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Research article

Chlorella Modulation of Cytokine Production in Obese Mice and Minireview of the Effects of the Alga in Obesity

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Abstract

In the present study, we present a minireview of the modulating effects of the alga Chlorella (CV) in experimental obesity, including our pioneer findings on the ability of the alga to restore the balance in the disturbed cytokine network in obese mice. These findings disclose a new field of interest concerning the possible clinical application of the alga in a large range of pathological conditions.

Keywords: *Chlorella; Cytokines; Obesity; Insulin resistance, Adipose tissue macrophages*

Introduction

Obesity is a worldwide epidemic resulting in enormous costs to health-care systems [1,2]. Data from the World Health Organization shows that its incidence worldwide has doubled since the 1980s [3]. Currently this disease can be treated by a limited number of medicines that, in spite of demonstrating some efficacy by reducing body weight and improving metabolic parameters, produce a good number of undesirable side-effects [4-6]. Therefore, the search for alternative therapies, particularly of natural products, able to modulate the disturbances observed in this disease is receiving increasing attention [5-8]. In this context, the alga CV has emerged as an alternative treatment and prophylactic agent against obesity-related complications.

CV is a complete food, containing all the ingredients necessary to promote human health. Its well-balanced nutrients include carbohydrates, proteins, nucleic acids, essential amino acids, fatty acids (ω-3 and ω-6), vitamins, dietary fiber, growth factors and antioxidants (lutein, α- and β-carotene, ascorbic acid and tocopherol) [9-11]. It is considered a biological response modifier [12], as demonstrated by its protective activities against different types of stress in normal and immunosuppressed mice [13-22]. In this context, an important mechanism of the alga is its ability to prevent the immunosuppressive effects of stress by inhibiting the elevation of endogenous corticosteroids [23].

Notably, the stimulation of the pool of hematopoietic stem cells and the activation of mature leukocytes are important aspects of Chlorella effects on the immune system of immunocompromised hosts [15,24,25]. In this respect, studies from our laboratory have demonstrated that Chlorella treatment induces a significant recovery in the reduced number of myeloid progenitor cells (colony-forming unit granulocyte–macrophage, CFU-GM) in tumor-bearing [26] infected [14], and obese mice [27]. A potential explanation for the myelosuppression induced by different types of stress might be related to the HPA-axis dependent production of glucocorticoid hormones [28]. Therefore, relevant to the prevention of myelosuppression induced by CV in the immunocompromised host is its ability to inhibit the elevation of endogenous corticosteroids during stress [23,19].

Altogether, our findings suggest that adjuvant colony-stimulating factors produced by the algae treatment, which act synergistically for highly enriched CFU-GM in combinations of modulatory cytokines, may inhibit the suppressive effects of different types of stress on critical pools of hematopoietic progenitor cells, thus potentiating immune surveillance.

We present here a mini review of the modulating effects of CV in obese mice, and complement our previous findings with our pioneer results on the adaptogenic ability of the alga to restore to normal values the distinctly disturbed profiles of cytokine response produced by diseases related to immunosuppression (tumor, infection, lead exposure) and to chronic inflammation (obesity). These findings disclose a new field of interest concerning the possible clinical application of the alga in a large range of pathological conditions.

Materials and Methods

Mice

Six-week-old male Balb/C mice were maintained under specific pathogen-free conditions in a regimen of 12 h dark/light cycles and a controlled environment (room temperature: 22 ± 3°C, humidity: 55 ± 5%). The animals were randomly divided into four groups (n = 6 mice per group) as follows: standard rodent chow and vehicle (control-CT), standard rodent chow and Chlorella (CV), high-fat diet and vehicle (HFD) and high-fat diet + Chlorella (HFD+CV). The HFD consisted of 55% calories from fat, 29% from carbohydrate and 16% from protein, as described previously [11,29-31]. The animals received water and their respective diets ad libitum for the whole period. Body weight and fasting blood glucose were measured weekly. At the end of the experiment, insulin and glucose tolerance tests were performed as previously described [11,31]. All animal studies were approved by the Animal Care and Use Committee at the State University of Campinas (process: 1987-1) and are in accordance with the guidelines for the Care and Use of Laboratory Animals.

Chlorella and treatment

The dried alga *Parachlorell beyerinckii* CK-5 (CV), previously identified as Chlorella vulgaris CK-5, a strain of unicellular green alga, was kindly provided by Research Laboratories, Chlorella Industry Co., Ltd., Fukuoka, Japan. The nutritional and fatty acid composition was previously demonstrated [11]. Contamination by endotoxin of material remaining in bottle after CV treatment, assayed by the Limulus amebocyte assay, was less than 0.06 ng/ml, which corresponds to the limit of detection of the assay. CV was prepared in distilled water and doses of 50 mg/kg/day were given orally once daily by gavage of 0.2 ml volume/mouse in a prophylactic/therapeutic manner. Treatment was given for 5 days prior to starting HFD administration and extended for the 12 weeks of the study. CT and HFD groups received vehicle (distilled water) only. In all groups, the experiments were performed in the morning, 24 h after the last administration of CV. The selection of CV dose was based on the preliminary dose-response studies performed in our laboratory [14,26]. The treatment schedule used here was standardized thenceforth to be used in all works produced with the alga by our group [11,26,19,32,33,22,26,34,17,18].

Serum samples

Mice were bled from the heart under deep halothane anesthesia. Within each experimental group, the blood was left at 37^0C for 30 min and the clots were allowed to retract overnight at 4^0C. Following centrifugation, the serum was removed and stored at -20^0C for determinations of cytokine production.

Splenocytes culture

Suspensions of splenocytes from all mice in each group were prepared by gently pressing aseptically removed spleen through a stainless steel mesh net, washed in red blood cells lysis buffer (NH_4Cl 0.17 mol/l–1) followed by phosphate buffered saline, and centrifuged for 10 min at 1500 rpm. Viability was determined by trypan blue exclusion and consistently exceeded 90%. The cells were suspended in enriched RPMI 1640 culture medium supplemented (with 5% FBS, penicillin, mercaptoethanol, and streptomycin), and 1×10^6 cells/ml were seeded in presence of 5 μg/ml Con A (Sigma). After 48 h incubation at 37^0C in 5% CO_2, cell-free supernatants were collected for determinations of cytokines (MCP-1 and IL-10) production.

Quantification of cytokine levels

Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1, macrophage migration inhibitory factor (MIF), monocyte chemoattractant protein (MCP)-1, interferon (IFN)-γ, transforming growth factor (TGF)-β and IL-10 were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) in microtiter plates (96-well flat-bottom maxisorp microplate-NUNC, Roskilde, DM) using the following kits: anti-

TNF-α, anti-IL-1, anti-MIF, anti-IFN-γ, anti-TGF-β and anti-IL-10 (BD Biosciences, San Diego, CA, USA), and anti-MCP-1 (R&D Systems, Minneapolis, MN, USA). The cytokine levels were determined according to the BD Biosciences cytokine ELISA protocol. Cytokine titers were expressed in pg per mL and were calculated by reference to standard curves constructed with known amounts of recombinant cytokines.

Statistical analysis

Data were analyzed for statistically significant experimental differences using analysis of variance (ANOVA) followed by the Bonferroni test to compare data among all groups. Statistical significance was reached when $p < 0.05$. In all cases, at least three independent experiments were conducted to warrant that the results were representative.

Results

Serum levels of proinflammatory and anti-inflammatory cytokines

As presented in Figure 1., a significant ($p < 0.05$) increase in the production of proinflammatory TNF-α, IFNγ, IL-1α, MIF, MCP-1 and TGF-β, concomitantly to a decrease in anti-inflammatory IL-10, was found in the serum of obese mice, compared to controls. Treatment with the alga restored to control values the increased levels of TNF-α, IFNγ, IL-1α, MIF, MCP-1 and TGF-β levels, as well as the reduced levels of IL-10 ($p < 0.05$). No changes were produced by the alga in the levels of all these cytokines in control animals.

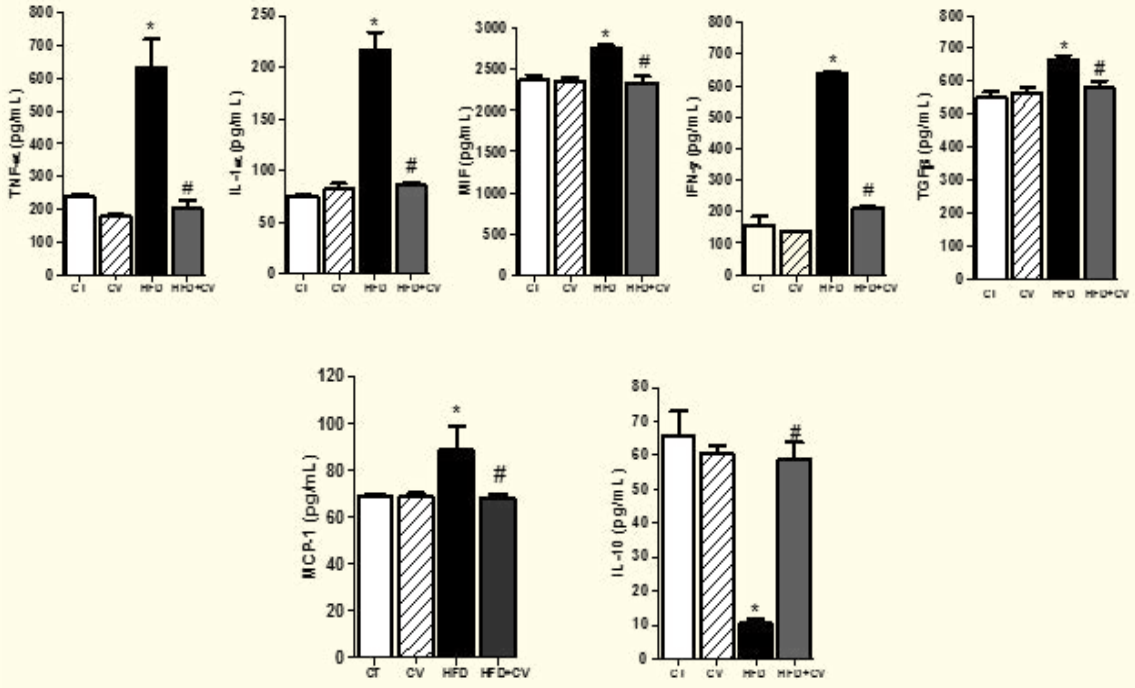

Figure 1: *Levels of pro- (TNF-α, IL-1α, MIF, IFNγ, TGF-β, MCP-1) and anti-inflammatory (IL-10) cytokines in serum.*

Levels of MCP-1 and IL-10 in the spleen of obese mice

As presented in Figure 2, a significant ($p < 0.05$) decrease in the levels of MCP-1 and IL-10 was observed in the spleen of obese mice, compared to controls. Treatment of these mice with Chlorella restored the production of both cytokines to control levels ($p < 0.05$). No changes in MCP-1 and IL-10 levels were produced by the alga in control mice.

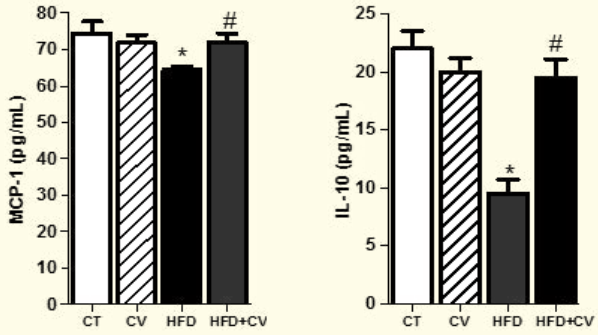

Figure 2: *Levels of pro- (MCP-1) and anti- inflammatory (IL-10) cytokines in the spleen.*

Discussion

It is well established that obesity is a state of chronic low-grade systemic inflammation deeply involved in insulin resistance and characterized by increased circulating concentrations of proinflammatory cytokines, in addition to the activation of inflammatory pathways [35-37].

Using different experimental models representative of immunosuppression, we have previously demonstrated that a core mechanism by which the alga Chlorella acts as a biological response modifier is by directly modulating the production of cytokines [34,18,19,20]. The common pattern of response in models of immunossupression [stress [19], exposure to lead [18], infection [34], and tumor [22]], is represented by a reduced production of proinflammatory cytokines (IFN-γ, IL-2 and TNF-α), simultaneously to increased production of the anti-inflammatory cytokine IL-10. Treatment with CV restored to normal levels the reduced production of the proinflammatory cytokines, as well as the increased production of IL-10, thus inducing a shift towards Th1 pattern of response. In the context of immunosuppression, the selection of the Th1 shift is important since this is the main mechanism that governs the resolution of pathological processes responsible for the recovery of the immunosuppressed host.

Conversely, in the present study, using a model of chronic low grade inflammation (obesity), in which the levels of proinflammatory cytokines are increased, and the anti-inflammatory response (IL-10) is reduced, we observed that the adaptogenic response of CV manifested as a down-modulation of the proinflammatory response and up-modulation of the anti-inflammatory response, leading, in both cases, to normal production of these cytokines, thus recovering a pattern of response that mitigates the obesity-associated state of chronic systemic inflammation. These adaptogenic effects of the alga on cytokine production in conditions of immunosuppression and inflammation are pioneer in the literature. These findings corroborate our previous studies [34,18,19,20] indicating that CV might have a direct adaptogenic effect through the induction of a normal pattern of endogenous cytokine production.

Increased production of proinflammatory cytokines is directly involved in the development of insulin resistance by disrupting the insulin signaling pathway through phosphorylation of insulin receptor substrate (IRS1) in the serine residue [35,38]. Of importance, our previous results show the ability of CV to prevent insulin resistance by increasing the phosphorylation of IRS1 on tyrosine residues in the liver, skeletal muscle and adipose tissue of obese mice. In addition, the alga lowered the phosphorylation levels of IRS-1^{ser307}, which is

used as a marker of insulin resistance in obesity [11]. This indicates that CV could regulate the IRS1 functions through a delicate balance between "positive" IRS1 tyrosine phosphorylation vs. "negative" IRS1 serine phosphorylation in combination with a modulatory effect in the production of cytokines.

In a pioneer study [27], we demonstrated a rapid decline in the number of granulocyte-macrophage progenitors (CFU-GM) in the bone marrow of obese mice, in association to a continuous migration of these cells into the spleen, a process characterized as extramedullary hematopoiesis (EMH). It is known that the migration, establishment and proliferation of hematopoietic cells into extramedullary tissues in the adult animal involve pathological changes in the hematopoietic stem cells, which include chronic inflammation, abnormal cytokine production, severe bone marrow failure and myelostimulation [39].

In this context, our present findings of increased circulating levels of MCP-1, a potent chemoattractant with an essential role in leukocyte trafficking and recruitment of proinflammatory macrophages (M1), concomitantly to its reduced levels in the spleen of obese mice suggest that, after differentiation, CFU-GM progenitors are attracted to leave the splenic microenvironment, being released into the blood and accumulated preferentially in the adipose tissue, where the levels of this cytokine are also increased [35,40].

Moreover, our present finding of increased serum levels of MIF, a specific attractant of activated macrophages, reinforces this assumption. It is known that accumulation of adipose tissue macrophages and consequent adipose tissue inflammation is a common feature in human and experimental obesity considered responsible for the majority of complications produced by this disease [39].

This expansion of inflammatory macrophages, along with the decrease in anti-inflammatory response in the visceral adipose tissue result in an imbalanced environment and is thought to drive insulin resistance and the progression to T2D in obese subjects [39]. Corroborating this hypothesis, our finding of increased expression of C-C chemokine receptor type 2 (CCR2) on granulocyte/macrophage progenitors in the spleen of obese mice points to an increased recruitment of monocytes, stem cell and progenitor cells to sites of inflammation [40,41]. Moreover, increased activity of hematopoietic cytokines (CSA) in the serum, in spite of reduced numbers of CFU-GM in the bone marrow, was also found in these mice. Importantly, treatment with CV restored to control values both bone marrow and spleen CFU-GM numbers, as well as the expression of CCR2, and further increased CSA in the serum.

Treatment with CV also restored to control levels the increased systemic levels of MIF and MCP-1, and the reduced production of MCP-1 in the spleen of obese mice. A possible mechanism involved in this modulating response of the alga might be that recruited proinflammatory macrophages switched to non-inflammatory macrophages (M2) on encountering a CV-driven local Th2 environment in adipose tissue. Consistent with this assumption, was the ability of the alga to restore to normal values the reduced levels of IL-10 (important M2 marker) in serum and spleen of obese mice. In addition, [43] reported that CV was able to lead to a dual activation of peroxisome proliferator activated receptors α/γ (PPARα/γ), which are known to play a key role in the activation of resident non-inflammatory macrophages in adipose tissue and liver, improving glucose tolerance, insulin levels, and reducing hyperlipidemia [44,45]. Moreover, recent studies [46] demonstrate the ability of the alga to modulate adipose tissue hypertrophy and adipokine secretion.

Together, these data suggest that one of the mechanisms which can explain the ability of CV to prevent the deleterious effects of inflammation in obesity is related to its ability to restore to control values the reduced CFU-GM numbers in the bone marrow, thus preventing the migration of these cells into the spleen, as demonstrated previously [27]. Therefore, the alga seems to prevent the development of extramedullary hematopoiesis and migration of inflammatory cells to target tissues, consequently avoiding the disruption of insulin signaling induced by the state of low-grade chronic inflammation induced by obesity.

A summary of the mechanisms discussed here on the consequences of the modulating mechanisms of CV on cytokine production in obesity relating to the present and previous findings from our laboratory, and others in the literature is presented in presented in Figure 3. Altogether our findings suggest that prevention by the alga of the deleterious effects induced by obesity is a good indicator for its use

as a prophylactic/therapeutic agent against obesity-related complications. The ability of CV to restore the physiological balance in the disturbed cytokine network with apparent lack of toxicity discloses a new field of interest concerning its possible clinical application in a wide range of pathological conditions, such as diabetes, atherosclerosis, metabolic syndrome, osteoarthritis, rheumatoid arthritis, among others [47-50], in which the unbalance of pro- and anti- inflammatory cytokines dictates the emergence and evolution of the pathological process. These results shed a new light on the triggering mechanisms responsible for adipose tissue macrophages accumulation induced by chronic low-grade systemic inflammation associated with a disturbed cytokine network during obesity. Moreover, the ability of the alga to modulate the shift in hematopoietic topographical hierarchy during inflammation is likely to have significant biological, diagnostic, and therapeutic implications in the treatment of insulin resistance.

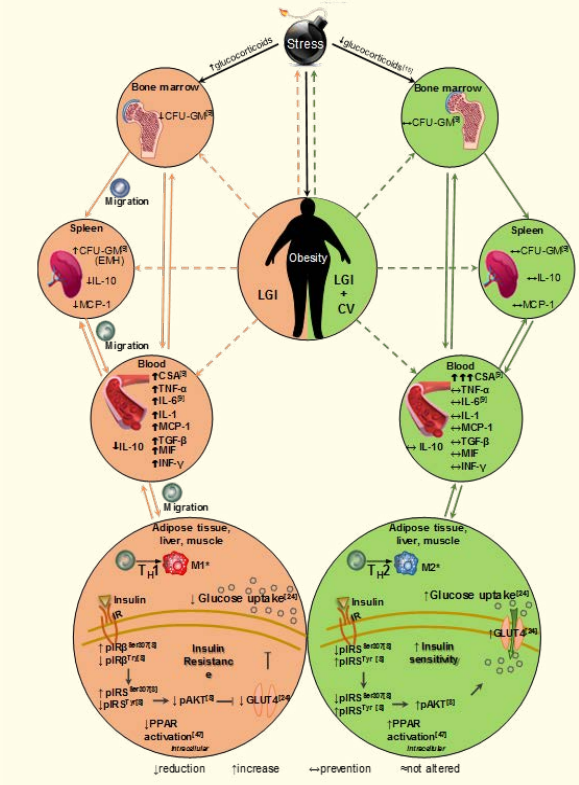

Figure 3: *Review of the consequences of the modulating mechanisms of CV on cytokine obesity.*

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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