Potential of polar lipids from bovine milk to regulate the rodent dorsal hair cycle

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ABSTRACT

Among the lipids in bovine milk, minor components such as conjugated linoleic acids and phospholipids are more attractive than triacylglycerols from the standpoint of biological activity. To explore novel functions of bovine milk polar lipids (MPL), topical application to murine dorsal skin was introduced as an assay system. The acetone-insoluble lipid fraction derived from bovine milk was dispersed in ethanol and applied to 9-wk-old C57BL/6N female mice for 3 wk. In combination with visual assessment of the dorsal pigmentation, the progression of the hair cycle was estimated by calculating the ratio of subcutis to dermis thickness. The administration of MPL led to earlier progression of the hair cycle compared with administration of the vehicle. In some cases, the extent of MPL-induced hair cycle progression was comparable to that in animals treated with minoxidil, the most well-known reagent that initiates anagen. These results indicate that the MPL preparation contains a dermal penetrative component that can regulate the hair cycle and, thus, this preparation possesses potential for cosmetic use.

Key words: milk polar lipids, hair cycle, anagen

INTRODUCTION

Most of the lipids in mature bovine milk are triacylglycerols; however, minor lipid components such as conjugated linoleic acids and phospholipids are more attractive from the standpoint of functional foods. Conjugated linoleic acids are expected to exhibit health benefits, including antiobesitic, anti-carcinogenic, antiatherogenic, antidiabetogenic, immunomodulatory, apoptotic, and osteosynthetic effects (Benjamin and Spener, 2009). The phospholipids in bovine milk include phosphatidylcholine (35%), sphingomyelin (SPM) (25%), phosphatidylethanolamine (22%), phos-

phatidylinositol (5%), and phosphatidylserine (3%; MacGibbon and Taylor, 2006). Sprong et al. (1998) studied the effect of phospholipid-rich buttermilk on the gastrointestinal survival and translocation of *Listeria* using the rat as an animal model system. They found that the phospholipids enhanced the gastrointestinal defense against the pathogen. Kivinen et al. (1992) conducted a human study to determine the effect of milk phospholipids on an aspirin challenge. The mucosal damage was almost completely absent when the phospholipids were administered, which suggested the involvement of components that are able to prevent gastritis. Nyberg et al. (2000) showed that the oral administration of SPM from bovine milk led to the inhibition of cholesterol absorption in rats. According to the study of Noh and Koo (2004), both milk and egg SPM markedly inhibit the absorption of cholesterol, fat, and other lipids. In addition, milk SPM was a more potent inhibitor than egg SPM, a result that was attributed to the higher degree of saturation and the longer chain length of the fatty acyl groups in milk SPM. In addition, Schmelz (2004) reviewed the prevention of tumor formation by dietary SPM. In recent studies, Haruta et al. (2008) and Haruta-Ono et al. (2012) used commercial milk ceramide MC-5 as a dietary SPM concentrate to investigate its effect on the epidermal function of hairless mice and noted an improvement of stratum corneum hydration and transepidermal water loss. For the investigation of dermatological function of edible phospholipids, topical application to a specific site of the skin is an alternative assay system because polar lipids with lower molecular masses are more able to penetrate into the epidermis and dermis than macromolecules such as proteins and peptides (Brown et al., 2008; Choi et al., 2012). Thus, we directly applied milk phospholipids to the dorsal skin of mice to determine if these compounds exhibit dermatological activity. Because the permeability of a substance into the skin depends on its inherent molecular characteristics and the delivery system adopted, we focused on the hair cycle, in particular anagen induction.

The growth of hair is characterized by 3 main cyclic phases of hair follicle transformation, including a rapid

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growth phase (anagen), an apoptosis-driven regression phase (catagen), and a relatively quiescent resting phase (telogen; Müller-Röver et al., 2001). Anagen is initiated by the activation of stem cells that reside in the bulge region, which is close to the epidermis in the dermis and is location of the point of insertion of the arrector pili (Tiede et al., 2007). During anagen, the hair bulb, including the dermal papilla cells and hair matrix cells, reaches the most proximal site to the subcutis. From the standpoint of the topical application to the epidermis, the distance required for penetration of the test reagent to reach the sites of interest is much less during the telogen phase than during the anagen phase. Therefore, the continuous application of a permeable reagent during the telogen phase might allow that reagent to exert its biological effect on the hair cycle; in particular, the initiation of anagen.

Considering this advantage, we conducted an *in vivo* study using the dorsal skin of mice and explored the potential of polar lipids from bovine milk to regulate the rodent dorsal hair cycle by topical application.

MATERIALS AND METHODS

Preparation of the Milk Polar Lipids

A mixture of methanol and chloroform (1:2) was added to the powder of Milk Ceramide MC-5 (a gift from Megmilk Snow Brand Co. Ltd., Tokyo, Japan) and kept at 25°C for 10 min to extract the lipid fraction. After centrifugation at $10,000 \times g$ for 20 min, the supernatant was filtered, and the filtrate was evaporated under vacuum. The resulting pellet was suspended in acetone, and the insoluble material was recovered on filter paper and then washed several times with fresh acetone. The material was dissolved in chloroform and concentrated by vacuum evaporation. The resulting pellet was suspended in ethanol and filtered to obtain the ethanol-soluble fraction, which was solidified by vacuum evaporation and used as the milk polar lipid (MPL) preparation. One gram of MPL was dissolved in 100 mL of ethanol (1% MPL) for use in the following studies.

Animals

Eight-week-old C57BL/6N female mice were purchased from Charles River, Japan, and provided standard rodent chow and water *ad libitum*. Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital, and then the dorsal part of the skin was shaved with an electric shaver. The animals were randomly divided into 3 groups ($n = 5-7$) and

Table 1. Summary of the topical application of test reagents on murine dorsal skin¹

Trial no.	Vehicle	Milk polar lipid	Minoxidil
1	5	6 ^a	5 ^a
2	6	7 ^b	
3	6	7	6
4	6	6	6 ^a
5	6	6 ^a	6 ^{a,c}
6	6	6 ^b	

^{a-c}The significance of the increased thickness of the subcutis-to-dermis ratio is noted with letters for the test reagents versus the vehicle (a: $P < 0.01$; b: $P < 0.05$) and for milk polar lipids versus minoxidil (Riup; Taisho Pharmaceutical Co. Ltd., Tokyo, Japan; c: $P < 0.01$).

¹Each number within the columns represents the number of animals used in the experiment.

maintained under the standard conditions of a 12-h light/dark cycle, 24°C, and 30% humidity. The care and handling of the animals were in accordance with Hokkaido University's (Sapporo, Japan) guidelines for the care and use of laboratory animals. The animal Resource Committee of Hokkaido University approved all animal experiments used in this study.

In Vivo Studies

After the period of adaptation to the new environment, the 63-d-old mice were treated with 1% MPL, with only ethanol as vehicle or with commercial 1% minoxidil, known as Riup (Taisho Pharmaceutical Co. Ltd., Tokyo, Japan). Each reagent (100 μ L) was applied topically on the dorsal skin for 6 consecutive days in a week for 3 wk.

Histological Evaluation

The posterior part of the dorsal skin was excised and maintained in 10% formalin buffered with PBS and embedded in paraffin blocks to obtain 4- μ m-thick longitudinal sections. The sections were stained with hematoxylin and eosin. To evaluate hair cycle progression, the hematoxylin/eosin-stained slides were photographed using a digital photomicrograph. Three slides per animal were selected, and 3 representative areas per slide were used to calculate the ratio of the subcutis and dermis thicknesses (S/D).

Statistical Analysis

Differences between the test reagent and vehicle groups were analyzed by nonparametric ANOVA using the Kruskal-Wallis test, followed by the Mann-Whitney U-test with the Bonferroni correction. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS AND DISCUSSION

The murine hair cycle during the first 14 wk after birth follows a rather precise time scale (Müller-Röver et al., 2001). In this period, anagen is induced in a wave-like pattern showing caudal-nuchal development and, as anagen advances, profound alterations occur in the architecture and thickness of skin compartments, in particular that of subcutis. In addition, in the C57BL/6 strain, anagen development is associated with a characteristic change in skin pigmentation from pink to black, owing to the strict coupling of follicular

melanogenesis and hair follicle cycling (Müller-Röver et al., 2001). Thus, anagen induction is readily assessed with the unaided eye. Taking advantage of this assessment method, we conducted a preliminary test in which MPL were applied to murine dorsal skin in the stable telogen phase at 63 d of age and visually compared the treated skin with that of the control (treated with the vehicle). Because the results obtained from the preliminary tests, which were performed twice, suggested an earlier onset of anagen (data not shown), we performed subsequent tests using histological analysis and performed 6 trials (Table 1). Minoxidil was also added

Figure 1. Box plot analysis of the ratio of subcutis-to-dermis thickness obtained from selected 4 trials: (A) trial 1, (B) trial 3, (C) trial 4, and (D) trial 5. V = vehicle; MPL = milk polar lipids; Min = minoxidil (Riup; Taisho Pharmaceutical Co. Ltd., Tokyo, Japan). Paraffin-embedded sections of representative anagen (E) and telogen (F) skin stained with hematoxylin and eosin are also shown. The largest and smallest observations for the box plot analysis were 90 and 10%, respectively. Color version available in the online PDF.

Figure 2. Induction of the anagen phase by the topical application of the test reagents. The results were obtained from trial 5. (A) Vehicle, (B) milk polar lipids (MPL), and (C) minoxidil (Riup; Taisho Pharmaceutical Co. Ltd., Tokyo, Japan). The dark gray areas of the reverse sides of the resected skin (derived from each mouse corresponding to the top image) indicate anagen induction.

to the additional experimental design in some cases because it is the most well-known reagent that initiates anagen (Messenger and Rundegren, 2004). Evaluation of the progression of the hair cycle was performed using the S/D of 84-d-old animals because from 14 wk onward, the murine hair follicle is ready to enter the anagen phase inherently.

Figure 1 shows the S/D results obtained from 4 selected trials. The S/D increased along with anagen progression; however, the magnitude of the test reagents depended on the trial. Although no significant difference due to the administration of MPL was noted in trials 3 and 4 (Figure 1B and 1C), an increasing tendency in the S/D was noted. Furthermore, an insignificant effect was observed even when minoxidil was applied, as shown in trial 3 (Figure 1B). Figure 2 shows the pigmentation of the dorsal skin in trial 5, where the difference between the treated groups was the most evident. The pigmentation of mice treated with minoxidil was more pronounced than that of mice treated with vehicle or MPL, a result that is consistent with the results presented in Figure 1D. Overall, the application of MPL significantly promoted anagen induction in 4 cases among the 6 trials, as summarized in Table 1.

Dermal papilla cells make up a cluster of specialized fibroblasts and are considered to play pivotal roles in the regulation of the hair follicle through the production of diverse regulatory factors, including insulin-like growth factor-I, hepatocyte growth factor, fibroblast growth factor-7 (**FGF-7**), endothelial growth factor, and transforming growth factor- β (Kim et al., 2010). Minoxidil contributed to the entry and duration of anagen, not only through the activation of the β -catenin pathway (Kwack et al., 2011), but also by stimulating the expression of FGF-7 via irritation of adenosine receptors on the dermal papilla cells (Iino et al., 2007). Although Kawano et al. (2005) showed that FGF-7 and FGF-10 are upregulated at the early stage of anagen, FGF-7 is preferentially accepted as

an anagen contributor (Stenn and Paus, 2001). Thus, we conducted reverse transcription-PCR using cDNA prepared from the skin of each mouse. The expression of FGF-7 in the skin was more frequently found when the animals were treated with MPL than with vehicle or minoxidil; however, the expression level of FGF-7 of the individual animals seemed to be inconsistent with the corresponding appearance of the skin pigmentation (data not shown).

To modulate the hair cycle, alternative agents have been screened; in particular, plant-based materials (Matsuda et al., 2003; Datta et al., 2009; Kim et al., 2010; Park et al., 2011). Although the data presented in this study are limited, this is the first report suggesting that MPL includes a dermal penetrative component that is able to regulate the hair, as demonstrated by the analysis of melanogenesis and skin thickness. In addition to triacylglycerols, phospholipids, ceramide, and glycosphingolipids, milk lipids consist of diverse minor constituents including glycerolipids, hydrocarbons, lipid-soluble vitamins, acylcarnitines, sterols, and steroidal hormones (Christie, 1995). It is of interest to identify the components in MPL that possess transdermal activity and the ability to stimulate certain cells, such as those present in hair follicles. Conducting further reliable *in vitro* studies would ensure the availability of further information to propose novel functions of milk lipids relevant to cosmetic use.

CONCLUSIONS

To explore a novel function of bovine milk polar lipids, these lipids were applied topically to murine dorsal skin. The acetone-insoluble lipid fraction derived from bovine milk was applied to 9-wk-old C57BL/6N female mice for 3 wk. Anagen induction was judged by visual assessment of the dorsal pigmentation and histochemical analysis of the skin thickness during anagen progression. Based on the results of the 6 consecutive

trials, MPL may have potential cosmetic uses because of the involvement of a dermal penetrative component that is able to regulate the hair cycle.

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