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Exogenous leptin restores in vitro T cell proliferation and cytokine synthesis in patients with Common Variable Immunodeficiency Syndrome

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Abstract

Common variable immunodeficiency (CVID) is a primary immunodeficiency characterized by hypogammaglobulinemia. Leptin has been implicated as an antiapoptotic compound as well as a stimulant of the immune response. Leptin administration is capable of reversing the immune deficiency that occurs upon starvation. We investigated a possible role for leptin in CVID; a condition associated with lowered plasma leptin levels. Thirty-eight patients were studied. Addition of leptin to the tissue culture media of PBMC from CVID patients increased the proliferative response of lymphocytes to mitogens and decreased activation-induced apoptosis of these cells. IL-2 and specially IL-4 production also increased significantly upon addition of leptin to the PBMC cultures. Our results suggest that leptin may be involved in some of the cellular defects observed in CVID and indicate a novel therapeutic strategy to improve immune function in these patients.
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Introduction

Leptin was originally characterized as an essential hormone governing satiety and hunger, energy expenditure and thermogenesis in order to control body fat stores [1]. Intricate mechanisms link leptin function to insulin levels and to other factors involved in homeostasis [2]. There is a positive correlation between leptin serum levels and body mass index (BMI); however, significant heterogeneity of individual indices points to other factors affecting regulation of leptin levels [3]. Besides thyroxin, and growth and sex hormones, leptin levels are also influenced, for instance, by diet. Acute starvation associated with low leptin levels causes thymus atrophy and reduces the delayed type hypersensitivity reaction to antigens in normal mice, resembling observations in ob/ob mice [4]. Leptin replacement reverses these effects [5,6]. Knockout mice (leptin or leptin receptor deficient) also show impaired T cell immunity [7,8]. The association of malnutrition [9] and/or starvation [10] with suppression of T cell immune responses has prompted further studies showing that leptin is capable

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of restoring proliferation and inflammatory T1 type responses in undernourished individuals [9,11]. Other studies show that leptin not only influences T cell function but also induces activation and secretion of IL-6 and TNF by peripheral monocytes [12,13]. Leptin affects in vitro proliferation and cytokine production in naive and memory T cells. In naive T cells, proliferation and IL-2 secretion are increased; but in memory T cells, there is hardly any proliferation or significant IFN-γ production. In addition, leptin seems to be necessary for maintenance of the T1 type oriented inflammatory response [5,14,15].

The effect of leptin on T cells from patients with primary immunodeficiencies has not been studied. Common variable immunodeficiency (CVID) is the most common primary immunodeficiency diagnosed in young adults, being characterized by low serum immunoglobulin IgG, IgA, and often IgM. The decreased antibody levels lead to bacterial and viral infections in the respiratory and gastrointestinal tracts. However, there are a number of other symptoms, including inflammatory conditions, allergic reactions, autoimmune disease, and the development of lymphomas and other types of malignancies that cannot be explained based solely on diminished immunoglobulin levels [16]. A number of defects in T cell function and deficits in the memory B cell pool have been identified, but the underlying causes remain unknown. Prognosis is often hindered by heterogeneity in the clinical manifestations, but established clinical and laboratory criteria allow the diagnosis of CVID patients [17].

In normal individuals, B lymphocyte activation, isotype switching, and differentiation to antibody-secreting plasma cells are dependent on cell to cell interactions and rely heavily on signals provided by T lymphocytes either directly or through professional antigen-presenting cells (APCs). Studies have uncovered defects in the expression of CD154 (CD40L) [18] and in the function of T cell costimulatory ligands such as CD28. An in vitro study has shown that stimulation through an anti-T cell receptor antibody in the presence of costimulatory anti-CD28 was incapable of inducing normal levels of IL-2 and IL-4 [19]. The interaction between CD27 and CD70, which is critical for plasma cell differentiation, has equally been reported to be affected in CVID patients [20].

B cells are influenced by a variety of cytokines. IL-2, IL-5, and IL-6 enhance proliferation and IL-4 protects B cells against apoptosis. Cytokines such as IL-6, IL-10, and IL-2 can dictate the outcome of differentiation into memory or plasma cells. Isotype switching is under the control of IL-4, IFN-γ, IL-5, and TGF-beta1 [21–23]. In fact, previous studies have revealed deficits in the production of IL-2, IL-4, IL-10, and IFN-γ by T cells from patients with CVID 2 [4,26]. Furthermore, it has been shown that long-term low-dose IL-2 replacement therapy increases T-cell-proliferative responses to mitogens, antigens, and to IL-2. It was also shown that IL-2 improves antigen-specific antibody synthesis in some of the treated patients, and although statistical significance was not achieved, patients treated with IL-2 recorded less days of bronchitis, diarrhea, and joint discomfort [27].

Taken together, the data in the literature suggest that the primary molecular defects leading to CVID may occur in B lymphocytes, T lymphocytes, APCs, or in all of these. Thus, T cell dysfunction may be an important factor leading to CVID. In our cohort, over 70% of the patients exhibited some degree of T cell dysfunction [24]. Because leptin has been associated with T cell function, we decided to investigate the effect of this molecule on T cells from these patients. Our findings uncover a previously unknown association between CVID and leptin deficiency. We observed highly significant changes in the in vitro PBMC responses of these patients upon leptin treatment. Furthermore, these results may help us understand disease progression in a subgroup of CVID patients that lose abruptly weight.

Patients and methods

Patients

CVID Patients (n = 38), with ages varying from 18 to 62 (mean age 33.32 years), from the Clinical Immunology and Allergy Division, University of São Paulo School of Medicine, were diagnosed according to the criteria defined by the WHO, PAGID, and LAGID [28]. All patients had a minimum follow-up of 1 year before sample collection and some of them have been followed for more than 8 years. The median time elapsed since the onset of symptoms and diagnosis varied from 6 months to 31 years (mean 10.5 years). Blood samples were obtained from noninfected patients immediately before intravenous immunoglobulin treatment. IgG levels were characteristically low; mean levels were 467.7 ± 78.8 g/l. Control samples (n = 40) were obtained from healthy, age- and weight-matched individuals referred to the hospital for investigation of ocular toxoplasmosis. B lymphocytes were counted by flow cytometry using monoclonal anti-CD19 antibody; values were within the expected range. Both CD4 and CD8 counts were lower in the patients, but abnormal values were more significant for CD4 T cells. CD4/CD8 ratios were thus altered in comparison to controls. A summary of the clinical data is shown in Table 1. The ethics committee in each of the institutions involved in this research approved all procedures.

Methods

Cell culture

Heparin-treated peripheral blood was obtained and mononuclear cells were separated by using gradient centrifugation (Isolymph, Gallard-Schlesinger, Cale Place, New York, USA) according to a previously described protocol, which yields 2–3 × 10^7 cells/10 ml of blood

Table 1
Characteristics of the studied population: clinical data for the complete set of CVID patients and control individuals

Parameter	Mean		SD		Minimum value		Maximum value		P
	CVID	CTRL	CVID	CTRL	CVID	CTRL	CVID	CTRL	
Age (years)	30.32	31.70	15.67	10.70	6	18	63	55	n.s.
Weight (kg)	53.97	67.12	14.12	11.65	20	50	77	103	<0.001
Height (m)	1.59	1.69	0.17	0.09	1.10	1.49	1.81	1.90	0.002
BMI	20.77	23.34	2.97	2.89	14.70	18.72	27.34	28.76	<0.001
IgG (mg/l)	467.7	1120	78.8	237	310	683	713	1800	<0.001
Serum leptin (ng/ml)	2.38	3.10	1.35	1.19	0.5	1.5	6.5	6.2	<0.001
CD4 (10E9/l)	869.1	1059.5	181.7	234.9	542	731	1278	1871	0.002
CD8 (10E9/l)	619.7	540.1	122.3	141.2	333	289	901	891	0.01
CD4/CD8	1.47	2.04	0.46	0.54	0.78	0.54	2.01	4.449	<0.001

Notes: CVID (n = 38): common variable immunodeficiency patients were diagnosed according to WHO, LAGID, and PAGID criteria; CTRL (n =40): control individuals; BMI: body mass index; P: statistical analysis for data calculated by chi-squared or Student's t tests.

[29]. Briefly, PBMCs were plated at 10^6 cells/ml in 96 flat-bottom well microtiter plates (Linbro-ICN/Flow, Costa Mesa, CA, USA) and cultured for up to 96 h in the absence or in the presence of 50 ng/ml recombinant leptin (R&D, Minneapolis, MN, USA) in 0.2 ml of DMEM (Life Technologies, Grand Island, NY, USA) enriched with 5% AB+ human serum pooled from blood donors (Biocell Laboratories Inc., Rancho Dominguez, CA, USA). Cultures were stimulated with 2.5 μg/ml of phytohemagglutinin-p (PHA-p) obtained from DIFCO (Detroit, MI, USA) and pulsed with 0.5 μCi per well of tritiated thymidine (Amersham Pharmacia Biotech, UK) 16–18 h before cell harvesting. Tritiated thymidine ([^{3}H]-Thy) uptake was measured by scintillation spectrometry of cells harvested onto glass fiber filters using a cell harvester (Wallac Oy, Turku, Finland). Cell proliferation, IL-4, and annexin-V measurements were performed after culturing for 48 h.

Flow cytometry

Leukocytes were obtained by treating peripheral blood with an erythrocyte lysing solution (FACSR Lysing Buffer, Becton Dickinson, Mountain View, CA) at room temperature for 10 min, protected from light. Cells were then washed with 2% human AB+ serum (Biocell Laboratories, Inc.) in PBS. An aliquot of the cell suspension of 100 μl (1.0×10^7 cell/ml) was incubated with anti-CD3/CD4/CD8 (BD/Immunocytometry Systems, San Jose, CA) for 30 min at room temperature, protected from light. Cells were washed three times with the 2% human AB+ serum PBS solution, and subsequently 10,000 events were counted in a FACScalibur cytometer (Becton Dickinson, Sunnyvale, CA). The results were analyzed using the CellQuest Program.

Cytokine analysis

Human IL-2 and IL-4 were measured by ELISA with paired antibodies (PharMingen/Becton Dickinson, San Diego) as described elsewhere [30].

Apoptosis in PBMC cell cultures

Apoptosis was measured by fluorescent recombinant annexin-V coupling to surface phosphatydilserine according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cells were washed and incubated for 10–15 min at room temperature with annexin-V, coupled to fluorescein in a Hepes buffer with Propidium iodide (PI), and analyzed on a flow cytometer using 488 nm excitation and a 515-nm band-pass filter for fluorescein detection and a filter >600 nm for PI detection.

Statistical analysis

Initially, the study was designed to select patients purposely matched to the controls as to body mass indices, serum leptin, and age in order to avoid a bias due to lower serum leptin or BMI values. However, mean and SD values in patients and controls were respectively 20.77 ± 2.97 and 23.34 ± 2.39 for BMI ($P < 0.001$) and 2.38 ± 1.35 and 3.10 ± 1.19 for leptin serum values ($P = 0.015$) (see Table 1). As we were not able to achieve the designed matching due to these differences, we excluded some of the patient (n = 9) and control samples (n = 8) that could not be matched for age and BMI and statistical analysis was applied to this matched subset. The results obtained with the matched set were in all instances comparable to the results on the complete set when the remainder of patients were included. Therefore, we chose to present here the results and conclusions referring to the whole cohort. The independent variables (sex, age, and BMI) were analyzed within patient and control groups by chi-squared test or Student's t test. Cell proliferation, annexin-V, IL-2, and IL-4 production were compared in two different time points (in the presence and in the absence of leptin) using ANOVA for repeated measurements. Since these variables showed significant differences between the CVID and control groups, a model was fitted to a general linear model adjusting for sex, age, and BMI. Stata 7.0 and SPSS 10.0 softwares were employed in all calculations.

Results

Irrespective of age, weight, and serum leptin levels of patients and controls, the overall comparison of control and

patients' T cell responses showed significant differences in all parameters analyzed, as shown in Fig. 1.

As expected, only 8 out of 38 CVID patients exhibited lymphocyte-proliferative response to PHA reaching the lower range observed for normal individuals. However, addition of leptin to the tissue culture media resulted in increased [^{3}H]-Thy uptake values by up to 1.5-fold (mean increase 36.87 ± 48.75%) in the proliferative response of lymphocytes from patients with CVID (Fig. 1A). This increase was observed in the majority of cases; but in a few instances, cpm values were either lower after the addition of the hormone, or no variation was observed. In contrast, proliferation of control lymphocytes did not vary when leptin was added to the culture medium (Fig. 1A). The same pattern was observed for annexin-V staining (Fig. 1B). Control values did not change in the presence of leptin with a mean of 16.9% positive cells in the presence or in the absence of leptin, ranging from of 9% to 27%. On the other hand, addition of leptin to lymphocyte cultures from patients with CVID resulted in a significant decrease in the number of annexin-V-stained cells, from approximately 39% to 24%. In some instances (eight patients), levels of annexin-V staining fell into the normal range. It is noteworthy that in patients where annexin staining values were close to the normal range, addition of leptin did not induce any change.

To further explore the effect of leptin on the immune response of CVID patients, we measured IL-2 and IL-4 secretion in response to PHA in the presence or absence of this hormone. The same pattern described above was repeated. Mean cytokine values for the patients were mostly below control levels. Both IL-2 and IL-4 productions by the patient's lymphocytes were increased in the presence of leptin, reaching the lower range of normal values in 44.7% and 71.0% of patients, respectively (Figs. 1C and D). Nevertheless, proliferation rates, apoptosis levels, and IL-2 or IL-4 production were not always normalized in the same patients. Only six cases showed correction of all parameters when cells were grown in the presence of leptin. In addition, the invariably low B lymphocyte count (around 1%) was unchanged in the presence of leptin, irrespective of leptin responsiveness (analysis of three patients, data not shown).

Univariate analyses of proliferation, annexin-V binding, and IL-2 and IL-4 production yielded the same results when the complete or the matched sets of data were employed. All

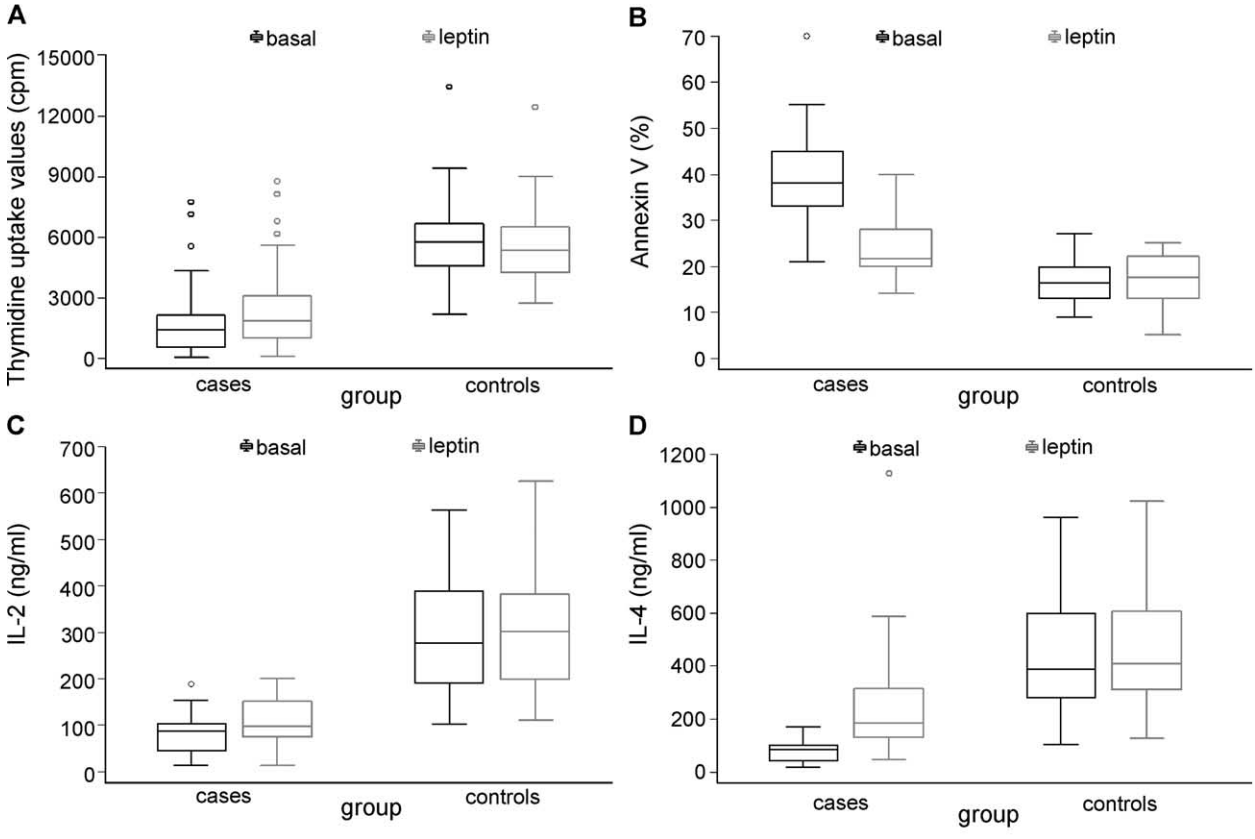

Fig. 1. Box plot presentation of results from CVID and control PBMC (10^6 cells), cultured for 48 h with 2.5 ng/ml PHA in the presence or absence (basal) of 50 ng/ml leptin. (A) Proliferation measured by thymidine uptake, values in cpm, (B) percentage of apoptosis measured by annexin-V binding using flow cytometry, (C) IL-2 production in ng/ml, and (D) IL-4 production in ng/ml, measured by ELISA. All analyses for the CVID group of patients were significant at $P < 0.001$ compared to controls.

parameters in the CVID group were significantly different ($P < 0.001$) when values with and without added leptin were compared. In the control group, there was no significant difference. Comparison of the values obtained for PBMC from CVID patients and controls, cultured with PHA only, showed significant differences in all 4 parameters ($P < 0.001$).

In our multivariate analysis, sex, age, and BMI were included as independent variables in addition to the four parameters of T cell function. Thus, eventual effects of the different BMI profile in the two groups were accounted for. In this weighted analysis, proliferation levels ($P = 0.07$) and IL-2 production ($P = 0.35$) lost significance. However, the two groups differed significantly in annexin-V binding ($P < 0.001$) and IL-4 production ($P < 0.001$).

Discussion

We have investigated the effect of leptin in CVID patients with a known impairment of both B and T cell function, as detected by clinical signs and quantified by low immunoglobulin G serum levels, CD4 and CD8 cell count, IL-2 production, and proliferative response to PHA [25]. We found that upon PHA stimulation of PBMC cultures, addition of leptin to the culture medium leads to a significant enhancement of cell proliferation and cytokine production. Leptin has also been identified as a cytokine with potent antiapoptotic effects on adipocytes [31], endothelial cells [32], T cells under stress [33], monocytes [34], as well as on other cell types [35,36]. Cells from healthy individuals did not exhibit any change in the level of apoptosis, corroborating the data from previous "in vitro" studies [15]. However, addition of leptin to the tissue culture medium significantly protected patients' cells from apoptotic cell death, as measured by annexin-V expression (mean ± SD, −14.07 ± 7.44%). The fact that leptin replacement restores, to some extent, T cell functions measured in cell cultures from patients with CVID suggests that a functional impairment of this hormone may be involved in the pathogenesis of the disease. Nevertheless, patient and control serum leptin levels in this study were essentially similar, although on the average, with values below those described in the literature for normal individuals [37]. The initial IL-4 production by the patients was, as expected, very low and in some instances even undetectable. Incubation with leptin increased IL-4 production in all but three patients to within normal range levels. Several studies have pointed out the deficit in IL-4 production in CVID patients upon a variety of stimuli [38,39]. To our knowledge, this is the first study that suggests a reversal of the typical low IL-4 secretion seen in this disease, implying a potential therapeutic role for leptin in the treatment of CVID patients in which no clear-cut B or T lymphocyte gene defect is identified. However, one should bear in mind that the number of B lymphocytes did not increase in the presence of leptin (measured in three patients), indicating that leptin alone may not be sufficient to correct for the functional defect in the B cell compartment.

It is noteworthy that proliferation and cytokine levels were always significantly lower in the patient group as compared to the control group and that leptin replacement only partially restored them to the lower end of the range exhibited by normal individuals for these parameters. These data suggest that in normal individuals the balance between available leptin and T cell function is optimal whereas in CVID patients it is shifted to require higher levels of this hormone. Because not all patients responded with an in vitro improvement of T cell function after exposure to leptin, the assertion may not be true for all individuals with CVID. The majority, however, does seem to respond to the hormone and some even exhibit a completely restored profile with respect to the response to PHA and cytokine production. Furthermore, in vivo studies in humans have shown the beneficial effects of recombinant leptin administration on CD4+ T cell count and impaired function in cases of congenital leptin deficiency [40].

The effects of leptin replacement on apoptotic cell death were striking. The addition of leptin to the cultures was very effective in decreasing apoptosis in most patients but did not induce any change in PBMCs from normal controls. Again, the improvement observed in the patients' cells only brought the percentages of apoptotic cell death close to control values since the basal levels for patients' cells were significantly higher (2.2-fold on average) than those of normal individuals. As in the case of proliferation and cytokine production, not all patients responded to leptin replacement since four individuals showed no effect on the rates of apoptosis. Further evidence for the complex nature of the defects in this disease comes from the fact that only six patients showed correction of all parameters when cells were cultured in the presence of leptin. The data also allow us to single out some patients for which leptin did not exert any visible effect on proliferation and/or cell death even when the initial values were abnormal.

Leptin receptors (Ob-R) are predominantly expressed on monocytes and a recent study using a monoclonal antibody against the extracellular domain of a recombinant leptin receptor has shown that expression on T lymphocytes is absent [41]. The issue is still controversial since in another study, employing polyclonal antibodies, leptin receptors were identified on T lymphocytes [14]. We confirmed the presence of messenger RNA for the long isoform of the leptin receptor in cell-sorted T cells from three patients (results not shown). Thus, we believe that the results observed in our assays were probably mediated by the direct effect of leptin on the T cells. Several studies have previously shown that stimulus through the leptin receptor leads to a T1-type inflammatory response with enhanced production of TNF and IL-6 by monocytes and of IL-2 and IFN-γ by T lymphocytes. We suggest that this effect may

suffice to partially restore function to T cells from CVID patients. The question as to why leptin does not have the same effects on cells from normal individuals remains open to discussion, but it may be explained by preferences established among the many redundant pathways that exist to induce T cell activation. In normal cells, the preferential pathways are in place and their presence inhibits the function of alternative pathways. In CVID patients, due to T cell defects, some or all of the preferential pathways might be impaired allowing alternative routes of activation to function, albeit not so efficiently. This situation would be analogous to the differences in behavior observed between knockout mice for a certain protein and normal mice treated with blockers for the same protein [42]. In fact, it has recently been confirmed that human leptin stimulates Janus kinase (JAK) signal transducer and activator of transcription (STAT), phosphatidylinositol 3-kinase (PI3K), and also mitogen-activated protein kinase (MAPK) pathways in PBMC [43]. Alternatively, we cannot exclude that as leptin structurally belongs to the long-chain helical cytokine family, which includes IL-2, IL-12, and GH [43,44], the high dose of leptin (50 ng/ml) employed in this study could have activated T cells through receptors belonging to the same class I cytokine receptor family, in this case, acting more like a cytokine than a hormone.

Taken together, the strong induction of IL-4 synthesis, the diminished levels of apoptosis, and the partial recovery of IL-2 secretion and T cell proliferation observed in this study further strengthens a role for leptin replacement as a possible strategy for treatment of CVID. IL-4 is a potent antiapoptotic factor for B lymphocytes, its signaling occurring through the STAT6 pathway and up-regulation of Bcl-xL [22]. We observed the increase in IL-4 but not in the number of B lymphocytes. Further studies should point out if this observation pertains to all CVID patients. Increased lymphocyte B count and T cell function is surely desirable in CVID patients and could eventually be achieved by a novel leptin-based therapeutic approach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clim.2004.09.002.

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