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Soy milk suppresses cholesterol-induced inflammatory gene expression and improves the fatty acid profile in the skin of SD rats

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ABSTRACT

Recently, an elevation in skin cholesterol level has been implicated in skin inflammation. Given the potential therapeutic effects of soy on low grade inflammatory diseases, we hypothesized that a CHOL diet could promote an inflammatory response in skin and that soy milk (SM) or fermented soy milk (F.SM) could prevent this cholesterol-induced skin inflammation. To test this hypothesis, freeze-dried SM or F.SM was provided as a protein replacement for 20% of the casein in the diets of Sprague–Dawley (SD) rats. The animals were divided into the following groups: (1) control group (CTRL), AIN76A diet without cholesterol, (2) high cholesterol (CHOL) group, AIN76A with 1% (w/w) cholesterol, (3) SM group, CHOL diet with freeze-dried SM, and (4) F.SM group, CHOL diet with F.SM. In the CHOL group, the expression levels of pro-inflammatory genes, including IL-1β, IL-1α, iNOS, and COX-2, were elevated. In comparison, the SM and F.SM groups displayed the lowered expression of IL-1β, COX-2, F4/80, and Cd68, an increase of a n-3/n-6 ratio, and a reduction in the estimated desaturase activities of delta 5 desaturase (D5D) and steaoryl CoA desaturase (SCD-1). In particular, F.SM significantly increased the proportion of dihomo-γ-linolenic acid (DGLA) in skin fatty acid (FA) composition compared with the CHOL group. Here we present evidence that SM or F.SM could alleviate the inflammatory response in the skin that is triggered by excess dietary cholesterol by reducing the expression of pro-inflammatory genes. This response could be partly associated with a decreased in macrophages in skin and/or by modulation of the skin's FA composition.

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1. Introduction

Skin acts as a barrier to protect against water loss and adverse environmental agents [1]. The proper maintenance of the skin layer requires balanced proliferation and differentiation of skin cells through the actions of the epidermis and the dermis [1]. Skin lipid homeostasis is important in the maintenance of the normal barrier function of skin. The outermost epidermis is surrounded by a lipid-rich extracellular matrix where ceramides, cholesterol, and free FAs are abundant [2]. When the tissue barrier is disrupted, the outermost epidermis increases the synthesis of cholesterol and FAs to provide new cell membrane lipids [3]. Though the precise contribution of dietary FAs to the skin lipid pool is not known, the dietary supplementation of n-3 polyunsaturated fatty acids (PUFA) was proven to improve the condition of skin disorders [4].

Skin inflammation occurs in the early stages of the normal immune response and provides protection against various foreign molecules. Abnormal regulation of this process can lead to acute and chronic inflammatory diseases such as psoriasis and atopic dermatitis, involving the induction of proinflammatory cytokines and eisocanoids [5,6]. Recently, skin cholesterol deposition was shown to be closely related to skin inflammation [7]. Genetically-engineered double knockout mice of the LDLR and ApoA1 genes showed a significant accumulation of cholesterol in their skin, which exhibited severe lesions as well as the extensive infiltration of macrophages [7]. The role of cholesterol in inflammation was also suggested by the reversible inflammatory response in mice liver upon the removal of cholesterol and cholate from an atherogenic diet [8]. Therefore excessive cholesterol has been implicated as playing a causative role in the induction of inflammation [9].

Soy is widely consumed worldwide. Its beneficial effects on various low-grade inflammation-related diseases are elicited by its high content of n-3 FAs and bioactive components including isoflavones and plant sterol [10–13]. Soy and/or soy constituents lowered the risk of allergic reactions and ameliorated dysregulated immune responses [12,13]. The substitution of soy protein for casein dramatically improved inflammation-related markers [14]. The bioactivity of soy isoflavones can be enhanced through fermentation by converting them into aglycones which are easier for intestinal absorption [15]. In particular, equol, a metabolite

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generated by the intestinal gut bacteria, shows a stronger potency than its precursor daidzein [16]. Thus, fermentation with probiotic bacteria can be a potential means to increase the bioavailability of soy constituents and increase the bio-effectiveness of soy products.

We hypothesized that a CHOL diet could initiate the inflammatory response in skin and that SM or F.SM might lessen this inflammation. To this end, we investigated the expression levels of proinflammatory and macrophage marker genes and the FA composition in the skin of rats fed CHOL diets with or without SM or F.SM.

2. Materials and methods

2.1. Animals

Five-week old SD rats were obtained from Koatech (Pyungtek, Korea). After a one-week adaptation period, the animals were divided into four groups containing eight animals and each fed an experimental diet *ad libitum* for six weeks. The CTRL group was fed a CTRL diet that is AIN76A diet which contains 50% sucrose by weight. The CHOL group was given a CHOL diet, which is an AIN76A diet supplemented with 1% (w/w) cholesterol. The SM group was put on a SM diet, which is a CHOL diet with 20% of its casein and 40% of its corn oil substituted with freeze-dried SM. The F.SM group was fed on a CHOL diet with freeze-dried F.SM replacing 20% of the casein and 40% of the corn oil. At the end of the experimental period, rats underwent a 12-h fast prior to sacrifice. After the animals were euthanized with diethyl ether, their skin tissue was excised, rapidly frozen with liquid nitrogen, and then stored in the freezer at −80 °C. All the experimental procedures were reviewed and approved by the Committee on Animal Experimentation and Ethics of Yonsei University.

2.2. Preparation of diets containing freeze-dried SM and F.SM

SM was provided from Yonsei Milk Corporation (Asan, Korea). SM was mainly composed of the following nutrients: 25 g carbohydrate/L, 35 g protein/L, 17.5 g lipids/L, 10 g dietary fiber/L, and 750 mg sodium/L. To prepare the F.SM, 1 L of SM was mixed with 20 g of galactooligosaccharide (Samyang, Seoul, Korea) and then was fermented using 0.1% (w/v) *Lactobacillus plantarum* (CellBiotech, Seoul, Korea) at 25 °C until the pH reached 5.1. The SM and the F.SM were filtered and the filtrates were dehydrated with a freeze dryer (ILShinBioBase, Dongduchen, Korea).

2.3. RNA extraction and quantitative RT-PCR

Skin tissue samples were homogenized in Trizol (Qiagen, CA) and the total RNA was extracted according to the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA using random hexamers and ImprompII reverse transcriptase (Promega, WI). Expression levels of the genes were measured using EvaGreen qPCR mix plus (Solis BioDyne, Estonia) and CFX96 Touch Real-time PCR Detection System (Biorad, CA). The relative mRNA level was calculated using 18SrRNA as a control and the difference in C_t values among the animals groups was expressed as the fold change.

2.4. Western blot analysis

Skin tissues were homogenized and lysed in RIPA buffer containing protease inhibitors (Sigma–Aldrich, MO). After centrifugation, the skin tissue lysates were boiled in Laemmli buffer. Equal amounts of protein lysates were loaded onto a SDS–polyacrylamide gel. Rabbit anti-COX-2, rabbit anti-IL-1β, and mouse anti-GAPDH (Santa Cruz Biotech., CA) were used to detect the corresponding proteins. Peroxidase-conjugated secondary antibodies (Santa Cruz Biotech., CA) were used to detect the proteins.

2.5. Fatty acids and cholesterol analysis using gas chromatography

Total lipids from the animal skins were extracted according to a modified Folch method [17]. Hexane and 14% boron trifluoride in methanol (Sigma–Aldrich, MO) were used for lipid extraction and methylation. Methylated samples were separated by gas chromatography (Agilent, CA) using capillary columns, with an Omegawax 250 column (Supelco, PA) used for fatty acid methyl esters and an Ultra 1 Methyl Siloxan capillary column (Agilent, CA) used for cholesterol methyl esters. Individual FAs and cholesterols were identified based on their peak retention times compared to those of known standards (PUFA-2, DGLA, and cholesterol, Sigma–Aldrich, MO). Skin FAs were expressed as the percentage of total FAs. The indices of desaturase activities were calculated based on the ratio of product to precursor FAs. SCD-1 activity index was defined by C16:1*n*-7/C16:0. The D6D activity index was C20:3*n*-6/C18:2 *n*-6. D5D activity index was C20:4*n*-6/C20:3*n*-6 [18].

2.6. Statistical analyses

SPSS software was used for statistical analysis (SPSS Ins., IL). The results were presented as means ± SE. The statistical significance was tested using one-way analysis of variance (ANOVA) and Duncan's multiple range test, with $p < 0.05$ as the criterion of significance.

3. Results

3.1. Skin cholesterol accumulation in rats fed high cholesterol diets with and without soy milk substitution

Food intake was not significantly different among the CTRL, SM, and F.SM groups, but the CHOL group showed a lowered food intake compared with the other groups (Fig. 1A). On the other hand, the CHOL, SM, and F.SM groups all displayed a similar elevation in free cholesterol levels in the skin as compared to the CTRL group (Fig. 1B). These data showed that SM or F.SM did not significantly affect the accumulation of cholesterol induced by the CHOL diet in the skin when it was provided *ad libitum*.

3.2. SM and F.SM reduced the expression of IL-1β and COX-2 genes and macrophage marker genes in skin tissues

CHOL significantly elevated IL-1β gene expression in terms of both mRNA and protein levels compared to CTRL (Figs. 1C and 2D) ($p < 0.05$). SM and F.SM decreased IL-1β mRNA to levels comparable to that of CTRL (Fig. 1C). At the protein levels, F.SM significantly reduced while SM showed a tendency for reduction of IL-1β (Fig. 2D). Pro-IL-1β protein showed a similar expression trend to those of IL-1β (Fig. 2C and D). When compared to CTRL, CHOL increased COX-2 mRNA levels but elicited an increased tendency in COX-2 protein levels. CHOL-elevated expression of COX-2 gene were down-regulated by SM and F.SM (Figs. 1D and 2B) ($p < 0.05$). The IL-1α and iNOS mRNA levels were increased by CHOL compared to CTRL ($p < 0.05$); SM and F.SM did not affect the expression of these genes (Fig. 1E and F). The abundance of TNF-α and IL-6 mRNA did not significantly differ among all tested groups (Fig. 1G and H). CHOL did not affect the transcript levels of macrophage marker genes, including F4/80 [19] and Cd68 [20], compared to CTRL (Fig. 2E and F). However, SM and F.SM reduced F4/80 mRNA levels but did not significantly affect Cd68 mRNA levels in the skin as compared to CTRL and CHOL (Fig. 2E and F). These

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