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Dietary effect of pomegranate seed oil on immune function and lipid metabolism in mice

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Abstract

Objectives: We evaluated the effects of dietary pomegranate seed oil (PSO), which contains high levels of punicic acid (9*c*, 11*t*, 13*c*-octadecatrienoic acid), on immune function and lipid metabolism in C57BL/6N mice.

Methods: Mice were fed experimental diets containing 0%, 0.12%, or 1.2% PSO for 3 wk.

Results: No significant differences were observed between growth patterns of the experimental groups. Splenocytes isolated from mice fed 0.12% or 1.2% PSO produced larger amounts of immunoglobulins G and M but not immunoglobulin A irrespective of stimulation with or without phorbol 12-myristate 13-acetate and the calcium ionophore A23187. Dietary PSO did not affect the percentages of B cells or CD4-positive or CD8-positive T cells in splenocytes. Levels of interleukin-4, interferon-γ, and tumor necrosis factor-α production from splenocytes were comparable among all dietary groups. Analysis of serum lipid parameters showed significant increases in serum triacylglycerol and phospholipid levels but not in total cholesterol in the PSO groups. Serum, liver, epididymal, and perirenal adipose punicic acid levels were high with increases in dietary PSO level. However, punicic acid was not detected in splenocytes for any dietary group. Interestingly, 9*c*, 11*t*-conjugated linoleic acid level could be detected in serum, liver, and adipose tissues in mice fed the 0.12% or 1.2% PSO diet.

Conclusions: These results suggest that PSO may enhance B-cell function in vivo.

Keywords: Pomegranate seed oil; Punicic acid; Immunoglobulin; Mice; Cytokine

Introduction

There are accumulating data indicating that dietary fat manipulates various immunologic functions. For example, modulation of dietary fat quality, such as dietary fat level and the balance of ω-6/ω-3 polyunsaturated fatty acids, strongly affects lymphocyte function [1,2]. The balance of dietary fatty acids and of trace fatty acids in food components is also expected to influence immune function. Conjugated linoleic acid (CLA) is a generic term for the positional and structural isomers of octadecadienoic acid. CLA has been reported to exert various beneficial physiologic functions at extremely low dietary levels. Our previous study showed that CLA strongly promotes immunoglobulin (Ig) production and modulates the production of various cytokines in rat and mouse splenocytes [3–6]. In addition, 10*trans*, 12*cis*-CLA promoted Ig production, whereas 9*cis*, 11*trans*-CLA promoted tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) production. These observations suggest that dietary conjugated polyunsaturated fatty acids exert potent and beneficial physiologic functions through

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their immunomodulatory effects. Further, pomegranate seed oil (PSO) contains large amounts of 9*cis*, 11*trans*, 13*trans*-conjugated linolenic acid (CLN), called punicic acid. Various CLNs have been shown to inhibit the growth of transplanted cancer cells or to exert cancer cell killing activity in vitro [7–9]. No evidence has yet been reported to suggest immunomodulatory functions of CLN. We studied the effects of dietary PSO on the function of splenocytes in mice.

Materials and methods

Pomegranate seed oil (fatty acid composition presented in Table 1) was prepared at the Plantech Research Institute (Yokohama, Japan), and soybean oil was purchased from Sigma (St. Louis, MO, USA). Male, 4-wk-old C57BL/6N mice ($n = 24$; Japan CLEA Inc., Tokyo, Japan) were fed a non-purified commercial pellet diet and given water ad libitum for 1 wk after their arrival. After acclimation, mice were assigned to one of three groups of eight animals each. Mice were kept at the Biotron Institute of Kyushu University (Fukuoka, Japan) under a 12-h light/12-h dark cycle (lights on from 8:00 AM to 8:00 PM) in an air-conditioned room (20°C and 60% humidity under specific pathogen-free conditions). This experiment was carried out according to the guidelines for animal experiments of the Faculty of Agriculture and the Graduate Course, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Japanese Government. As presented in Table 1, experimental diets were manufactured according to AIN-93G standards [10] and contained 0%, 0.12%, or 1.2% PSO. At the end of the feeding period, mice were killed by exsanguination from the abdominal aorta under light anesthesia with diethyl ether. Immediately after excision, each tissue was weighed and lymphocytes were isolated from the spleen.

Preparation of spleen lymphocytes was performed according to a method described previously [4] without removing adhesive cells, such as macrophages and mononuclear cells. Briefly, a cell suspension prepared from the spleen was rinsed three times with RPMI-1640 medium (Nissui, Tokyo, Japan). Then 5-mL aliquots of cell suspension were added to Lympholyte-mouse (Cedarlane, Hornby, Canada) to isolate the lymphocytes and again washed three times with RPMI-1640 medium. Lymphocytes were cultured at 2.5×10^6 cells/mL in RPMI-1640 medium containing 10% fetal bovine serum (Intergen, Purchase, NY, USA) with or without 8 nM phorbol 12-myristate 13-actate (PMA) and the calcium ionophore A23187 at 150 nM, followed by incubation at 37°C for 24 h.

Table 1
Fatty acid composition of pomegranate seed oil

16:0	3.1
18:0	1.8
18:1 (ω-9)	5.4
18:1 (ω-7)	0.4
18:2 (ω-6)	5.3
18:3 (punicic acid)	83.1
20:0	0.4
20:1	0.5

Measurement of Ig concentration in the cultured medium was performed by sandwich enzyme-linked immunosorbent assay. Rabbit anti-mouse IgA (Zymed, San Francisco, CA, USA), goat anti-mouse IgG (H+L; Zymed), and rabbit anti-mouse IgM (μ-chain specific; Zymed) were used to fix each Ig. These antibodies were diluted using 10% Block Ace (Dainihon Pharmaceutical Co., Osaka, Japan), added to 96-well plates, and incubated for 1 h at 37°C. Aliquots containing 300 μL of 10% (25% for IgE) Block Ace were added and kept at 4°C overnight, and then samples (50 μL) were added to each well for 1 h at 37°C. Each well was treated with a solution of peroxidase (POD)–conjugated goat anti-mouse IgA (Zymed), POD-conjugated goat anti-mouse IgG (H+L; Zymed), or POD-conjugated rabbit anti-mouse IgM (Zymed) to detect the respective Ig and incubated for 1 h at 37°C (20 min at 4°C for IgE). Plates were rinsed with phosphate buffered saline containing 0.05% polyethylene sorbitan monolaurate (Nacalai Tesque, Kyoto, Japan) between each step. Then a 10:9:1 mixture of 1.8 mmol/L of hydrogen peroxide in 0.2 mol/L of citrate buffer (pH 4.0), H_2O, and 11.7 mmol/L of 2,2′-azinobis (3-ethylbenzothiazoline sulfonic acid) was added. Absorbance at 415 nm was measured after the addition of 160 mmol/L of oxalic acid to stop the coloring reaction.

We measured levels of interleukin-4 (IL-4), TNF-α, and IFN-γ from the supernatants of spleen lymphocytes cultured with PMA and A23187. TNF-α and IFN-γ levels were measured by sandwich enzyme-linked immunosorbent assay as reported previously [4]. Briefly, rabbit anti-mouse/rat IFN-γ (BioSource), anti-mouse TNF-α (Endogen, Woburn, Sunnyvale, MA, USA), or anti-mouse IL-4 (BD Bioscience, Franklin Lakes, NJ, USA; 1:500 dilution) were used to fix these cytokines for 1 h at 37°C (overnight at 4°C for IL-4). Then blocking was performed using 25% Block Ace at 37°C for 1 h. In the following step, each well was treated with 50 μL of appropriate cultured supernatant for 2 h at 37°C and then the plate was treated with a diluted solution of biotinylated anti-mouse IFN-γ (Genzyme, Cambridge, MA, USA; 1:500 dilution), biotinylated anti-mouse/rat TNF-α (Genzyme; 1:250 dilution), or biotinylated anti-mouse/rat IL-4 (BD Bioscience) for 1 h at 37°C. Streptavidin-conjugated POD (Zymed) diluted with 10% Block Ace was added to each well and incubated for 1 h at 37°C. Plate washing between each step and the coloring reaction was performed as described for the Ig measurement protocol. The coloring step was performed by the same method as described for quantification of Ig.

CD45R (a B-cell marker), CD4, and CD8 (T-cell subpopulation markers) cell surface expressions were analyzed by flow cytometry as reported previously [4]. After isolation of lymphocytes from the spleen, cells were washed three times with