Short Communication: Effects of *Lactobacillus helveticus*-Fermented Milk on the Differentiation of Cultured Normal Human Epidermal Keratinocytes

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ABSTRACT

Effects of *Lactobacillus helveticus*-fermented milk whey on the differentiation of normal human epidermal keratinocytes were studied. Analysis using real-time reverse transcription-polymerase chain reaction revealed that addition of *Lactobacillus helveticus*-fermented milk whey to the culture medium enhanced mRNA expression of keratin 10, an early differentiation marker, as well as involucrin, a late differentiation marker. Whey of artificially acidified milk, prepared by the addition of DL-lactic acid to milk instead of fermentation, also promoted expression of both markers, but *Lactobacillus helveticus*-fermented milk whey was more effective in increasing expression of those markers. These results indicate that milk whey has the potential to induce multiple stages of keratinocyte differentiation and that fermentation with *Lactobacillus helveticus* increases that activity. Furthermore, we examined the expression of profilaggrin, which increases with epidermal terminal differentiation, and found that *Lactobacillus helveticus*-fermented milk whey enhanced expression of profilaggrin mRNA in a dose-dependent manner. Expression also occurred to a greater extent than with artificially acidified milk whey or other whey samples prepared with several lactic acid bacterial species. Because the proteolytically processed form of profilaggrin, filaggrin, is very important for normal epidermal hydration and flexibility, our results indicate that *Lactobacillus helveticus*-fermented milk whey has the potential to enhance the production of filaggrin-related natural moisturizing factor, because of its effect on the induction of epidermal differentiation, and is expected to be a useful skin moisturizing agent.

Key words: keratinocyte, epidermal differentiation, lactobacillus, fermented milk

In many parts of the world, fermented milk has been consumed as a valuable food source for centuries. Many

studies have shown several beneficial effects of fermented milk on health, including improvement of gastrointestinal conditions, antitumor effects, and antihypertensive effects, as reviewed (Rašić and Kurmann, 1978; Takano et al., 1985; Takano and Yamamoto, 2002). In relation to skin, fermented milk has been used for clinical treatment of wound healing and burn injuries (Rašić and Kurmann, 1978), and is now being used widely as a cosmetic material. No studies, however, have reported the effects of ingestion or topical application of fermented milk on skin homeostasis. In addition, scientific data are lacking regarding direct effects of these materials on cells constituting the skin. On the other hand, a few reports have revealed the physiological effects of milk or other fermented products on the skin. One report showed that a whey extract of bovine milk stimulates wound repair activity in vitro and promotes healing of rat skin incisional wounds (Rayner et al., 2000), whereas other reports revealed that a *Bifidobacterium*-fermented soy milk extract stimulated hyaluronic acid production in skin cells and its topical application improved cutaneous physiological properties (Miyazaki et al., 2003, 2004). These observations led us to speculate that milk itself may have physiological effects on the skin system and that its fermentation may produce new function(s) or effects.

Human epidermis is made up of multiple layers of keratinocytes that are in a continual process of cell replacement. Within the epidermis, proliferation takes place in the basal layer, whereas desquamation occurs at the skin surface; this balance between cell production and loss coordinates epidermal turnover (Potten and Booth, 2002). In this process, morphological and biochemical changes occur in keratinocytes: the cells stop proliferating; they produce new proteins including specific cytokeratins and cornified envelope precursors; and they progressively flatten to result in the production of terminally differentiated cells, termed corneocytes. Composite of billions of corneocytes covers the skin surface and protects against pathogens and exogenous stimuli such as physical stress and ultraviolet light. In addition, corneocytes have multilamellar structures consisting of intercellular lipids in the extra-

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cellular matrix that play a role in the barrier against transpiration (Madison et al., 1987). As noted above, keratinocyte differentiation occurs continuously in the epidermis and is essential for skin homeostasis, especially with respect to epidermal turnover and barrier function. In this study, we investigated the effects of fermented milk products on keratinocyte differentiation.

Because *Lactobacillus helveticus* CM4 (**CM4**), selected from a starter culture of Calpis sour milk (Calpis, Tokyo, Japan), has a very potent protease activity and can efficiently produce milk protein-derived biologically active peptides (Nakamura, 2004), we expected that fermented milk prepared with this strain might show some effects on skin cells. We tested that concept in this study. Nine percent (wt/wt) reconstituted skim milk (Yotsuba Milk Products Co., Ltd., Hokkaido, Japan) medium, pasteurized at 105°C for 10 min, was used in all preparations. The CM4-fermented milk was obtained by incubation at 37°C for 24 h. As a control, artificially acidified milk was obtained by adding the same amount of DL-lactic acid [2.29% (wt/wt), Wako Pure Chemical Industries Co. Ltd., Osaka, Japan] to the skim milk medium to achieve the same acidity as in the CM4-fermented milk. These samples were separated into the whey by centrifugation at $20,000 \times g$ for 10 min; these fractions were named CM4-fermented milk whey (**CFMW**) and artificially acidified milk whey (**AAMW**). To compare the activity of several types of fermented milk whey, fermented milks were also obtained by incubation at 37°C for 24 h (*Streptococcus thermophilus* and *Lactobacillus lactis*) and at 37°C for 48 h (*Lactobacillus casei* and *Lactococcus lactis*); wheys from these fermented milks were obtained by the same process used for CFMW and AAMW. We determined the concentration of calcium, which affects keratinocyte differentiation, in the whey series using a Fuji Drychem 7000I analyzer (Fuji Medical System Co. Ltd., Tokyo, Japan).

Normal human epidermal keratinocytes (Kurabo, Osaka, Japan) were seeded in 60-mm dishes in Humedia-KG2 medium (Kurabo) with a cell density of 1×10^4 cells/cm², and were cultured at 37°C in an atmosphere of humidified 95% O₂ and 5% CO₂. After 24 h of culture, when the keratinocytes were at the subconfluent stage, whey samples were added to the culture medium to a final concentration of 0.03 to 1%. Cells were then cultured for 0, 1, 2, 4, 6, or 8 d after the sample addition, and then were washed with HEPES buffer, after which total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and subjected to real-time reverse transcription-polymerase chain reaction (**RT-PCR**). For comparisons of the activities of several whey samples, total RNA were harvested

at 24 h and used. All experiments were performed in triplicate.

Real-time RT-PCR was performed using a Smart Cycler II System (Cepheid, Sunnyvale, CA) with the One-Step SYBR RT-PCR kit (Takara Bio Inc., Shiga, Japan). The primers used were: keratin 10, sense primer: GG ATGAGCTGACCCTGACCAA, antisense primer GCA GCATTCATTTCCACATTCAC; involucrin, sense primer: TAACCACCCGCGAGTGTAAG, antisense primer: CACCTAGCGGACCCGAAATAAG; profilaggrin, sense primer: CAGACAATCAGGCACTCGTCA, antisense primer: ACTGGACCCTCGGTTTCCAC; glyceraldehyde-3-phosphate dehydrogenase, sense primer: GCACCGTCAAGGCTGAGAAC, and antisense primer: ATGGTGGTGAAGACGCCAGT. The thermal cycle profile for one-step RT-PCR consisted of a 15-min RT step at 42°C and 2 min of *Taq* polymerase activation at 95°C, followed by 45 cycles of PCR at 95°C for 5 s (denature), and 60°C for 20 s (annealing and extension). Following amplification, melting curve analyses were performed to verify the correct products by their specific melting temperatures. Data were obtained in triplicate and all values were expressed as the mean $\pm$ SEM of relative mRNA expression normalized to glyceraldehyde-3-phosphate dehydrogenase. Statistical analyses were performed using the Dunnett's test for comparison with nonstimulated cells or using the Student-Newman-Keuls test for CFMW vs. AAMW.

To examine the effects of fermented milk on epidermal keratinocyte differentiation, changes in mRNA expression of keratin 10, a marker of early differentiation, as well as involucrin, a late marker, were analyzed. As shown in Figure 1, addition of CFMW or AAMW to the culture medium to a final concentration of 1% enhanced the mRNA expression of both differentiation markers. When compared with AAMW, CFMW induced greater amounts of gene expression of keratin 10 after 4 d ($P < 0.05$) and involucrin at 2 d ($P < 0.01$). These findings were further supported by immunocytochemical analysis, in which enhancement of both markers also was observed at the protein level (data not shown). These results indicate that the unfermented milk whey sample induces multiple stages of keratinocyte differentiation, and that its fermentation with CM4 further strengthens the effect. This is the first report that milk or fermented milk samples have an effect on keratinocyte differentiation.

Moreover, we investigated the changes in mRNA expression of profilaggrin, another late differentiation marker. As shown in Figure 2A, CFMW increased ($P < 0.05$) profilaggrin mRNA expression in a dose-dependent manner at 24 h, although AAMW showed a slight effect. This activity was then compared with several

Figure 1. Effect of fermented milk whey on mRNA expression of differentiation markers, keratin 10 (A) and involucrin (B) over time. Cells were nonstimulated (×), stimulated with 1% CM4-fermented milk whey (CFMW, ●), or stimulated with artificially acidified milk whey (AAMW, ○); CFMW differed (* $P < 0.05$ or ** $P < 0.01$) from AAMW.

types of fermented milk whey, prepared by other species of lactic acid bacteria (*Streptococcus thermophilus*, *Lactobacillus casei*, *Lactobacillus lactis*, and *Lactococcus lactis*). The CFMW showed greater ($P < 0.01$) activity, increasing expression of profilaggrin mRNA more than 1.8 times, whereas the other whey samples tested showed similar activities to AAMW, increasing expression around 1.4 times (Figure 2B).

The possible mechanism underlying these effects was initially thought to be the calcium present in the samples because milk is rich in that mineral. It is well known that calcium is a potent inducer of keratinocyte differentiation and that calcium gradients, which rise from 0.5 mM in the epidermal basal layer to >1.4 mM in the stratum granulosum layer, are critical to normal epidermal homeostasis (Lansdown, 2002). We analyzed the calcium concentrations of these samples, and found

Figure 2. Effect of fermented milk whey on the expression of profilaggrin mRNA: A) Comparison of the activity between CM4-fermented milk whey (CFMW; black bars) and artificially acidified milk whey (AAMW; white bars). B) Comparison of the activity among several types of fermented milk whey. Cells were nonstimulated (1), stimulated with 0.3 or 1% AAMW (2), or stimulated with 0.3 or 1% fermented milk whey prepared with CM4 (CFMW; 3), *Streptococcus thermophilus* (4), *Lactobacillus casei* (5), *Lactobacillus lactis* (6), or *Lactococcus lactis* (7). * $P < 0.05$ and ** $P < 0.01$ (differed from nonstimulated cells); CFMW differed († $P < 0.05$ and †† $P < 0.01$) from AAMW.

that the final estimated concentrations of calcium in the various culture media were almost identical (Table 1). Thus, we suppose that component(s) other than calcium may be the effective agent(s) in the augmentation of keratinocyte differentiation by CFMW.

Milk contains several nutritional components, including minerals, amino acids, and lactose. In addition, its fermentation increases the doses of various milk protein-derived peptides, which are mainly cleaved from casein, the major protein in milk (Mayo, 1993). *Lactobacillus helveticus* can efficiently produce milk protein-derived peptides during fermentation because it has a potent protease activity. In such peptides, Val-Pro-Pro and Ile-Pro-Pro have been shown to act as anti-hypertensive agents because of their inhibition of angio-

Table 1. Calcium concentrations in milk wheys used in this study¹

Sample name	Calcium concentration (mM)
Artificially acidified milk whey	0.34
<i>Lactobacillus helveticus</i> CM4-fermented milk whey	0.31
<i>Streptococcus thermophilus</i> -fermented milk whey	0.30
<i>Lactobacillus casei</i> -fermented milk whey	0.35
<i>Lactobacillus lactis</i> -fermented milk whey	0.34
<i>Lactococcus lactis</i> -fermented milk whey	0.33

¹Data were obtained in duplicate and all values are expressed as the mean concentration when samples were added to the culture medium at a final concentration of 1%.

tensin I-converting enzyme, which is important in the regulation of blood pressure (Yamamoto et al., 1994; Nakamura et al., 1995). The possibility that other biologically active peptides are produced during the fermentation is reasonable, and we speculate that one or more of those elements are responsible for the effects of CFMW.

The characteristic finding of this study was that CFMW further strengthened the upregulation of involucrin expression relatively early (on 2 d), whereas upregulated keratin 10 expression was delayed (after 4 d) compared with AAMW (Figure 1). We speculate that this is characteristic of this material (i.e., strong promoting effect on keratinocyte differentiation, especially in the later stages of differentiation). This might lead to a potent profilaggrin-inducing effect, which was prominent among the fermented milk whey series prepared with other lactic acid bacteria (Figure 2). It should be noted, however, that it is still unclear whether CFMW-induced profilaggrin enhancement is *L. helveticus*-specific or is CM4 (strain)-specific. Further studies will be necessary to resolve this and to elucidate the mechanism(s) involved.

During normal epidermal differentiation, large amounts of profilaggrin are expressed in keratinocytes. Profilaggrin is proteolytically processed to filaggrin and functions to aggregate keratin proteins to form the tightly aligned macrofibril bundles characteristic of cornified cells (Dale et al., 1978). In the stratum corneum, filaggrin is further degraded by proteases and its amino acids are chemically modified to produce a mixture of hygroscopic compounds that are believed to be crucial to normal epidermal hydration and flexibility (Rawlings et al., 1994). On the other hand, dry skin (such as atopic dermatitis and ichthyosis vulgaris) exhibits reduced or absent levels of profilaggrin mRNA and protein compared with normal skin (Sybert et al., 1985; Seguchi et al., 1996). Therefore, the control of filaggrin production should be a valid approach to improve some skin mala-

dies such as skin dryness, and the use of CFMW might be very effective.

In conclusion, the present study revealed that fermented milk prepared with CM4 promotes keratinocyte differentiation, which also increases the expression of the differentiation-related element profilaggrin at the mRNA level. From these results, CFMW is expected to be a useful skin moisturizing agent that functions to increase filaggrin-related natural moisture factor.

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