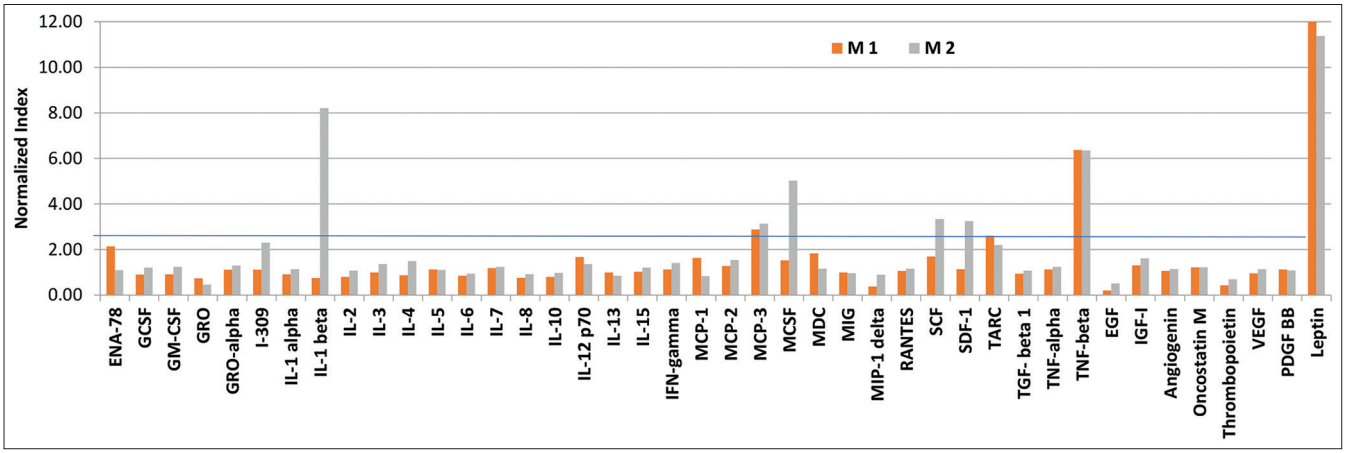

Figure 1: Evaluation of 42 analytes from melanoma patients' serum (M1 and M2) using protein microarray technology. Expression of analytes is normalized to normal values of healthy donors pooled serum (green line at index 1)

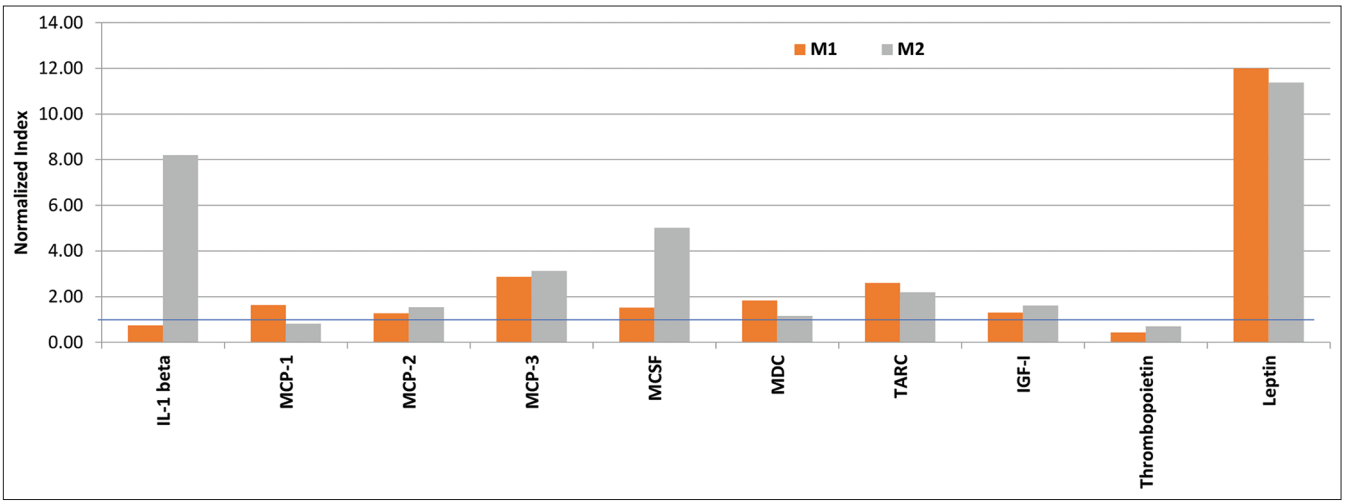

Figure 2: Evaluation of the group of ten analytes that are over-expressed in both investigated cases of melanoma patients (M1 and M2). Expression of analytes is normalized to normal values of healthy donors' pool (green line at index 1)

from the panel of circulating immune molecules to concomitantly evaluate the levels of LEP in comparison to other circulatory molecules.

Although the number of investigated patients is very low the technology showed that in both investigated cases, the circulatory levels are high, actually LEP has the highest expression index in comparison to all other investigated molecules. According to the published results in experimental animal models of melanoma, LEP is an angiogenic and mitogenic factor promoting melanoma growth [21]. Earlier studies performed in melanoma patients using ELISA have shown that serum leptin can predict sentinel node metastasis [22]. In our case, patient M2 with high circulating levels of both LEP and IL-1 displayed also high S100 serum value and developed metastases of the lung and brain 6 months post-testing. Moreover, in the same case, the LEP high circulatory values were correlated with the IL-1 high values as shown previously in other pathologies [11], [12].

Chemokines were found increased in both patients and the finding is in accordance with the previous reports [23] showing that chemokine gradients are chemokine receptor up-regulations are metastatic-related mechanisms in melanoma [24] and in cancer in general making them novel targets of therapy [25], [26]. Much to our knowledge our report is the first one showing the high serum circulatory levels of chemokines in relation to the high LEP concentration. We found ten analytes that show clear abnormal values and in patient M2 high LEP concentration is correlated with high IL-1, MCP-2, MCP-3, MCSF, and TARC serum concentrations. As LEP is known to be correlated in metastatic patients with IL-1, the finding that MCP-2 is also correlated shows that inflammatory cells like monocytes, T-cells, and NK cells are activated and sustain the pro-inflammatory systemic milieu. MCP-3 is one of the most potent chemokines as it binds to multiple receptors from the innate immune cells triggering high pro-inflammatory processes in monocytes and neutrophils [27].

The growth factor MCSF was found elevated just in M2 case and it is known to increase the phagocytic and chemotactic activity of monocytes and macrophages and up-regulate tumor cell cytotoxicity. In diabetic patients and normal individuals subjected to glucose tolerance test, the LEP plasma level was found correlated with MCSF level [28]. TPO levels were found by us under normal values, correlated with low regulatory IL-6 levels. Although up to now literature in melanoma research does not link TPO and LEP, it should be noted that there is a physiological significance of leptin-TPO-1 crosstalk. In animal models of leptin deficient ob/ob mice that are characterized by obesity, severe hepatic steatosis and insulin resistance liver regeneration was studied. It was reported that TPO administration in leptin deficient ob/ob mice was inefficient in 55% of mice suffering liver resection. This data shows that metabolic abnormalities, that is, lack of leptin, cannot be overridden by a pleiotropic substance, like TPO [29], [30].

The study has as limitations the low number of patients, but it is compensated by the high number of serum analytes identified spanning an important range of immune and non-immune molecules. Further, investigation on this subject would bring new data regarding the clinical outcome of patients in correlation with the levels of circulating leptin.

Conclusion

Leptin displays multiple biological functions and is engaged in complex immune and non-immune networks [30]. Leptin is a pleiotropic adipocytokine that was proven to be involved in various diseases, including

skin cancer, as melanoma. As it is involved in favoring inflammation and further tumorigenesis, future effective leptin-based therapeutic approaches would open new possibilities of adjuvant therapy in skin cancers.

Authors' Contributions

Monica Neagu and Carolina Constantin designed and performed the study. Mihaela Surcel performed the experiments and wrote the paper. Monica Neagu, Mihaela Surcel, and Carolina Constantin wrote and revised the final form of the paper.

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