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Chlorella vulgaris culture supernatant (CVS) reduces psychological stress-induced apoptosis in thymocytes of mice

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Abstract

A glycoprotein prepared from *Chlorella vulgaris* culture supernatant (CVS) is a biological response modifier (BRM) which exhibits protective activities against tumor metastasis and 5-fluorouracil-induced immunosuppression. We here show that oral administration of CVS prevented significantly the apoptosis of thymocytes in mice undergoing psychological stress in a communication box. Mice were exposed to the emotional stress for 14 days by witnessing other mice being exposed to foot-shock. The numbers in thymocytes, especially $CD4^+CD8^+$ population, were decreased significantly and apoptotic cells, as assessed by Annexin V expression, were reciprocally increased after the exposure to the psychological stress. *C. vulgaris* culture supernatant (CVS) administration significantly suppressed the increase in serum corticosterone level in the psychologically stressed mice. These results suggest that CVS prevents psychological stress and maintain homeostasis in the face of external environmental changes.

Keywords: *Chlorella vulgaris* culture supernatant; Psychological stress; Apoptosis

1. Introduction

Chlorella, a unicellular green algae, can divide into four cells every 16–20 h, utilizing sunlight for photosynthesis. *Chlorella* cells contain 55–67% protein, 1–4% chlorophylls, 9–18% dietary fiber and large amounts of minerals and vitamins. At present, *Chlorella* is widely sold as a health supplement in Japan, the US and other countries. Recently, we have reported that a glycoprotein-rich substance was released from *Chlorella vulgaris* strain CK-22 cells into the culture medium

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[1]. The substances, designated as *C. vulgaris* culture supernatant (CVS), is a glycoprotein with an approximate molecular weight 63 100 amu and contains 6-linked β-(1–6) galactopyranose-rich carbohydrate (66.9%) and protein (35.2%). It was reported that intra-tumor administration of CVS showed anti-tumor effects on both spontaneous and experimentally induced metastasis in mice [2].

It is generally accepted that immune, nervous and endocrine systems are linked biochemically and functionally. Interactions between the neuroendocrine and immune systems aim at maintaining homeostasis in the face of external environmental changes. There are various studies concerning the effects of stress on immune system. Stress affects the numbers and functions in monocytes, lymphocytes and CD4/CD8 ratio and impaired immune responses including antibody production, natural killer activity and lymphocyte responses to mitogen stimulation [3,4]. The effects of stress on immune responses have been attributed mainly to the adrenocortical hormones, glucocorticoids, which induce apoptosis in immunocompetent cells including thymocytes and peripheral T lymphocytes [5]. Suppressed immunity following the death of a spouse has implicated previously in the increased morbidity and mortality associated with bereavement. Not only physical stress but also psychological stress may thus weaken host defense against external pathogens and stimuli and internal tumor development.

Hara and Ohta have established the communication box method as an experiment tool for studying psychological stress [6]. They showed with the communication box that animals witnessing other animals being exposed to foot-shock developed gastric lesions and loss of appetite [7]. In the present study, we tested the ability of CVS to prevent the psychological stress responses in mice using the communication box. Oral administration of CVS prevented significantly the cell death by apoptosis in thymocytes and splenocytes accompanied by suppressed glucocorticoid level following a psychological stress. Implication of these findings for prophylactic and therapeutic use of CVS to maintain homeostasis in the face of external environmental changes during psychological stress.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice at 8 weeks of age were purchased from Charles River, Japan and kept in an animal facility. Mice were raised in a housing facility with constant temperature and humidity, and exposed to a 12/12-h light/dark cycle. Food and water were provided ad libitum.

2.2. Apparatus

Psychological stress was imposed using the communication box for mice. The floor of the communication box was equipped with grids for electrification. The inside was divided into 36 compartments (10×10 cm), consisting of foot-shock compartments with a grid floor and non-foot-shock compartments with a grid floor covered by insulated boards. The non-foot-shock compartments were arranged to surround the foot-shock compartments. The mice in foot-shock compartments received a foot-shock of 5 s duration at intervals of 15 s for 1 h per day. The electric current for the shock was 1–2 mA. Psychologically stressed mice (responder mice) were exposed to the emotional responses of foot-shock mice (sender mice) daily for 14 days. Sender mice were changed on alternate days to prevent a reduced emotional response based on adaptation or learned spiritlessness due to repeated electric stimulation.

2.3. Preparation of CVS diet

C. vulgaris strain CK-22 was cultured under aseptic conditions. *C. vulgaris* culture supernatant (CVS) was prepared from the culture fluid of *Chlorella* by centrifugation ($6200 \times g$ for 30 min) and ultrafiltration (cut-off MW 10 000, Nihon Millipore Co., Tokyo, Japan). Lyophilized CVS was added to CL-2 diet (Clea Japan, Tokyo, Japan) at a concentration of 2% (w/w). Solid chows containing CVS were given to C57BL/6 mice for 2 weeks before the psychological stress burdening and also during the 14 days of stress administration.

2.4. Flow cytometric (FCM) analysis

Freshly isolated thymocytes and splenocytes were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Lyt-2 (CD8) monoclonal antibody (mAb; PharMingen, San Diego, USA), phycoerythrin (PE) -conjugated anti-mouse L3T4 (CD4) mAb (Becton-Dickinson, CA, USA), or biotin-conjugated anti-mouse NK1.1 mAb (PharMingen, San Diego, USA) followed by FITC-conjugated streptavidin (Dako, Glostrup, Denmark). Cells were washed two times with Hank's balanced sodium solution (HBSS) (containing 2.5% Nu-serum (Collaborative Research Inc., MA, USA) and 0.1% sodium azide, and analyzed using a FACScan™ flow cytometer (Becton-Dickinson, CA, USA). With regard to the expression of Annexin V, freshly isolated thymocytes were stained with Annexin V-FITC Apoptosis Detection Kits™ (PharMingen, San Diego, USA). The percentage of apoptosis in thymocytes cultured with RPMI 1640 medium supplemented with 10% heat-inactivated fecal bovine serum (FBS, Equitech Bio, TX, USA), penicillin (100 mg/l), kanamycin (200 mg/l), 5 mM Hepes and 4.5 mM 2-mercaptoethanol was analyzed by FACScan™ and ModFit LT™ software (Becton-Dickinson, CA, USA). In the case of blood analysis, 100 μl of whole blood diluted three-fold with PBS was stained with FITC-conjugated anti-mouse F4/80 mAb (PharMingen, San Diego, USA) and biotin-conjugated anti-mouse Mac-1 mAb (PharMingen, San Diego, USA) followed by FITC-conjugated streptavidin (Dako, Glostrup, Denmark). Afterwards, red blood cells were lysed by adding 1 ml of Immunolyse™ (Coulter, Hialeah, FL) for 60 s, and cells were fixed with 250 μl of Fixative™ (Coulter, Hialeah, FL, USA). Cells were then washed two times in PBS and analyzed by FACScan™ (Becton-Dickinson, CA, USA).

2.5. Plasma corticosterone radioimmunoassay

Total plasma corticosterone was measured by a radioimmunoassay using rabbit antiserum [8]. Antiserum against corticosterone was produced in rabbits immunized with corticosterone-21-hemisuccinate conjugated to bovine serum albumin. Total plasma corticosterone levels are expressed in units of ng of corticosterone per ml serum.

2.6. Detection of cytokine mRNA by reverse transcriptase chain reaction (RT-PCR)

After solid chows containing 2% CVS were given to normal mice for 2 weeks, RT-PCR analysis of cytokine mRNA in the adherent PEC from the mice. Total RNAs were extracted from the adherent PEC and reverse-transcribed. The cytokine mRNA expression patterns of IL-1α, TNFα, GM-CSF, and IL-10 were determined by PCR using the respective murine cytokine-specific primers. PCR primer pairs specific for murine β-actin were synthesized at Takara Shuzo (Tokyo, Japan) according to published primer sequences [9]. PCR primer pairs specific for murine IL-1α, TNFα, and GM-CSF were purchased from Stratagene (La Jolla, CA, USA). PCR primer pairs specific for murine IL-10 were purchased from Clontech (Palo Alto, CA, USA). The methods of cDNA preparation and PCR analysis followed by electrophoresis were described previously [10]. Relative quantification of the amounts of the RT-PCR products was performed using a computing densitometer and MasterScan™ software.

2.7. Statistical analysis

The statistical significance of the data was determined by Student's *t*-test. A P value of less than 0.05 was taken as significant.

3. Results

3.1. Effects of CVS administration on the cell numbers of thymocytes and splenocytes in psychologically stressed mice

The numbers of thymocytes and splenocytes were measured on day 14 after a psychological stress using the communication box. As shown in Fig. 1, the number of thymocytes was decreased

significantly in the psychologically stressed mice, whereas such stress-induced decrease was not evident in mice given CVS orally. Similar results were obtained in the splenocytes (Fig. 1). Thus, CVS administration prevented significantly the atrophy of thymus and spleen induced by the psychological stress.

3.2. Effects of CVS administration on the cell populations in thymus, spleen and blood in psychologically stressed mice

We next examined the expression of cell surface antigens on the thymocytes, splenocytes and blood leukocytes in the psychologically stressed mice by FCM analysis. The absolute number of each population was calculated by multiplying the whole number by the percentage. As shown in Fig. 2, the numbers of $CD4^+CD8^+$ cells in the thymus and $CD4^+$ cells in the spleen were decreased markedly in the psychologically stressed mice, whereas the numbers of $CD4^+CD8^+$ cells in the thymus and $CD4^+$ cells in the spleen were not decreased in CVS-administrated mice after the psychological stress. It is well known that the number of NK cells and the NK cell activity are decreased by various stresses. Consistent with these findings, the number of $NK1.1^+$ cell in the spleen was significantly decreased in the psychologically stressed mice, whereas such a decrease was only marginal in CVS-administered mice. To determine the change of granulocytes and macrophages following the psychological stress, we examined the expression of Mac-1 and F4/80 on blood leukocytes in the psychologically stressed mice. As shown in Fig. 2, the percentage of $Mac\text{-}1^+/F4/80^-$ cells corresponding to granulocytes in blood leukocytes was decreased in the psychologically stressed mice, whereas the percentage of $F4/80^+$ cells, representative of mature macrophages, was not changed after the psychologically stressed mice (data not shown). Oral administration of CVS prevented significantly the reduction of the granulocytes in blood after psychologically stressed mice (Fig. 2).

3.3. Effects of CVS administration on the expression of Annexin V of thymocytes in the psychologically stressed mice

It is well known that stress induces the cell death by apoptosis in thymocytes [11,12]. We next analyzed early apoptotic thymocytes by staining with Annexin V, which indicate nuclear change such as DNA fragmentation [13]. As shown in Fig. 3, the percentage of thymocytes expressing

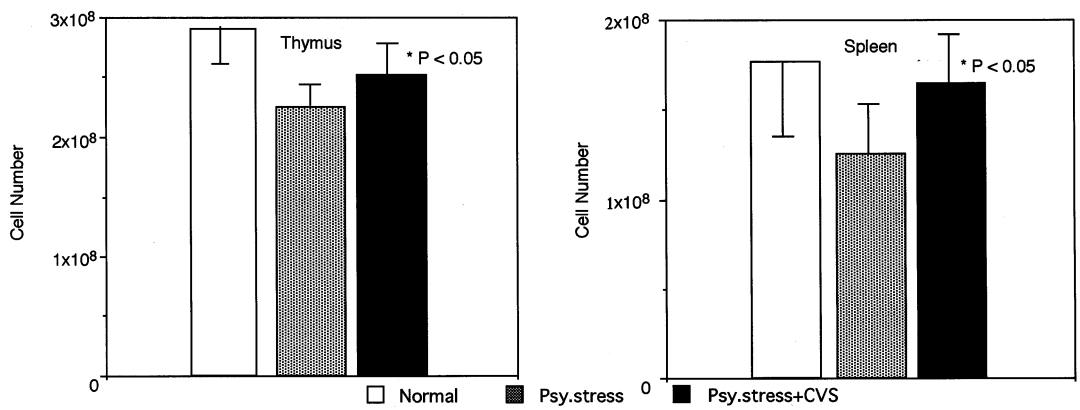

Fig. 1. Effects of CVS on the cell numbers of thymocytes and splenocytes in psychologically stressed mice. Female 8-week-old, C57BL/6 mice were burdened with psychological stress using the communication box on day 1 to day 14. Mice were sacrificed by decapitation on day 14. Thymocytes and splenocytes were isolated and the number of viable cells was counted. Solid chows containing 2% CVS (w/w) were given to the mice burdened with stress on day -14 to day 14. The data represent mean $\pm$ S.D. for six mice. The asterisk indicates the statistical significance ($^*P < 0.05$) as compared with the value of psychologically stressed mice by Student's *t*-test.

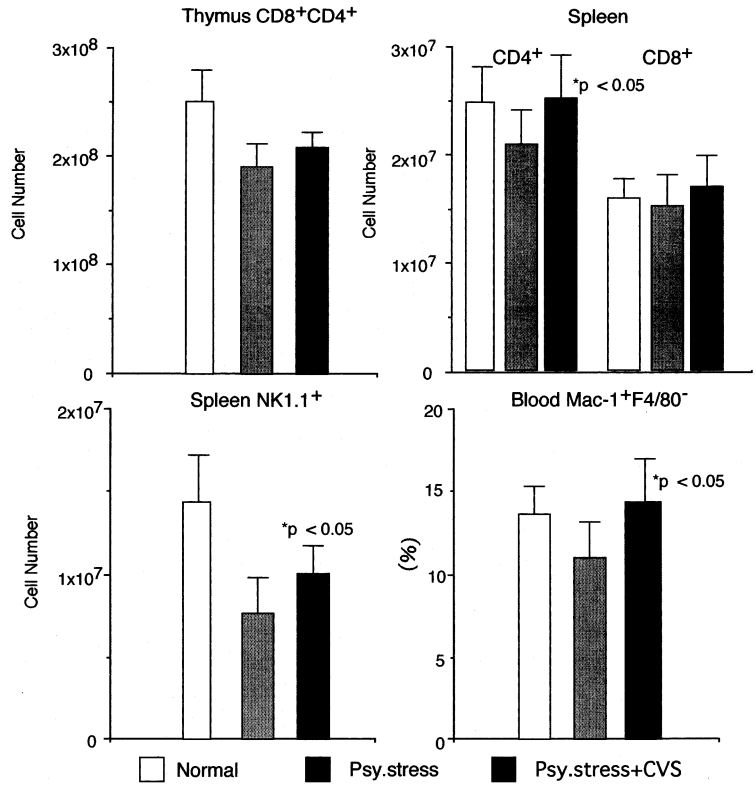

Fig. 2. Effects of CVS on the numbers of thymocyte, splenocyte, and blood leukocyte subsets after psychological stress using the communication box for 14 days. Freshly isolated thymocytes and splenocytes were stained with FITC-conjugated anti-mouse Lyt-2 (CD8) mAb, and PE-conjugated anti-mouse L3T4 (CD4) mAb or biotin-conjugated anti-mouse NK1.1 mAb followed by FITC-conjugated streptavidin, respectively. Blood leukocytes were stained with FITC-conjugated anti-mouse F4/80 mAb and biotin-conjugated anti-mouse Mac-1 mAb followed by FITC-conjugated streptavidin. Solid chows containing 2% CVS (w/w) were given to the mice burdened with stress on day -14 to day 14. The data represent mean $\pm$ S.D. for six mice. The asterisk indicates statistical significance ($^*P < 0.05$) as compared with the value of psychologically stressed mice by Student's t-test.

Annexin V^+PI^-, which represent the early apoptotic cells, was increased in the psychologically stressed mice, whereas oral administration of CVS inhibited the increases in the apoptotic cells in the thymus after psychological stress. We examined further the frequencies of apoptotic cells after 24-h in vitro culture of thymocyte from the psychologically stressed mice. The thymocytes were cultured in RPMI 1640 medium supplemented with 10% FBS for 24 h and the percentage of apoptotic cell was analyzed by ModFit LT™ software. As shown in Fig. 4, the percentage of apoptotic cells in thymocytes from normal control mice was 23.5% after the in vitro culture and the percentage markedly increased in the psychologically stressed mice. On the other hand, such increase was only marginal in CVS-administered and the psychologically stressed mice. These results suggested that CVS prevented significantly the apoptosis in thymocytes in the psychologically stressed mice.

3.4. Effects of CVS administration on the serum corticosterone level in the psychologically stressed mice

It was reported that stress enhanced secretion of corticosterone by the adrenal cortex, and corticosterone induced a significant degree of apoptosis [11]. Therefore, we next examined the serum

corticosterone level in the psychologically stressed mice. As shown in Table 1, the serum level of corticosterone increased markedly in the psychologically stressed mice, whereas CVS-administered and the psychologically stressed mice did not show any increase of serum corticosterone. Thus, CVS administration inhibited the increase of corticosterone level following the psychological stress.

3.5. Effects of CVS on the expression of cytokine mRNA in the adherent PEC from normal mice

We examined the effects of oral administration of CVS on cytokine gene expression in macrophages in mice just before psychological stress. Total RNAs extracted from adherent PEC from normal mice or mice given CVS orally for 2 weeks were reverse-transcribed and amplified with cytokine-specific primers, as described in Section 2. As shown in Fig. 5, the elevated levels of mRNAs specific for IL-1α, TNFα, and GM-CSF were detected in the adherent PEC from CVS-administered mice, while the IL-10 levels seemed to be decreased slightly by CVS administration. These results suggest that oral administration of CVS may activate macrophages to produce inflammatory cytokines such as IL-1α, and TNFα.

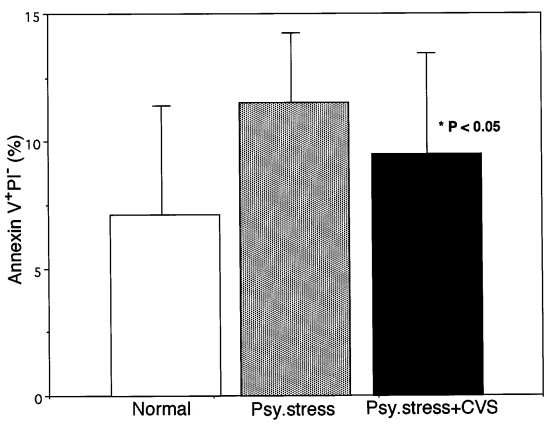

Fig. 3. Effects of CVS on the expression of Annexin V of thymocytes in psychologically stressed mice. Female 8-week-old C57BL/6 mice were burdened with psychological stress using the communication box on day 1–14. Mice were sacrificed by decapitation on day 14. Freshly isolated thymocytes were stained with Annexin V-FITC Apoptosis Detection Kits™ (PharMingen), and analyzed by FACScan™ (Becton-Dickinson). Solid chows containing 2% CVS (w/w) were given to the mice burdened with stress on day −14 to day 14. The data represent mean ± S.D. for six mice. The asterisk indicates statistical significance ($*P < 0.05$) as compared with the value of psychologically stressed mice by Student's *t*-test.

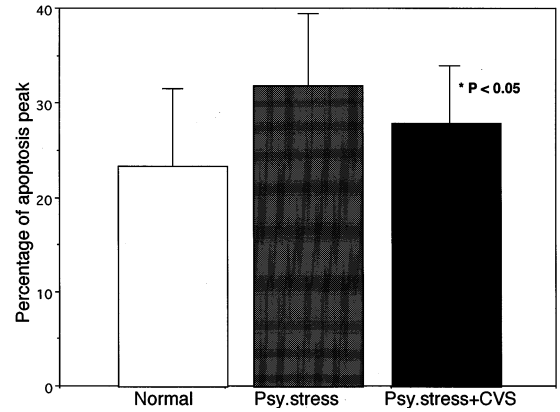

Fig. 4. Effects of CVS on the percentage of apoptosis of thymocytes in psychologically stressed mice. Female 8-week-old, C57BL/6 mice were burdened with psychological stress using the communication box on day 1–14. Mice were sacrificed by decapitation on day 14. The thymocytes were isolated, and cultured with RPMI 1640 medium supplemented with 10% FBS for 24 h. After 24 h, the percentage of apoptosis of thymocytes were analyzed by ModFit LT™ software (becton-Dickinson). Solid chows containing 2% CVS (w/w) were given to the mice burdened with stress on day −14 to day 14. The data represent mean ± S.D. for six mice. The asterisk indicates the statistical significance ($*P < 0.05$) as compared with the value of psychologically stressed mice by Student's *t*-test.

Table 1
Effects of CVS on serum corticosterone level in psychologically stressed mice[a]

Group	Serum corticosterone level (ng/ml)
Normal	261.3 ± 41.6
Psychological stress	557.0 ± 55.4
Psychological stress + CVS	229.3 ± 56.2**

[a] Female 8-week-old C57BL/6 mice burdened with psychological stress repeatedly on day 1–14. Mice were sacrificed by decapitation on day 14. The serum corticosterone level was measured by radioimmunoassay. Solid chows containing 2% CVS (w/w) were given to the mice burdened with stress on day −14 to day 14. The data represent mean ± S.D. for six mice. The asterisks indicate statistical significance ($**P < 0.01$) as compared with the value of psychologically stressed mice.

4. Discussion

Stress is defined as a complex dynamic condition in which normal homeostasis is disturbed. The disturbed state may be induced by many complicated physical stressors, including electric stress, water emersion stress and restraint stress. The hypothalamic pituitary adrenal (HPA) axis releasing endogenous glucocorticoids is thought to play a crucial role in the stress responses including apoptosis of thymocytes and mature T cells [14]. We here show the first evidence indicating that the psychological stress applied using the communication box induced apoptosis in thymocytes and splenocytes accompanied by increased levels of serum glucocorticoid. Notably, administration of CVS inhibited significantly the increases in serum corticosteroid levels and prevented apoptosis in thymocytes in the psychologically stressed mice. These results suggest that CVS, a biological response modifier (BRM), can be applied for a prophylactic approach to control the psychological stress.

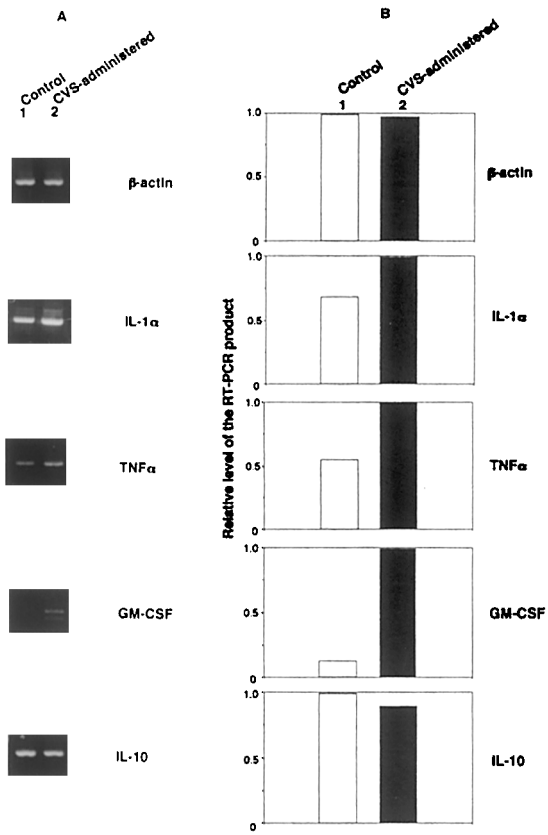

Fig. 5. RT-PCR of analysis of cytokine mRNA expression in the adherent PEC from mice given CVS. Solid chows containing 2% CVS were given to the normal mice for 2 weeks previous to RNA extraction. Total RNAs were extracted from the adherent PEC and reverse-transcribed. The cytokine mRNA expression patterns of IL-1α, TNFα, GM-CSF, and IL-10 were determined by PCR using murine cytokine-specific primers. The PCR products were separated in a 2% agarose gel and visualized by ethidium bromide staining. Relative quantification of the amounts of the RT-PCR products was performed using a computing densitometer and MasterScan™ software.

It is well known that exogenous and endogenous glucocorticoid induce cell death by apoptosis in thymocytes, mature T cells and intestinal intraepithelial cells (i-IEL) [15,16]. Administration with glucocorticoid induced thymic involution through the enhancement of apoptosis, influencing primarily the $CD4^+CD8^+$ thymocytes. It has been shown that apoptosis of thymocytes is also induced by elevated endogenous glucocorticoids in chicken injected by supernatant of mitogen-induced spleen cells [17] and in the physically stressed rats [11,12]. Glucocorticoid also induced programmed cell death in mature T cells [11,18]. We have reported previously that apoptosis of i-IEL can be induced under stressed condition [16]. In mice subjected to water immersion stress, an increase in the DNA fragmentation of i-IEL and a subsequent decrease in the number of viable i-IEL were observed in similar kinetics as the in vivo administration of glucocorticoids. The elevated corticosterone levels in the plasma of water-emersion stressed mice suggest that endogenous glucocorticoids might be a major factor inducing apoptosis of i-IEL after stress. Similarly, we have found that apoptosis of thymocytes can be induced in the psychologically stressed mice accompanied by elevated levels of serum corticosteroids. The physiological stress model has demonstrated that animals' witnessing other animals being exposed to foot-electric shock result in development of gastric lesions and depression of appetites [7]. The communication box is divided into small compartments consisting of the foot-shock compartments and the non-foot-shock compartments by transparent plastic boards. The mice placed in

the foot-shock compartment are exposed to electric shock, while, the mice placed in the non-foot-shock compartments cannot avoid perceiving the emotional behavior, such as shrieking, the smells of feces and urine, and jumping reaction of the mice subjected to foot-shock. Using this stress model, we found an elevated serum glucocorticoid in the psychologically stressed mice. Although there is no direct evidence, it is possible that endogenous glucocorticoids are involved in induction of apoptosis in thymus and splenocytes in the psychologically stressed mice.

Notably, CVS administration inhibited significantly the elevation of serum glucocorticoid levels after the repeated psychological stress and, concurrently, prevented the decrease in number of thymocytes, neutrophils and spleen cells due to cell death by apoptosis. Thus, CVS prevents the psychological stress as assessed by apoptosis of thymocyte and serum glucocorticoid level. At present, the mechanism, whereby CVS suppressed the apoptosis of thymocytes induced by the psychological stress, remains unknown. It is most likely that elevated endogenous glucocorticoids induce the apoptosis of thymocytes in the psychological stress. *C. vulgaris* culture supernatant (CVS) may prevent the apoptosis through suppressing the elevation of endogenous corticosteroid.

C. vulgaris culture supernatant (CVS) is known to stimulate macrophages to produce pro-inflammatory cytokines such as IL-1β, TNF-α and IL-12, of which transcriptions are required for NF-kb activation [10]. Therefore, such an activity of CVS to induce cytokine production may be involved in suppression of elevation of stress-induced corticosterone production. Certain pro-inflammatory cytokines such as IL-1β activate the hypothalamic-pituitary-adrenal axis for glucocorticoid release [19]. In contrast, in our results, CVS suppressed significantly the elevation of serum corticosteroids after psychological stress. There are several possibilities to explain the discrepancies. Although IL-1β are well-known to stimulate the hypothalamic pituitary corticotropin-releasing hormone (CRH)/adrenocorticotropic hormone (ACTH) system [20], thereby evoking secretory responses by the adrenal cortex, chronic stimulation with such cytokines induced by CVS may make the HPA axis refractory against the psychological stress. Alternatively since TNF-α and IL-1α are reported to depress glucocorticoid synthesis by adrenal cortex [21], CVS directly suppresses the activity of adrenal cortex to produce glucocorticoids. IL-1α is reported to reduce glucocorticoid receptor translocation and function [21]. Therefore, it is also possible that CVS may reduce glucocorticoid receptor function on thymocytes and splenocytes beside suppression of glucocorticoid production. Servatius et al. reported that elevated serum cholesterol levels have been observed in rats given stress exposure [22]. The results suggest that glucocorticoid and cholesterol metabolism are influenced by stress. It has been reported that *Chlorella* has cholesterol-lowering effects in rats fed a high cholesterol diet and found that *Chlorella* prevented the absorption of endogenous and exogenous cholesterol in bile and increased the excretion of cholesterol from the body [23]. *Chlorella* may be involved in the inhibition of absorption of exogenous steroids and promotion of turnover of bile acids in rats. Therefore, it is also possible that CVS administration accelerates the turnover of cholesterol metabolism and, consequently, suppresses the elevation of stress-induced glucocorticoid production. However, these are only speculations and further experiments are needed to clarify the mechanisms whereby CVS prevented the psychological stress.

In acute infection, pro-inflammatory cytokines induced glucocorticoid via stimulation of HPA axis and this hormone can protect against cytokine-mediated pathologies [24]. On the other hand, long lasting psychological stress such as the death of a spouse may weaken the host defense against external pathogens, surveillance of cancer development and stimuli for a long time [25–27]. Suppressed immunity, following the long-lasting psychological stress, has been implicated in the increased morbidity and mortality associated with bereavement. Oral administration of CVS may be useful not only for potentiating immune surveillance but also for preventing stress-induced immunosuppression.

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