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A novel mechanism for improvement of dry skin by dietary milk phospholipids: Effect on epidermal covalently bound ceramides and skin inflammation in hairless mice

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ABSTRACT

Background: Dietary milk phospholipids (MPLs) increase hydration of the stratum corneum and reduced transepidermal water loss (TEWL) in hairless mice fed a standard diet. However, the mechanism by which MPLs improve skin barrier functions has yet to be established.
Objective: This study was designed to examine the mechanism by which MPLs may affect covalently bound ceramides and markers of skin inflammation and improve the skin barrier defect in hairless mice fed a magnesium-deficient (HR-AD) diet.
Methods: Four-week-old female hairless mice were randomized into four groups (n = 10/group), and fed a standard (control) diet, the HR-AD diet, the HR-AD diet supplemented with either 7.0 g/kg MPLs (low [L]-MPL) or 41.0 g/kg MPLs (high [H]-MPL).
Results: Dietary MPLs improved the dry skin condition of hairless mice fed the HR-AD diet. MPLs significantly increased the percentage of covalently bound ω-hydroxy ceramides in the epidermis, and significantly decreased both thymus and activation-regulated chemokine (TARC) mRNA and thymic stromal lymphopoietin (TSLP) mRNA levels in skin, compared with the HR-AD diet. Furthermore, the MPL diets significantly decreased serum concentrations of immunoglobulin-E, TARC, TSLP, and soluble P-selectin versus the HR-AD diet.
Conclusion: Our study showed for the first time that dietary MPLs may modulate epidermal covalently bound ceramides associated with formation of lamellar structures and suppress skin inflammation, resulting in improved skin barrier function.

1. Introduction

Skin provides an effective barrier between the organism and their environment, helping to reduce the risk of physical, chemical, and microbial damage. Oral intake of dietary components is known to play a beneficial role in improving skin barrier functions [1–6]. Bovine milk lipids contain approximately 0.5–1% of phospholipids. Milk phospholipid concentrates cause increased hydration of the stratum corneum (SC) and reduce transepidermal water loss (TEWL) in hairless mice fed a standard diet [3,4]. However, the mechanisms by which milk phospholipids (MPLs) improve skin barrier functions have yet to be established.

Reduced barrier function seems to be a consequence of inadequate structural and metabolic conditions in the epidermis. Skin barrier properties are primarily localized in the SC, the outermost layer of the epidermis. The SC consists of corneocytes surrounded by a neutral lipid-enriched intercellular matrix. Ceramides, which comprise approximately 50% of the intercellular lipids, play an important role in retaining epidermal water and, in combination with cholesterol and free fatty acids, they influence permeability of the epidermal barrier [7–9]. Recent research in NC/Nga mice demonstrated that dietary sericin [1] or gromwell [2] improved epidermal skin dryness due to increased levels of non-protein bound glucosylceramides and ceramides and up-regulation of glucosylceramide synthase, β-glucocerebrosidase, and

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acidic sphingomyelinase. The epidermis also contains covalently bound ω-hydroxy ceramides that are most frequently bound to a structural protein in the epidermal cornified envelope by an ester linkage. Levels of covalently bound ω-hydroxy ceramides were shown to be significantly decreased after ultraviolet-B (UV-B) irradiation, tape-stripping, or treatment with sodium dodecyl sulfate, whereas the levels of non-bound ceramides remained unchanged [10]. Lipid species of this type are therefore thought to play a crucial role in the formation of lamellar structures, and are involved in the maintenance of the skin barrier function [11,12].

Dry skin conditions, such as atopic dermatitis (AD) and psoriasis vulgaris, cause chronic skin inflammation. Although the pathogenesis of dry skin conditions is not completely understood, it is thought to involve a Th2 cell-mediated allergic inflammatory cascade. Skin injury, caused by environmental allergens, scratching, or microbial toxins, activates keratinocytes to release proinflammatory cytokines and chemokines that induce the expression of adhesion molecules on the vascular endothelium and facilitate the extravasation of inflammatory cells into the skin [13]. Serum levels of thymus and activation-regulated chemokine (TARC) [14], thymic stromal lymphopoietin (TSLP) [15], and soluble P-selectin (sP-selectin) [16] appear to be significantly higher in patients with AD than in people without this type of skin condition. Thus, serum markers of chemokines and platelet activator appear to be useful for assessing the severity of dry skin conditions. However, few studies have been able to demonstrate that the oral intake of dietary components can modulate both covalently bound ω-hydroxy ceramides and skin inflammation associated with skin dryness.

It is well known that feeding hairless mice a magnesium-deficient diet (HR-AD) for an extended period of time causes a skin barrier defect characterized by an increase in TEWL and a decrease in skin hydration [6,17–19]. Fujii et al. have previously reported that hairless mice fed the HR-AD diet develop skin inflammation accompanied by a skin barrier defect and itch-related scratching [17,19]. This study was designed to examine the effect of dietary MPLs on covalently bound ω-hydroxy ceramides and skin inflammation markers in hairless mice fed the HR-AD diet with the aim of elucidating a novel mechanism by which MPLs may improve skin barrier function.

2. Materials and methods

2.1. Animals

Forty four-week-old female hairless mice (Hos:HR-1, Nippon SLC Inc., Shizuoka, Japan) were used in this study. All mice were housed individually in plastic cages in a temperature- and humidity-controlled room (22 ± 1 °C and 50 ± 10% relative humidity, respectively) and maintained on a 12 h light-dark cycle. All of the animal experiments in this study were approved by Meiji Co., Ltd. Institutional Animal Care and Use Committee, and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by Meiji Co., Ltd.

After acclimatization for seven days, mice were randomized into one of four groups (n = 10/group), and fed either a standard (control) diet (F-2; Funabashi Farm, Chiba, Japan), the HR-AD diet (Norsan Corp., Kanagawa, Japan), or the HR-AD diet supplemented with either 7.0 g/kg (low [L]-MPL) or 41.0 g/kg (high [H]-MPL) of MPLs (Phospholipid concentrate 700, Fonterra Co-operative Group Ltd., New Zealand). Mice were allowed free access to food and water. The MPLs consisted of 16.0% sphingomyelin, 31.0% phosphatidylcholine, 3.0% phosphatidylserine, and 8.7% phosphatidylethanolamine. The remaining components included 25.8% other lipids, 2.5% moisture, 6.5% lactose, and 6.0% minerals. The magnesium content in the experimental diets was 200 mg/g (control), 17.6 mg/g (HR-AD), 17.5 mg/g (L-MPL), and 17.2 mg/g (H-MPL).

TEWL and the water content of the SC was measured at baseline (time 0) and after 2, 4, 6, and 8 weeks of feeding. After eight weeks of feeding, all mice were euthanized under isoflurane anesthesia. Blood samples were collected and subsequently centrifuged at 1000 g at 4 °C; serum samples were stored at −80 °C until analysis. Skin was excised quickly and immediately frozen at −80 °C until the time of assay.

2.2. Measurement of TEWL and water content of the SC

The TEWL and water content of the SC were assessed under standardized conditions. Both parameters were measured with a Tewemeter MPA580 (Courage and Khazaka Electronic GmbH, Cologne, Germany) and a Corneometer® (Courage and Khazaka Electronic GmbH, Cologne, Germany), respectively.

2.3. Histological analysis and measurement of epidermal thickness

Dorsal skin sections were stained with hematoxylin and eosin (H&E). The thickness of the epidermis (the distance from the bottom of the basal layer to the top of the granular layer) was measured with a biomicroscope BX-2 (Olympus Corporation, Tokyo, Japan) and a CCD camera DP-72 (Olympus Corporation, Tokyo, Japan). Sections were digitally assessed by image measurement and analysis with WinROOF software (Mitani Corporation, Tokyo, Japan).

2.4. Analysis of covalently bound ω-hydroxy ceramides

Extraction of covalently bound ceramides was carried out using a modification of the method reported by Macheleidt et al. [20]. Epidermal sheets were obtained from skin samples by treating them with Dispase®II (Roche, IND, USA) at 4 °C overnight. Tissues were homogenized in chloroform/methanol (2:1, v/v) using a glass homogenizer. After removal of the supernatant by centrifugation, a chloroform/methanol solution was added to wash the protein residue twice. After drying, protein pellets were incubated in 1 M KOH in 95% methanol at room temperature overnight to release the lipids covalently bound to the stratum corneum by ester-like bonds. The methanolic layer was removed after centrifugation and neutralized with 1 N HCl. The protein pellets were washed using chloroform/methanol (2:1, v/v). The organic phases were combined, dried, and redissolved in methanol. The protein pellet was immersed into 0.1 M sodium hydroxide solution containing 1% sodium dodecyl sulfate and incubated at 60 °C for 2 h to solubilize the protein. After incubation, the solution was neutralized with 1 N HCl. The protein concentration was assayed using a commercial kit (Micro BCA assay kit, Pierce Biotechnology, Inc., IL, USA).

Covalently bound ceramides in the mice epidermis were identified using a high performance liquid chromatography system coupled to a tandem mass spectrometer (HPLC-MS/MS) (Quattro premier XE, Waters Corporation, Milford, MA, USA). All the analyses were performed on a 2 mm × 100 mm column with a particle size of 1.7 μm (ACQUITY UPLC® BEH C18, Waters Corporation). Mobile phase A consisted of 5 mM ammonium acetate in 95% methanol, whereas mobile phase B consisted of 5 mM ammonium acetate in acetonitrile. The initial eluent composition was 100% A, followed by an increase to 100% B for 30 min, 100% B for 2 min, and then a reduction to 0% A for 3 min. Total running time was 35 min. The eluent flow was 0.4 mL/min and the column temperature was set at 40 °C. Analytes were detected using electrospray ionization in the positive mode. Multiple-reaction-monitoring (MRM) was performed using characteristic