Effects of highly ripened cheeses on HL-60 human leukemia cells: Antiproliferative activity and induction of apoptotic DNA damage

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ABSTRACT

To establish cheese as a dairy product with health benefits, we examined the multifunctional role of cheeses. In this report, we clarify whether different types of commercial cheeses may possess antiproliferative activity using HL-60 human promyelocytic leukemia cell lines as a cancer model. Among 12 cheese extracts tested, 6 (Montagnard, Pont-l'Évêque, Brie, Camembert, Danablu, and Blue) revealed strong growth inhibition activity and induction of DNA fragmentation in HL-60 cells. Based on the quantification of nitrogen contents in different cheese samples, a positive correlation between the ripeness of various cheeses and their antiproliferative activity tested in HL-60 cells was displayed. Four varieties of Blue cheese ripened for 0, 1, 2, or 3 mo demonstrated that the Blue cheese ripened for a long term was capable of causing the strong suppression of the cell growth and the induction of apoptotic DNA damage as well as nucleic morphological change in HL-60 cells. Collectively, these results obtained suggest a potential role of highly ripened cheeses in the prevention of leukemic cell proliferation.

Key words: cheese ripening, antiproliferation, apoptosis, leukemia

INTRODUCTION

Cheese is a well-known fermented dairy product that is widely produced throughout the world from milk of cows and other ruminants. Because of enriched ingredients (e.g., proteins, peptides, fatty acids, and unidentified bacterial metabolites), numerous studies have been conducted to clarify the health benefit of the daily intake of these fermented milk products (Woo et al., 1984; Collins et al., 2003; Meisel, 2004). During milk fer-

mentation, bacterial proteolytic and lipolytic enzymes provide more bioactive compounds (Matar et al., 1996, 2003; Roy et al., 1999; Sousa et al., 2001; Collins et al., 2003; Inayat et al., 2003). A casein-derived peptide, Asp-Lys-Ile-His-Pro-Phe, generated during ripening of Edam cheese has been found to demonstrate inhibition activity in the intestinal absorption of β -LG (Tanabe et al., 2003), whereas other casein-derived peptides (e.g., Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met and Tyr-Pro) generated in milk-fermented products have been shown to serve as antioxidative (Kudoh et al., 2001) and antihypertensive (Yamamoto et al., 1999) agents, respectively.

With regard to cancer prevention and treatment, a considerable number of studies have indicated that dairy and natural food products may possess anticancer activity (Ip et al., 2003; Kandaswami, 2005; Parodi, 2007). Proteolytically digested skim milk or fermented milk cultured with various lactic acid bacteria and yeast can exhibit differential suppressive activities on the cell growth of human leukemia cells (Ito et al., 1998; Roy et al., 1999). By serving as an apoptotic inducer for tumor development, compounds found in bovine milk (e.g., lactoferrin and lactoferricin) can suppress the growth of cancer cells in which DNA damage is also involved in vitro and in vivo (Roy et al., 2002; de Moreno de LeBlanc et al., 2005; Mader et al., 2005). A recent report has indicated that 1) cysteine and cysteine-enriched proteins, 2) peptides or γ -glutamylcysteine dipeptides originated from whey protein, which are efficient substrates for glutathione synthesis, or 3) both can contribute to suppress tumorigenesis (Parodi, 2007). We therefore were interested in examining the differential antiproliferative effects of cheeses and clarifying the role of ripeness for cancer prevention.

In this study, we investigated the potential role of commercial cheese products on the cell growth and induction of DNA fragmentation in HL-60 human promyelocytic leukemia cells as a cancer model. The HL-60 cell line was chosen because of a valid and useful model

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for discovery of cancer chemopreventive or chemotherapeutic agents from natural products (Gorczyca et al., 1993; Suh et al., 1995; Roy et al., 1999, 2002; Kawaii and Lansky, 2004; Yu et al., 2008). A systematic investigation of the differential antiproliferative activities of the 12 cheese samples as well as Blue cheese with varying ripening periods was performed. Moreover, the evaluation of associations between the ripeness of individual cheese and the antiproliferative activity examined in HL-60 cells was carried out.

MATERIALS AND METHODS

Materials

Eleven commercial cheese products including 2 wash type (Montagnard and Pont-l'Eveque), 3 mold-ripened type (Brie, Camembert, Danablu), 2 propionic acid bacteria-ripened type (Emmental and Gruyere), and 4 lactic acid bacteria-ripened type (Parmesan, Edam, Gouda, and Mozzarella) cheeses imported from overseas were purchased from retail stores (Kumamoto, Japan). Another mold-ripened type cheese (Blue) with 4 varying ripening periods (0, 1, 2, or 3 mo) was kindly provided by a domestic manufacturer, Snow Brand Milk Products Co. Ltd. (Saitama, Japan). These cheese products were stored at -20°C until use. The HL-60 human promyelocytic leukemia cells (JCRB0085) were obtained from Japanese Collection of Research Bioresources (Tokyo, Japan). Cisplatin, RPMI 1640 medium, RNase A, and ethidium bromide were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum, penicillin G, and streptomycin sulfate were purchased from Invitrogen (Carlsbad, CA). Cell counting kit-8 containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), and 1-methoxy-5-methyl-phenazinium-methyl-sulfate and Hoechst 33258 solution were products of Dojindo Labs (Kumamoto, Japan). DNA 200-bp ladder was from Takara Biotech (Tokyo, Japan). Proteinase K was purchased from Wako Chemicals (Osaka, Japan). Dimethyl sulfoxide (DMSO), 8% glutaraldehyde solution, and isopropanol were products from Nacalai Tesque Inc. (Kyoto, Japan). Advantec no. 3 filter paper was obtained from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). All other chemicals were of the highest grade commercially available.

Preparation of Cheese Extracts

The cheese extract was prepared from individual samples by the following method. Briefly, dried cheese samples were first prepared from approximately 5 g (wet weight) of manufactured cheeses by lyophilization

and crushed. Thereafter, 1 g of dried cheese samples was mixed with 16 mL of methanol on ice. The cheese suspension therein was homogenized for 30 s for 3 times on ice using a Polytron PT20 (Kinematica AG, Lucerne, Switzerland). After the centrifugation at $30,000 \times g$ for 20 min at 4°C , 100 μL of the supernatant was collected and allowed to dry. Subsequently, the resulting cheese extracts were redissolved in a same amount of DMSO for the cell proliferation assay.

Proliferation Assay of HL-60 Cells

The HL-60 human promyelocytic leukemia cells were routinely maintained under a 5% CO_2 atmosphere at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin G (100 U/mL), and streptomycin sulfate (100 $\mu\text{g}/\text{mL}$). For the cell proliferation assay, HL-60 cells were seeded in individual wells of a 96-well culture plate at a density of 2×10^4 cells/100 μL per well in the same medium. The cell counting kit-8 was used to measure the activities of dehydrogenase enzyme(s) in viable cells according to the manufacturer's instructions. Briefly, 1 μL of cheese extracts, reconstituted in DMSO, was added to each well. After incubation for 24 h, 10 μL of 5 mM WST-8 solution containing 0.2 mM 1-methoxy-5-methyl-phenazinium-methyl-sulfate and 150 mM NaCl was added to each well, followed by another 3 h of incubation. The reduction of WST-8 was determined colorimetrically at 450 nm using an automatic microplate spectrophotometer (model 550, BioRad Laboratories Inc., Hercules, CA).

Analysis of DNA Fragmentation in HL-60 Cells

For the visualization of DNA fragmentation, HL-60 cells grown in a 30-mm tissue culture dish (density at 2×10^5 cells/mL) were incubated with 2 mL of culture medium in the presence of cheese extracts at 1%. After 6 h of incubation, the cells were collected after centrifugation, washed twice with 1 mL of PBS, and lysed for 10 min on ice in 130 μL of lysis buffer (10 mM Tris-HCl buffer, pH 7.4, 10 mM EDTA, and 0.5% Triton X-100). Upon centrifugation at $20,000 \times g$ for 20 min at 4°C , 100 μL of supernatant containing DNA was collected and treated with 400 $\mu\text{g}/\text{mL}$ of RNase A for 1 h at 37°C and with 300 $\mu\text{g}/\text{mL}$ of proteinase K for another 30 min at 50°C . Thereafter, DNA was precipitated with an additional 120 μL of isopropanol plus 20 μL of 5 M NaCl overnight at -20°C . The DNA precipitates, dissolved in Tris-EDTA buffer containing 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA were applied onto a 2% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining.

Measurement of Nitrogen Contents in Cheese Samples

The amounts of water-soluble nitrogen (SN) and total nitrogen (TN) of the cheeses were determined by a macro-Kjeldahl method using a Kjeltex system as described in the previous report (Higashio and Yoshioka, 1983). Briefly, 20 g of each solid cheese that was homogenized in 50 mL of 0.5 M trisodium citrate solution and adjusted to 200 mL with MilliQ water (Millipore, Billerica, MA) was used for the analysis of TN. In the case of SN, 100 mL of the cheese for TN analysis was adjusted to pH 4.4 and filled up to 200 mL with MilliQ water, and the filtrate was prepared with an Advantec no. 3 filter paper. For determination of nitrogen content, digestion and distillation of samples were carried out using a 2200 Kjeltex Autodistiller unit (Foss Tecator AB, Hoganas, Sweden). Ripeness of individual cheese sample was calculated based on the percentage of SN against TN content in cheese extracts as determined above.

Detection of Nuclear Morphological Changes in HL-60 Cells

Apoptotic cell death was also evaluated by fluorescence microscopy after staining with Hoechst 33258. Cells grown in a 30-mm tissue culture dish (density at 2×10^5 cells/mL) were incubated with 2 mL of culture medium in the presence of Blue cheese extract at 1%. After 0, 6, or 12 h of incubation, the cells were collected in a micro tube, fixed with PBS containing 1% glutaraldehyde, and kept at room temperature for 30 min. Thereafter, the cells suspended in 20 μ L of PBS were stained with 4 μ L of 1 mM Hoechst 33258 following the observation of nuclear morphological changes using an Olympus BX51 fluorescent microscope at 200 $\times$ magnification (Olympus Optical Co. Ltd., Tokyo, Japan). Digital photo data taken by a Photometric Sensys camera were analyzed using Sensys MetaMorph software (Roper Scientific Inc., Tucson, AZ).

Statistical Analysis

The values are expressed as the means $\pm$ standard deviation derived from 3 or 4 parallel experiments from individual cheese extracts. All data were analyzed using a statistical add-on software program (Statcel, OMS Co., Saitama, Japan) for Microsoft Excel 2004 (Microsoft Corp., Redmond, WA). Statistical differences between control and test sample-treated cells were considered significant at $P < 0.05$, $P < 0.01$, or $P < 0.001$ using Student's *t*-test. Evaluation of the associations between ripeness (SN:TN ratio, %) and growth inhibi-

tion rate examined was carried out using Pearson correlation test. $P < 0.0001$ or $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The objective of the current study was to investigate the antiproliferative effects of commercial cheese products and the potential role the products have in induction of DNA damage in HL-60 human promyelocytic leukemia cells as a cancer model. With regard to cancer prevention and treatment, a considerable number of studies have indicated that dairy and natural food products may possess anticancer activity (Ip et al., 2003; Kandaswami, 2005; Parodi, 2007). We therefore were interested in examining the differential antiproliferative effects of various commercial cheeses with different ripening rates on cancer cell growth in vitro.

Correlation of Cytotoxic Activity and Ripeness of Commercial Cheeses

In this study, the antiproliferative effect of methanol extracts prepared from 12 different cheeses was first investigated using HL-60 human promyelocytic leukemia cells. The HL-60 cell line was chosen because of a valid and useful model for discovery of cancer chemopreventive or chemotherapeutic agents from natural products (Gorczyca et al., 1993; Suh et al., 1995; Roy et al., 1999, 2002; Kawaii and Lansky, 2004; Yu et al., 2008). As shown in Figure 1, the data calculated from colorimetric analysis of viable cells revealed that 6 (Emmental, Gruyere, Parmesan, Edam, Gouda, and Mozzarella) cheese extracts exhibited virtually no inhibition activities in HL-60 cell growth. In contrast, the other 6 (Montagnard, Pont-l'Eveque, Brie, Camembert, Danablu, and Blue) cheese extracts displayed significant strong activities at more than 50% in the growth inhibition. The 2 (Emmental and Gruyere) and 4 (Parmesan, Edam, Gouda, and Mozzarella) that demonstrated no growth inhibition activities are classified, respectively, as propionic acid bacteria-ripened type and lactic acid bacteria-ripened type cheeses. Two cheeses categorized as wash type (Montagnard and Pont-l'Eveque) and 4 cheeses as mold-ripened type (Brie, Camembert, Danablu, and Blue) also showed strong activities. In this experiment, 2×10^4 cells in 100 μ L of culture medium were exposed to the methanol-soluble extract from 62.5 μ g of dried cheese. In another experimental setting, a dose-dependent growth inhibition was observed in 3 cheeses (Blue, Camembert, and Pont-l'Eveque), whereas no activity was found in 2 other cheeses (Gruyere and Gouda; data not shown). Previous studies have demon-

Figure 1. Antiproliferative activities of 12 different commercial cheeses in HL-60 human promyelocytic leukemia cells. Control indicates the cell group treated with dimethyl sulfoxide as a vehicle. Growth inhibition rate was calculated based on the cell viability measured by WST-8 assay. Data shown represent mean $\pm$ standard deviation derived from 4 determinations of individual cheese samples. Asterisk indicates statistical significance versus the inhibition rate of control ($P < 0.001$).

strated that proteolytically digested skim milk or fermented milk cultured with various lactic acid bacteria and yeast can exhibit differential suppressive activities on the HL-60 cell growth (Ito et al., 1998; Roy et al., 1999). It is thought that biologically active molecule(s) for antiproliferative activity against cancer cells in these cheeses tested can be generated by the action of different types of fungi or other microorganism(s) during their manufacture.

To gain insight into the involvement of cytotoxic effects of cheese extracts in HL-60 cells, the induction of apoptotic DNA fragmentation was analyzed. As shown in Figure 2, apoptotic DNA fragmentation patterns in HL-60 cells were visualized when cells were incubated in the presence of Montagnard, Pont-l'Eveque, Brie, Camembert, and Blue cheese extracts. Among them, the intensity of fragmented DNA was prominent in the cells grown with Pont-l'Eveque cheese extract. Another important issue is the extent of the induction of apoptotic DNA damage in HL-60 cells after incubation with the cheese extract. A parallel experiment was performed in the presence of Blue cheese or cisplatin, an apoptosis-inducing agent (Gorczyca et al., 1993). As shown in Figure 3, there was an increase of fragmented DNA when cells were incubated in the presence of Blue cheese at the amount of 1 $\times$ or 2 $\times$. The visualized DNA fragmentation pattern shown at 2 $\times$ Blue cheese was seemingly similar to the pattern demonstrated at

50 μ M cisplatin. It is to be noted that the circulating levels of cisplatin in humans reach approximately 12.5 μ M (Untch et al., 1994; Andreotti et al., 1995). It has been reported that bovine milk and its fermented products have potential cytotoxic properties against different types of tumor cells in vitro and murine breast cancer cells in vivo (Ito et al., 1998; Mader et al., 2005; de Moreno de LeBlanc et al., 2005). Moreover, skim milk digested with a proteolytic enzyme from yeast can strongly inhibit the proliferation of HL-60 cells and induce apoptotic cell death, including the formation of apoptotic bodies and DNA fragmentations (Roy et al., 1999). A recent report has indicated that 1) cysteine and cysteine-enriched proteins 2) or peptides or γ -glutamylcysteine dipeptides originated from whey protein, which are efficient substrates for the glutathione synthesis, or 3) both may contribute to suppress tumorigenesis (Parodi, 2007). It is therefore conceivable that the antiproliferative activities of the cheese extracts shown in Figure 1 may be because of the active molecule(s) present in these cheeses. Whether these active molecules from cheeses may be present at enough concentration to exert cancer-preventive action will be an issue for further investigation. It is presently unclear whether the antiproliferative action of cheese constituent(s) enterogastrically digested (e.g., by several proteolytic and lipolytic enzymes) is markedly enhanced or attenuated at the circulation in the body.

Figure 2. Analysis of the induction of DNA fragmentation by 12 different commercial cheeses in HL-60 cells. Sample analyzed corresponds to Montagnard (lane 1), Pont-l'Eveque (lane 2), Brie (lane 3), Camembert (lane 4), Danablu (lane 5), Blue (lane 6), Emmental (lane 7), Gruyere (lane 8), Parmesan (lane 9), Edam (lane 10), Gouda (lane 11), Mozzarella (lane 12) cheeses, and control (lane 13). Lane M shows the DNA size markers coelectrophoresed during agarose gel electrophoresis. The figure is illustrative of 3 independent repetitions.

Figure 3. Comparison of induction of DNA fragmentation by Blue cheese and cisplatin in HL-60 cells. Sample analyzed corresponds to control (lane 1), dimethyl sulfoxide (lane 2), 1× and 2× concentration of Blue cheese (lanes 3 and 4, respectively), PBS (lane 5), and 10 and 50 μ M (lanes 6 and 7, respectively). Lane M shows the DNA size markers coelectrophoresed during agarose gel electrophoresis. The figure is illustrative of 3 independent repetitions.

In view of the fact that FFA are also thought to be key compounds in directly regulating the apoptotic cell death as well as lipid metabolism-mediated cell death in HL-60 cells (Kuhajda et al., 1994; Yu et al., 2008), it is important to investigate whether specific digestion of the cheese compound(s) by proteolytic and lipolytic enzymes in the body may provide more useful information and strategy in potentiating prevention of cancer.

Figure 4 shows the ripeness exhibiting a highly significant positive correlation with the growth inhibition of various cheeses ($r = 0.943$; $P = 0.0000140$). This result implies that highly ripened commercial cheeses may indeed possess antiproliferative activity against HL-60 cells rather than the low-ripened cheeses in vitro. In a previous report, we demonstrated a significant correlation between bioavailability of cheeses (e.g., antioxidative activity) and peptide generation during

fermentation (Igoshi et al., 2008). We therefore were interested in examining the relationship between the antiproliferative activity of cheeses and their aspect of ripeness. Determination of SN and TN contents in individual cheese samples was performed using a macro-Kjeldahl method to generate the SN:TN ratio, defined as a ripeness index.

Correlation of Cytotoxic Activity and Ripeness of Blue Cheese

To further investigate the mechanism of their antiproliferative activities in HL-60 cells, the effects of ripening periods in Blue cheese on cell growth were analyzed. Figure 5 shows that ripeness displayed a significant positive correlation with the growth inhibition of the Blue cheeses ($r = 0.985$; $P = 0.016$). It is therefore an interesting issue whether ripeness can be considered as a key factor for dictating the antiproliferative activity of fermented milk food products. It is of note that ripeness and peptide contents of various cheese samples demonstrate significant positive correlations with their antioxidative properties (Igoshi et al., 2008).

To examine whether the induction of DNA damage in HL-60 cells by the Blue cheese ripened for a long

Figure 4. Relationship of 10 different commercial cheeses to the ripening rate and the growth inhibition rate determined in HL-60 cells. Each data point corresponds to cheese samples as follows: Pont-l'Eveque (a), Brie (b), Camembert (c), Blue (d), Danablu (e), Montagnard (f), Gouda (g), Parmesan (h), Gruyere (i), Emmental (j), and Mozzarella (k). Data represent mean values derived from 3 experiments of individual cheese samples. $P < 0.0001$ was considered statistically significant. SN = water-soluble nitrogen; TN = total nitrogen.

Figure 5. Relationship of Blue cheese with various ripening periods to the ripening rate and the growth inhibition rate determined in HL-60 cells. Each data point corresponds to Blue cheese samples after 3 mo (a), 2 mo (b), 1 mo (c), and 0 mo (d) ripening. Data shown represent mean values derived from 3 experiments of individual cheese samples. $P < 0.05$ was considered statistically significant. SN = water-soluble nitrogen; TN = total nitrogen.

term occurs in the context of the antiproliferative activity, the involvement of apoptotic DNA fragmentation and morphological changes of nuclei in the cells was examined. As shown in Figure 6, there was an increase of the fragmented DNA when the cells were incubated for 6 h in the presence of Blue cheeses fermented for 2 and 3 mo. To clarify how many cells undergo the apoptotic nuclear damage, apoptosis-characteristic morphological changes (e.g., chromatin condensation, blebbing, and fragmentation) were displayed and scored (Figure 7). The ratios of apoptotic cells after 6 h of incubation in the presence of Blue cheese at the amount of 0.5×, 1×, and 2× were 3.8, 14.6, and 38.6%, respectively, whereas those of apoptotic cells in the presence of cisplatin at 10 and 50 μM were 12.3 and 27.3%, respectively. A time-dependent increase of apoptotic cells was prominent when cells were treated with 50 μM cisplatin. It should be emphasized that the distinct antiproliferative activity of cheese extracts needs to be quantitatively evaluated in a wide range of apoptotic indices (e.g., activation of caspase cascades, changes in mitochondrial membrane permeability and other apoptosis-related signal pathways) as well as necrotic indices. Another important issue is with regard to the cell type or tissue-specific induction of apoptosis by cheese components. Nevertheless, further investigation should be given to whether the antiproliferative

effect of cheeses found is specific not to normal cells but cancer cells. Several reports have shown that bacterial proteolytic and lipolytic enzymes provide more bioactive compounds during milk fermentation (Matar et al., 1996, 2003; Roy et al., 1999; Sousa et al., 2001; Collins et al., 2003; Inayat et al., 2003). It is therefore conceivable that the chemically active molecule(s) that was (were) capable of triggering apoptosis might be generated during a long-term ripening in Blue cheese and perhaps other cheeses as well.

CONCLUSIONS

In this report, we demonstrated for the first time a potential antiproliferative effect of commercial cheeses using HL-60 human promyelocytic leukemia cells as a model. Cell proliferation assay and the electrophoretic patterns of DNA revealed that highly-ripened cheeses have strong activities on the growth inhibition and induction of DNA fragmentation in HL-60 cells. Whether

Figure 6. Effect of ripening period of Blue cheese on the induction of DNA fragmentation in HL-60 cells. Sample analyzed in lanes C, 0, 1, 2, and 3 corresponds to control, 0-, 1-, 2-, and 3-mo ripened Blue cheeses, respectively. Lane M shows the DNA size markers coelectrophoresed during agarose gel electrophoresis. The figure is illustrative of 3 independent repetitions.

Figure 7. Detection of nuclear morphological changes in HL-60 cells after incubation in the presence of Blue cheese or cisplatin. Cells were incubated in the presence of 3-mo ripened Blue cheese at the concentration of 0.5×, 1×, and 2× or cisplatin at 10 and 50 μM for 6 h (gray column) or 12 h (black column). Control indicates the cells before test sample treatment (white column). Fluorescent nuclei were screened for normal or apoptotic nuclei (fragmented and condensed nuclei) as marked by arrowheads. The insets show a part from representative fluorescence photomicrographs (observed area: $455 \times 365 \mu\text{m}$; magnification: 200×) taken for the nuclei of cells stained with a Hoechst 33258 a) before, b) after 6 h of incubation with 1× Blue cheese, or c) after 12 h of incubation with 50 μM cisplatin. A white scale bar indicates 10 μm . Data represent mean $\pm$ standard deviation derived from 3 independent determinations, each carried out in duplicate. Statistical significance versus the ratio of apoptotic cells in control is indicated by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). DMSO = dimethyl sulfoxide.

the bioactive compound(s) present in these cheeses are responsible for the antiproliferative effect in the leukemic cell model remains to be entirely clarified. These results may dictate a potential role of cheese products in suppressive effects on cancer cell growth, and ripeness can be considered as a key factor. Extensions of this work are warranted to fully elucidate the beneficial contributions of fermented dairy products in cancer prevention.

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