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Elevated serum leptin levels in patients with systemic lupus erythematosus

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Previous studies have indicated that leptin and the soluble leptin receptor (SLR) might influence inflammatory and immune processes in autoimmune diseases, but this remains unclear in systemic lupus erythematosus (SLE). The aim of our study was to assess if leptin and SLR are involved in the etiopathology of SLE and the possible mechanism of immune regulation. We studied 87 patients with SLE and 85 matched subjects. We assessed the levels of serum leptin and SLR, tested the long isoform leptin receptor (Ob-Rb) mRNA levels in SLE patients and a control group. Furthermore, we measured Th1 and Th2 percentage in SLE patients' lymphocytes and examined lymphocytes activation and proliferation assays with leptin stimulation *in vitro*. The study found a higher level of serum leptin in SLE patients, however, no difference was found in serum SLR levels or Ob-Rb mRNA levels between SLE patients and the control group. The percentage of Th1 cells decreased and Th2 cells increased after treatment with glucocorticoids in SLE patients. Leptin stimulated the proliferation of T cells *in vitro*, and differentiation to Th1 cells increased. The present study demonstrated that leptin may play an important role in the pathogenesis of SLE, inducing dysfunction of autoimmune processes.

1. Introduction

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease affecting virtually any organ system of the patients. People of all genders, all ages and all ethnic groups are susceptible but females are more commonly affected than males and most of the patients are women of the child-bearing age group. The prognosis of the disease is not good and it is not curable until now. Even though the etiology of SLE is unknown, many predisposing factors have been found, including environmental, genetic, microbiological, and hormonal factors (Talaat et al. 2015).
Recent studies showed that adipose hormones play an important role in the pathogenesis of SLE. Leptin is one of the most important adipose derived hormones. It has long been recognized as a key factor in regulating food intake and energy balance through leptin receptors. In addition to its critical involvement in these physiological functions, leptin is also involved in modulating immune responses (Ahima et al. 2000; Fantuzzi et al. 2000). Several studies implicated that leptin could influence the development of rheumatoid arthritis, type 1 diabetes and autoimmune encephalomyelitis (Tian et al. 2014; Friedman et al. 1998; Vadacca et al. 2011). Few studies have described elevated serum leptin levels in patients with SLE and closely related to the SLE Disease Activity Index (SLEDAI) score, osteoporosis, and cardiovascular diseases (Kim et al. 2010; Vadacca et al. 2013; McMahon et al. 2011; Al et al. 2009). Just two studies provided information regarding leptin receptors. Bagheri et al. (2012) found soluble leptin receptors (SLR) decreased in SLE patients and concluded that this may act as a negative regulator of leptin activity. However, Afroze' results were inconsistent with that (Afroze et al. 2015).
Leptin could protect T lymphocytes from apoptosis and regulates T-cells activation and proliferation, and influences cytokine production from T lymphocytes (Fujita et al. 2014; Amarilyo et al. 2013). These studies all used mice as research subjects. The effect of leptin and SLR, whether and to what extent they regulate T lymphocytes of SLE patients still remains unclear. Therefore, we assessed serum leptin and SLR levels, measured Th1 and Th2 percentage of lymphocytes and detected lymphocyte activation and proliferation after stimulation by leptin in SLE patients and healthy controls. This way, we tried to explore whether leptin and SLR are involved in the etiopathology of SLE and the possible mechanism of immune regulation.

2. Investigations and results

2.1. Serum leptin and SLR levels in patients with SLE

In this study, we included a total of 87 SLE patients and 85 healthy controls. Serum leptin levels were significantly higher in patients with SLE than in controls (7.06 ± 3.19 *vs* 5.36 ± 2.66, $P < 0.05$). No statistically significant differences of serum SLR

Table 1: Serum leptin levels and SLR levels in SLE patients and control groups (ng/ml, mean ± SD)

Groups (No.)	Lp	sLR
SLE patients (87)	7.06 ± 3.19*	7.56 ± 4.16
Healthy controls(85)	5.36 ± 2.66	7.44 ± 3.68
p-value	0.000	0.835
SLE patients		
LN (47)	7.13 ± 3.18	7.77 ± 4.69
Without LN (40)	6.99 ± 3.25	7.31 ± 3.48
p-value	0.839	0.613
SLE patients		
Active(40)	6.96 ± 3.13	7.70 ± 4.45
Inactive(47)	7.15 ± 3.28	7.44 ± 3.94
p-value	0.779	0.769

*$P < 0.05$,VS. Healthy controls.

levels were found between SLE patients and the control group. There were no significantly differences of serum leptin levels and SLR levels in SLE individuals with lupus nephritis (LN) or active in comparison with SLE patients without LN or inactive ($P > 0.05$) (Table 1).

2.2. *The levels of Ob-Rb mRNA in patients with SLE versus controls*

Ob-Rb mRNA was expressed in PBMCs, the gray scan of PCR production in SLE patients and healthy controls were 1.01 ± 0.92 and 0.88 ± 0.41, respectively. There was no significant difference in Ob-Rb mRNA expression between SLE patients and healthy controls ($P > 0.05$) (Fig. 1).

2.3. *Th1 and Th2 percentage in SLE patients' lymphocytes*

The ratio of Th/lymphocytes, Th1/Th and Th2/Th in SLE patients were 4.27 ± 3.18%, 14.43 ± 12.76% and 69.97 ± 23.17%, respectively, lower than that of the healthy controls 8.14 ± 2.99%, 22.73 ± 10.37% and 96.27 ± 2.27% respectively ($P < 0.05$). The ratio of Th1/Th in newly diagnosed SLE patients was 16.98 ± 16.92% which was higher than that of the patients after treatment 11.45 ± 8.15% ($P < 0.05$). The ratio of Th2/Th in revisiting SLE patients were 70.96 ± 27.43% which was higher than that of the newly diagnosed SLE patients 58.86 ± 12.03% ($P < 0.05$) (Table 2).

2.4. *Lymphocytes activation and proliferation assays in vitro*

After three days culture, the ratios of Th/lymphocytes were 6.82 ± 2.27% in the high-dose leptin stimulated group and 5.54 ± 2.04% in the low dose leptin stimulated group, both were higher than the control group 2.29 ± 1.67% ($P < 0.05$). The ratios of Th2/Th were 19.09 ± 8.96% in the high-dose leptin stimulated group and 31.04 ± 3.43% in the low dose leptin stimulated group, both were lower than the control group 45.65 ± 7.14% ($P < 0.05$). The ratio of Th1/Th were 9.52 ± 1.94% in the high-dose leptin stimulated group and 11.50 ± 1.99% in the low dose leptin stimulated group, both were higher than the control group 5.53 ± 1.89% ($P < 0.05$). However, there was no significant difference between low dose leptin stimulated group and high dose stimulated group (Table 3).

After five days culture, the OD value of lymphocytes costimulated with leptin and PHA was 0.80 ± 0.02, which was higher than that of the PHA incubation group (0.69 ± 0.04; $P < 0.05$),which was in turn higher than that of the control group (0.46 ± 0.09; $P < 0.05$).

3. Discussion

Since its discovery in 1994, leptin has attracted increasing interest in the scientific community for its pleiotropic actions. One of these functions is the relationship between nutritional status and immune competence. Several studies have shown that imbalance in the levels of leptin and its receptor is involved in the development of SLE. The present study also evaluated the role of leptin in SLE and found that leptin levels were significantly higher in patients with SLE than in controls, which was in agreement with most reports (Kim et al. 2010; Vadacca et al. 2013; McMahon et al. 2011; Al et al. 2009). Opposite to our results, Wisłowska et al. (2008) found no statistically significant difference between serum leptin levels in SLE and a control group. A recent study reported this serum leptin levels correlated positively with SLEDAI scores (Vadacca et al. 2013). Serum leptin levels in SLE patients with arthritis and central nervous system (CNS) involvement ($P < 0.05$) were significantly lower in comparison with SLE patients without arthritis and CNS involvement (Wisłowska et al. 2008). Some studies found that serum leptin levels were closely related to osteoporosis, and cardiovascular complications of SLE (McMahon et al. 2011; Al et al. 2009). In our study, no correlation was found between serum leptin levels and the SLEDAI score which was in agreement with previous studies in adults with SLE (Kim et al. 2010; Al et al. 2009; Wisłowska et al. 2008) .The different study results may be explained in part by the small number of patients, variable disease activity, different disease pathogenesis, and different patient inclusion criteria such as age and steroid dosage. The human leptin receptor has three isoforms, ob-Ra,ob-Rb,ob-Rc. According to the length of its protein cytoplasmic region, there is a long isoform and a short isoform receptor. Ob-Rb is a long isoform receptor with signaling function. The long isoform of the leptin receptor is expressed in the lymphocytes, endothelial cells and hematopoietic stem cells (Chua et al. 1997; Ge et al. 2002).

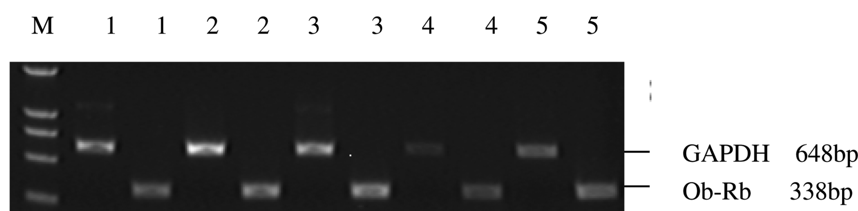

Fig. 1: Gel electrophoresis quantitative PCR amplicons of Ob-Rb mRNA. No significantly difference was found between SLE patients and healthy controls. (SLE patients:1,3,5. healthy controls:2,4).

Table 2: Ratio of Th/ lymphocytes, Th1/ Th and Th2/Th in SLE patients

	Healthy controls	SLE patients	SLE patients (newly diagnosed)	SLE patients (after treatment)
N	20	20	10	10
Th(%)	8.14 ± 2.99	4.27 ± 3.18*	5.78 ± 5.23	$3.73 \pm 2.25^{\#}$
Th1(%)	22.73 ± 10.37	14.43 ± 12.76*	16.98 ± 16.92	$11.45 \pm 8.15^{\#}$
Th2(%)	96.27 ± 2.27	69.97 ± 23.17*	58.86 ± 12.03	$70.96 \pm 27.43^{\#}$

$^{*}P < 0.05$, VS. Healthy controls. $^{\#}P < 0.05$, VS. SLE patients (newly diagnosed).

Table 3: Leptin increased Th1 cell frequency and decreased Th2 cell frequency in vitro

	Controls	LP (0.5nM)	LP (5nM)
Th%	2.92 ± 1.23	5.45 ± 1.61*	6.82 ± 1.86*
Th1%	5.53 ± 1.89	11.50 ± 1.99*	9.52 ± 1.94*
Th2%	45.65 ± 5.83	31.04 ± 2.80*	19.09 ± 11.39*

$^{*}P < 0.05$, VS. controls. Controls group (PHA: 2 μg/ml); low dose leptin group (PHA: 2 μg/ml, LP: 0.5 nM); high-dose leptin group (PHA: 2 μg/ml, LP: 5 nM). There was no significant difference between low dose leptin group and high dose group.

Only two studies investigated levels of SLR in SLE patients. One study showed decreased serum levels of SLR in patients with SLE, the other did not show a significant difference between SLE patients and controls (Bagheri et al. 2012; Afroze et al. 2015). The present study detected not only the levels of SLR in serum, but also Ob-Rb mRNA levels in PBMC of SLE patients. We found no significant difference between SLE patients and healthy controls in both assays. Our study suggested that serum leptin receptor levels may be of minor importance in the pathophysiology of SLE. Further studies on greater number of patients are necessary to confirm these issues.

In our study, the percentage of Th1 cells decreased and Th2 cells increased after treatment with glucocorticoid in SLE patients, which implied skewing of T-cells toward Th1 cells in SLE patients before glucocorticoid therapy. Previous studies reported that glucocorticoids can accelerate the apoptosis of lymphocytes. Our findings illustrated that glucocorticoids may be selective for lymphocytes subtype which is worth to be studied further in the future.

Recent studies indicated that leptin intereferes with inflammation and immunity. Leptin could stimulate the proliferation of T cells *in vitro*, promotes Th1 response and protects T cells from corticosteroid-induced apoptosis (Lord et al. 1998; Busso et al. 2002; Howard et al. 1999). Congenital leptin deficiency is associated with a decreased number of circulating $CD4^{+}$T cells and decreased cytokine release (Farooqi et al. 2002). *In vitro*, leptin was shown to stimulate the production of proinflammatory cytokines by monocytes and macrophages. Administration of leptin to fasted mice reversed the impairment of T cell function (Liu et al. 2012). In our study, we demonstrated that leptin could stimulate the proliferation of T cells *in vitro*, and increase differentiation to Th1 cells. Recently, leptin was reported to suppress regulatory T cells and enhance Th17 cells *in vitro* and to exacerbate collagen-induced arthritis. Ob/ob mice, which have a genetic deficiency of leptin due to a mutation in the leptin gene, have a reduced susceptibility to develop autoimmunity and display elevated numbers of peripheral regulatory T cells (Fujita et al. 2014).

In summary, we showed that serum leptin levels in SLE patients were higher than in healthy controls, and leptin could promote switching the phenotype of Th toward a Th1 response in SLE. Although the mechanisms by which this occurs remain to be determined, our findings suggest that blockade of leptin signaling may be of therapeutic benefit in patients with SLE.

4. Experimental

4.1. Study population

The study was done prospectively during May 2010 to December 2013 at the First Affiliated Hospital of Dalian Medical University (Dalian, China). Eighty-seven consecutive SLE patients (76 females, 11 males), with mean age 43.14 ± 14.70 years (18 y to 78 y), mean body mass index (BMI) 21.25 ± 2.37 kg/m^2, who fulfilled the 2012 derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for the diagnosis of SLE were included in the present study (Petri et al. 2012). Disease activity was evaluated according to the SLEDAI score (Active: SLEDAI score ≥ 10; inactive: SLEDAI score < 10). Eighty-five healthy subjects (71 females, 14 males) with appropriate age, sex, and BMI were included in the study as a control group. Patients with hypertension, diabetes, cardiovascular diseases, pregnancy, active or chronic infections, or other connective tissue diseases, were excluded. Daily glucocorticoids doses of SLE patients were lower than 5 mg prednisone equivalent. The study was approved by the Medical Ethical Committee of the First Affiliated Hospital of Dalian Medical University and informed consent was obtained from all patients.

4.2. Evaluation of serum leptin and soluble leptin receptor levels

Venous blood was withdrawn from patients and healthy subjects at 7 a.m. after an overnight fasting. Serum and peripheral blood mononuclear cells (PBMCs) were isolated and kept at −80 °C until processed. Serum leptin and SLR levels were measured by a commercially available, enzeme-linked immunosorbent assay (ELISA) kits (KeyGEN Co. Ltd, Nanjing, China) according to the instructions of manufacturer. The sensitivity of the assay was 0.1 ng/ml.

4.3. RNA extraction and determination of the long isoform leptin receptor (Ob-Rb) mRNA levels

Total RNA was extracted from PBMCs of patients and controls using Trizol reagent (Takara Biotechnology, Dalian, China). The quantification and the quality of RNA were assessed using a NanoDrop® ND-2000C spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Total RNA was reverse-transcribed by cDNA Synthesis kit (RNA PCR kit (AMV) Ver.3.0, Takara Biotechnology), which included heating to 30 °C for 10 min, 42 °C for 10 min, 99 °C for 5 min, 5 °C for 5 min, and stored at − 20 °C. The mRNA levels of Ob-Rb (sense: 5'-ATAGTTCAGTCACCAAGTGC-3'; antisense 5'-GTCCTGGAGAACTCTGATGTCC-3') in ratio to the housekeeping gene, GAPDH (sense: 5'-CCACATCGCTCAGACACCA-3'; antisense 5'-GCATCGCCCCACTTGA-3'), were determined by RT-PCR. PCR conditions were denaturation 4 min at 94 °C, 30 cycles of 45 s at 94 °C, 40 s at 57 °C, 1 min at 72 °C. These were followed by a final extension step at 72 °C for 5 min. Amplified DNA fragments were electrophoretically separated on 1% agarose gels containing 0.5 μg/ml ethidium bromides. The lengths of the products were 338 bp and 648 bp, respectively. Ob-Rb and GAPDH PCR products were semiquantified by densitometric scanning using the gel imaging analysis system.

4.4. Measurement of Th1 and Th2 percentage in SLE patients' lymphocytes by flow cytometry

Heparinized peripheral blood (100 μl) of SLE patients was aliquoted into polystyrene tubes with 10 μl FITC-conjugated anti-CD4 and incubated for 10 min in the dark at room temperature (RT), and then erythrocytes were removed using red cell lysing buffer. After undergoing washing and rupture of membranes procedure, the cells were stained with PE-conjugated anti-IFN-γ or PE-conjugated anti-IL-4 for 30 min in the dark at RT. All

the antibodies were bought from eBioscience, San Diego, CA, USA. After staining, the cells were washed again and analyzed by flow cytometry (FACSCalibur and CellQuest software;Becton Dickinson). Cells were logically gated on CD4 vs SSC and FSC vs SSC. IFN-$\gamma^+$and IL-4^- cells were defined as Th1 cells; IFN-γ^- and IL-4^+ cells were defined as Th2 cells.

4.5. Lymphocytes activation and proliferation assays in vitro

PBMCs were isolated from venous blood of healthy controls by Ficoll gradient centrifugation. PBMCs were cultured briefly in flat–bottom wells for 2 h (37 °C) to deplete monocytes. Nonadherent peripheral blood lymphocytes (PBL) were resuspended in RPMI–1640 medium containing 10% fetal calf serum and cultured in 6 –well plates (3×10^6 cells/well) with phytohemagglutinin (PHA) at a final concentration of 2 μg/ml and human leptin (Rochy Co. Ltd, America) in decreasing concentrations (5 nM, 0.5 nM, 0 nM) at 37 °C, 5% CO_2 for 72 h. After 68 h of culturing, monesin (1.7 μg/ml) was added. Th1, Th2 cells were detected by flow cytometry.
Aliquots of 100 μl lymphocytes suspension (1×10^5cells/well)were added to 96-well microculture plates. Lymphocytes were stimulated in the presence of PHA, or costimulated with leptin (5 nM) and PHA (2 μg/ml) in duplicate for 5 days. Control lymphocytes were cultured in the absence of PHA and leptin. After 5 days of culturing, lymphocyte proliferation was evaluated by MTT assay. The optical density (OD)was measured at 490 nm with an ultraviolet spectrophotometer. Proliferation/viability was expressed as: OD of stimulated – OD of non-stimulated cultures.

4.6. Statistical analysis

Statistical analysis was performed by using SPSS software, version 17 (SPSS 17.0; SPSS, Inc., Chicago, IL). All data were expressed as mean ± SD. Differences between groups were evaluated by one-way ANOVA and paired-samples t test.The P values < 0.05 was considered to be significant (two-tailed analysis).

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