Effects of Bovine Milk Fermented by Culturing with Lactic Acid Bacteria and a Yeast on the Proliferation Activity of Human Carcinoma Cells

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The effects of aged fermented bovine milk produced by a mixture of various lactic acid bacteria and a yeast, or a mixture of the lactic acid bacterium, *Bifidobacterium longum*, and a yeast on the proliferation activities of human carcinoma cells (DLD-1, MKN-1, and HL-60 cell lines) and human normal fibroblast cells (Fb) were investigated. Nonfermented bovine milk suppressed the proliferation activity in various degrees depending on the cell lines tested, suggesting that bovine milk may contain a component regulating *in vivo* proliferation of certain cells. The fermented milk produced by a mixture of various lactic acid bacteria and a yeast selectively reduced the proliferation activity of DLD-1 and MKN-1 cells. In contrast, the fermented milk produced by a mixture of *B. longum* and a yeast was selectively toxic to HL-60 cells, and cell proliferation activity was completely lost. Analysis of this cytotoxicity was revealed to be due to the induction of apoptosis which was demonstrated by the formation of apoptotic bodies and the fragmentation of DNA in treated cells. These findings strongly suggest that the cytotoxic factors affecting the viability of transformed and nontransformed cells are present in latent form in bovine milk, and these factors could be produced from milk by digestion using microbial enzymes.

Keywords: milk, lactic acid bacteria, fermented, HL-60, apoptosis

Kefir is an aged fermented milk produced by Kefir granules in which various species of lactic acid bacteria, yeasts and some species of acetic acid bacteria co-exist (Adachi *et al.* 1990). Kefir is believed to contain many functional substances. Recently, the effective utilization of enzymes by combining various enzymes from different microorganisms has received attention, because such a multi-enzyme system is expected to produce novel physiologically active substances. *Saccharomyces cerevisiae* cannot grow in bovine milk alone, but it can grow if lactic acid bacteria are present, because lactic acid bacteria provide a source of nitrogen (amino acids) for the yeast by hydrolyzing milk proteins with proteases which they secrete (Ezzat *et al.* 1985; Kok 1990; Pritchard & Coolbear 1993). In such a mixed-culture medium, it is thought that a much wider variety of substances is produced from milk components than in the case of a single-culture medium. On the basis of this, we investigated, in a previous study, a process for the manufacture of aged fermented milk using a mixture of various lactic acid bacteria and a yeast (Tamai *et al.* 1995). The fermented milk produced by a mixed culture of various strains of lactic acid bacteria and a yeast exhibited more effective antimutagenicity against a wider range of mutagens than milk produced using a single lactic acid bacterium. In the present study, we investigated the effect of fermented milk on the proliferation activity of human carcinoma cell lines and provided preliminary evidence of the cytotoxicity of fermented milk to HL-60 cells. Prior to this study, the effects of aged fermented bovine milk produced by

a mixture of various lactic acid bacteria and a yeast on cell proliferation had not been investigated.

Materials and Methods

Materials The cell titer 96 aqueous non-radioactive cell proliferation assay system (MTS assay system) was purchased from Promega. RPMI medium, Dulbecco's PBS(–), BL-agar medium, and GAM-broth were purchased from Nissui (Tokyo). All other chemicals used were of the purest grade available.

Preparation of fermented milk Two kinds of fermented milk were prepared. One fermented milk prepared by culturing with various lactic acid bacteria and a yeast corresponded to a fermented milk No. 4 in a previous paper (Tamai *et al.* 1995) and was abbreviated as F.M.1 in this paper. The lactic acid bacteria used for the mixed culture were *Lactococcus lactis* subsp. *lactis* SMK 111, *Lactococcus lactis* subsp. *cremoris* SMK 118, *Streptococcus lactis* subsp. *diactylactis* SMK 413, *Streptococcus salivarius* subsp. *thermophilus* SMK 447, *Leuconostoc cremoris* SMK 308, *Lactobacillus delbrueckii* subsp. *bulgaricus* SMK 750, *Lactobacillus casei* SMK 709, and *Lactobacillus helveticus* SMK 745. The yeast was invertase-deficient *Saccharomyces cerevisiae*. All strains were from the culture collection of the Research Laboratory of Shikoku Milk Product Co., Ltd (Ehime). Another fermented milk was prepared with *Bifidobacterium longum* SMK 595 and a yeast according to the method used for preparing fermented milk No. 5 in a previous paper (Tamai *et al.* 1995), except that the culture temperature was 35°C and culture time was 14 d. The culture temperature and time accelerated more

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growth of *B. longum* than before. This fermented milk was abbreviated as F.M.2 in this paper.

Cell cultures Human stomach carcinoma (MKN-1), human colon carcinoma (DLD-1), and human acute myeloid leukemia (HL-60) cells were obtained from the Japanese Cancer Research Resources Bank (JCRB), Tokyo. Human dermal fibroblast (Fb) cells were purchased from Cell System Corporation (Kirkland, WA). The former cell lines were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 1.4 mg/ml NaHCO_3 at 37°C in a humidified atmosphere containing 5% CO_2 . The latter were cultured in a fibroblast cell growth medium system (CS-3FO-250) at 37°C in a humidified atmosphere containing 5% CO_2 .

Effect of fermented milks on cell proliferation activity Counting viable cells: Prior to the proliferation activity test,

two kinds of fermented milk (F.M.1 and F.M.2) were adjusted to pH 7.0 by adding a small amount of 8 N NaOH. The resulting precipitates were removed by centrifugation at $27,000\times g$ for 10 min. The supernatants were sterilized by filtration through filter paper ($0.45\ \mu\text{m}$), and 1 ml of each was added to 9 ml of culture media containing 10^6 cells. As a control, bovine milk was supplemented with lactic acid until the pH of the milk reached 3.6 and then adjusted to pH 7.0 by adding 8 N NaOH. The precipitate was removed, sterilized by filtration and used. Samples incubated in the absence of milk were also used. At 24, 48 and 72 h after the start of incubation, viable HL-60 cells in suspension culture were counted using the trypan blue dye exclusion method. In those cases where cells adhered to the flasks (DLD-1, MKN-1, and Fb cells), the culture supernatants were discarded, the flasks were washed twice with phosphate-buffered saline (PBS(-)), and the cells

Fig. 1. Effects of milk on the growth of human transformed and nontransformed cells by counting viable cells. Human cells were cultured in the absence or presence of fermented or nonfermented bovine milk. Cells were counted at 24, 48 and 72 h. $\square$, in the absence of milk. $\diamond$, in the presence of nonfermented bovine milk. $\circ$, in the presence of fermented milk (F.M.1). $\triangle$, in the presence of fermented milk (F.M.2).

were detached by trypsin treatment and counted. The cells that remained attached to the flasks after washing were regarded as viable cells.

Cell titer 96 aqueous non-radioactive cell proliferation assay (MTS assay) Cells (5×10^3 cells) were suspended in 100 μ l of media in 96-well plates, to which 10 μ l/well of fermented or nonfermented milk which had been treated as previously described was added, and the plate was incubated at 37°C. At 4, 24 and 48 h after the start of incubation, 22 μ l of the MTS/PMS-solution (20:1, v/v) was added to each well, the incubation was continued for 4 h, and then the absorbance at 490 nm of the solution in each well was measured using a micro-plate reader (BioRad, Model 450). The absorbance has been confirmed to be correlated with cell viability (Bartrop & Owen 1991; Tada *et al.* 1986).

Morphological examination HL-60 cells incubated for

48 h in the presence or absence of fermented milk (F.M.2) were observed under a light microscope.

Analysis of DNA fragmentation HL-60 cells (5×10^5 cells/200 μ l) cultured for 12 h and 24 h in the absence or presence of fermented milk (F.M.2) were supplemented with 10 μ l of 10 mg/ml proteinase K, 10 μ l of 10 mg/ml RNase A, and 20 μ l of 10% SDS and incubated at 37°C for 30 min. Three hundred microliters of NaI solution containing 6 M NaI, 13 mM EDTA, 0.5% sodium N-lauroyl sarcosinate, 10 mg/ml glycogen, and 20 mM Tris-HCl, pH 8.0, was then added to each culture. After incubation at 60°C for 15 min, the cultures were mixed with 500 μ l of 100% isopropanol, and DNA fragments were prepared according to the procedure described by Ishizawa *et al.* (1991). The DNA fragments were loaded onto a 1.5% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) and electrophoresed at a

Fig. 2. Effects of milk on the proliferation activity of human transformed and nontransformed cells measured by the MTS assay. Human cells were cultured in the absence or presence of fermented or nonfermented bovine milk. At 4, 24, and 48 h after the start of culture, the MTS/PMS-solution was added. After a further incubation for 4 h, the absorbance at 490 nm was measured. The symbols are the same as those in Fig. 1.

constant voltage of 100 V. The gel was stained with ethidium bromide and photographed on a UV transilluminator.

Results and Discussion

The sensitivities of human carcinoma cells and normal cells to the nonfermented and two kinds of fermented bovine milk were assayed. Figure 1 shows the number of viable cells which proliferated in the presence and absence of the milks. The fermented milk cultured with various lactic acid bacteria and a yeast (F.M.1) exhibited strong proliferation inhibition activity toward two cell lines, DLD-1 and MKN-1. In contrast, the effect of this fermented milk on the proliferation activity of HL-60 cells was the same as that of nonfermented bovine milk, suggesting that the sensitivity to this fermented milk is different among the cell lines, transformed cells and nontransformed cells. Almost the same results were observed using the MTS assay (Fig. 2). The fermented milk cultured with a single lactic acid bacterium, *B. longum*, and a yeast (F.M.2) exhibited marked proliferation inhibition activity toward HL-60 cells, but not toward the other cells tested. The inhibition of the proliferation activity of HL-60 cells measured by the MTS assay also decreased with incubation time. That a viability decrease with culture time was specific to HL-60 cells suggested that HL-60 cells were likely to lyse by the F.M.2 during culturing. On the other hand, the

nonfermented bovine milk exhibited a moderate proliferation inhibition activity toward the DLD-1 and HL-60 cells and a weak inhibition activity toward the MKN-1 and Fb cells, although the effect of nonfermented bovine milk on cell proliferation was much less when the activity was measured by the MTS assay. Recently, Hakansson *et al.* (1995) reported that a multimeric form of α -lactalbumin present in human milk induces cell death in various cell lines *in vitro*. Our results suggest that bovine milk may also have a suppressive effect on the proliferation of certain cell lines.

While investigating the effect of fermented milk on the proliferation activity of cells, we observed morphological changes in HL-60 cells incubated with milk fermented by culturing with a mixture of *B. longum* and a yeast. Figure 3 shows the morphological change in HL-60 cells incubated for 48 h in the presence of F.M.2. The cells exhibited shrinkage and the formation of apoptotic bodies, which are compatible with the occurrence of apoptosis (Kerr *et al.* 1987). Untreated HL-60 cells maintained their normal morphology during the test period. The DNA of HL-60 cells was then examined. DNA fragmentation is known as a characteristic feature of apoptosis (Wyllie 1980; Vaux & Strasser 1996). As shown in Fig. 4, oligonucleosome-length DNA fragments (DNA laddering), characteristic of apoptosis were observed in HL-60 cells incubated for 24 h in the presence of F.M.2.

The milk fermented with a single strain of lactic acid bacterium alone, such as *Bifidobacterium longum*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, or *Streptococcus salivarius* subsp. *thermophilus* had no significant effect on the proliferation of HL-60 cells (data not shown). Fermented milk products such as yogurt are commonly manufactured by fermentation within a day. In contrast, F.M.2 was prepared

Fig. 3. Apoptosis in HL-60 cells induced by incubation with fermented milk (F.M.2). (A) Cells 24 h after incubation in the absence of milk. (B) Cells 24 h after incubation in the presence of fermented milk (F.M.2).

Fig. 4. Agarose gel electrophoresis of DNA extracted from HL-60 cells. Lane 1, molecular size markers. Lane 2, DNA from HL-60 cells cultured for 24 h in the absence of milk. Lane 3, DNA from HL-60 cells cultured 12 h in the absence of milk. Lane 4, DNA from HL-60 cells cultured 24 h in the presence of fermented milk (F.M.2). Lane 5, DNA from HL-60 cells cultured for 12 h in the presence of fermented milk (F.M.2).

after a long incubation of over 14 d, suggesting that autolysis of yeast cells will occur during incubation. This would lead to the participation of various intracellular enzymes of yeast in the fermentation of milk. On the other hand, raw bovine milk has not been reported to induce apoptosis in several cell lines (Hakansson *et al.* 1995). These observations, combined with our present results, indicate that bovine milk contains latent factors which can induce apoptosis in HL-60 cells and the factors would be produced from the milk by cooperative digestion of the milk components using enzymes from both microorganisms, *B. longum* and *S. cerevisiae*.

The tryptic hydrolyzates of bovine casein have been known to have various biological activities (Chiba & Arai 1988). However, only a few experiments have been performed on the antitumor activity of fermented milk directly on cultured human cell lines. Biffi *et al.* (1977) reported that the milks fermented with *B. infantis* and *L. acidophilus* effectively inhibited the growth of MCF7 cells. Nagaune (1995) also described that tryptic peptide (P-10) from κ -casein exhibited proliferation inhibition activity in BALB/c3T3 cells *in vitro*. The milk fermented by a mixed culture with various lactic acid bacteria and a yeast (F.M.1) selectively exhibited proliferation inhibition activity toward two human carcinoma cell lines (DLD-1 and MKN-1), but not toward human leukemia HL-60. These results suggest that the cell proliferation inhibition substances derived from milk might be different between F.M.1 and F.M.2, and bovine milk must contain a wide variety of latent factors that regulate the proliferation of various types of animal cells. We are currently isolating and identifying the substances that induce apoptosis in HL-60 cells with a view toward clinical application of the factors.

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