Selective induction of apoptosis in human gastric cancer cells by Lactobacillus kefiri (PFT), a novel kefir product

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Abstract. The present study was undertaken to evaluate the effect of Lactobacillus kefiri (PFT), a novel kefir product, on apoptosis of gastric cancer cells (AGS), breast cancer cells (4T1), and human peripheral blood mononuclear cells (PBMCs). Cells were cultured with PFT and apoptosis was determined by flow cytometry using 7-AAD dye and cytospin preparation. Mitochondrial dysfunction and expression of Bcl2 were monitored by flow cytometry. Results showed that PFT induced apoptosis in AGS gastric cancer cells in a dose-dependent manner. Apoptosis was detected at a concentration of 0.3 mg/ml (20.8%), increased to 25.8% at 0.6 mg/ml, 37% at 1.2 mg/ml, 53.1% at 2.5 mg/ml, and peaked at 66.3% at 5.0 mg/ml. Apoptosis is associated with the decreased polarization of mitochondrial membrane potential (MMP) and decreased Bcl2 expression. PFT-treated AGS cells manifested membrane blebbing, nuclear condensation, and fragmentation as identified in cytospin cytocentrifuge Giemsa stained preparations. On the other hand, flow cytometry analysis showed that PFT did not induce apoptosis in 4T1 breast cancer cells nor in PBMCs. These results suggest that PFT is safe for white blood cells and selectively induces apoptotic effects in gastric cancer cells. Hence, it may have potential as a therapeutic agent for the treatment of gastric cancers.

Introduction

Globally, stomach cancer is the fifth most common type of cancer and the third leading cause of cancer deaths, making up 7% of cases and 9% of deaths (1). In 2012, 950,000 people contracted stomach cancer worldwide, leading to 723,000 deaths (1). In 2014, the United States recorded an estimated 22,220 new cases and 10,990 deaths from stomach cancer (2). Outcomes are often poor with a <10% 5-year survival rate globally, and a 28% 5-year survival rate in the United States (3). Gastric cancer is a multifactorial disease. The most common cause is infection by the bacteria Helicobacter pylori, which is responsible for 65-80% of gastric cancers (4), though other factors such as genetics, smoking, and diet (especially eating pickled vegetables) have also been shown to play an important role in the development of gastric cancer. Surgery, chemotherapy, radiation therapy, and targeted therapy are commonly used to treat this disease (5), and if treated late, palliative care may also be advised (4).

Chemotherapy aims to initiate apoptosis in gastric cancer cells (6,7); however, these drugs can be toxic. Therefore, recent studies have made an effort to investigate alternative treatments that have fewer and less potent side effects. Lactic acid bacteria (LAB) may represent a useful approach for the treatment of cancer. LAB are present in many foods such as yogurt and have been shown to elicit antitumor effects. Probiotics have been shown to act preventatively in in vitro studies and during carcinogenesis in studies on animals bearing tumors. For example, multiple studies show various LAB strains exert inhibitory effects on the growth of different types of tumors in rodents (8-11).

Earlier studies also reveal that different strains of LAB have anti-proliferative effects against human cancer cell lines. For example, Lactococcus lactis ssp. lactis (L.lac CF) induces apoptosis on the human colon cancer cell line SNUGC2A (12), L. casei rhamnosus induces apoptosis in the human monocytic leukemia cell line THP-1 (13), and L. reuteri enhances tumor necrosis factor (TNF)-induced apoptosis in human chronic myeloid leukemia-derived cells (14). Furthermore, probiotic consumption might be associated with reducing the incidence of colon tumors, as shown in epidemiological studies (15). This data suggests that fermented milk products and/or the fermentative bacteria themselves may have chemoprotective effects without the toxic side effects of conventional therapeutic drugs.

Probiotics Fermentation Technology (PFT), a kefir grain product, is a natural mixture composed primarily of Lactobacillus kefiri P-1F, a specific strain of L. kefiri with unique growth characteristics. Our recent studies have demonstrated the ability of PFT to induce apoptosis on human MDR myeloid leukemia (HL60/AR) cells in vitro (16). The present study was designed to examine the possible apoptotic effect of PFT against other types of cancer, specifically the human gastric cancer AGS cells, and murine breast cancer 4T1 cells, in vitro. It was of interest to note that PFT selec-
tively exerts apoptotic effects on AGS cells, but not on 4T1 cells. Furthermore, PFT showed no apoptotic effect on human PBMCs. The mechanism underlying the effect of PFT was examined.

Materials and methods

Tumor cell lines. Two cancer cell lines were used in the present study, namely: AGS, a human gastric adenocarcinoma cell line, and 4T1, a murine breast cancer cell line. The cells were purchased from American Tissue and Culture Collection (ATCC) (Manassas, VA, USA). AGS tumor cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, and 100 µg/ml streptomycin and penicillin. 4T1 tumor cells were maintained in RPMI-1614 (Invitorgen Corp.) supplemented with 10% FBS and 2 mM glutamine, and 100 µg/ml streptomycin and penicillin. Cells were routinely maintained in log phase in a humidified incubator at 37˚C with 5% CO₂.

Probiotics Fermentation Technology (PFT) kefir grain product. PFT is a mixture that consists mainly (~90%) of a freeze-dried form of heat-killed *L. kefiri* P-IF. In addition, PFT contains ~2-3% each, of one bacterial strain *L. kefiri* P-IF. As well as the yeast strains *Kazachstania turicensis*, *Kazachstania unispora* and *Kluveromyces marxianus*. P-IF is a specific strain of LAB that has a unique DNA sequence, and PET scans show a 99.6% homology with regular kefrires. Its characteristics were recently reported (16). PFT was provided by Paitos Co., Ltd., yokohama, Kanagawa, Japan.

**PFT induces apoptosis on cancer cells - flow cytometry study.** 7-Aminoactinomycin D (7-AAD staining) was used to detect cancer cell viability. AGS cells were cultured in the presence or absence of PFT at different concentrations (0.0, 0.3, 0.6, 1.2, 2.5 and 5 mg/ml) for 3 days and the percentage of dead cancer cells was examined by 7-AAD (BD Biosciences, San Diego, CA, USA) technique using a FACSCalibur (Becton-Dickinson). A decrease in signal of difference in the percent changes of apoptotic significance of difference in the percent changes of apoptotic

**Mechanism underlying PFT effect**

*a) Expression of Bcl-2.* For detection of Bcl-2, cells were first fixed and permeabilized with ice-cold 70% methanol. Cells were then stained with FITC-labeled anti-Bcl-2 or isotype control (Dako Corp., Carpinteria, CA, USA), washed and analyzed by FACSCalibur. The percentage of cells expressing Bcl-2 and mean fluorescent intensity (an indicator of density of the molecules/cell) was determined.

*b) Detection of mitochondrial membrane potential (MMP).* Variations of the mitochondrial transmembrane potential ∆Ψm during apoptosis were studied using tetramethylrhodamine ethylester (TMRE, Molecular Probes, Eugene, OR, USA). After treatment with PFT for 3 days, cancer cells (5x10⁵ cells/ml) were incubated with 50 nM TMRE for 30 min at 37˚C, washed with PBS, and analyzed with FACSCalibur. The side scatters were used to gate and exclude cellular debris using a FACSCalibur. The cells were excited at 488 nm and the emission was collected on the FL2 channel. Five thousand cells were analyzed. The data were acquired and analyzed using CellQuest software (Becton-Dickinson). A decrease in red fluorescence indicates loss of membrane potential ∆Ψm.

**Effect of PFT with human peripheral blood mononuclear cells (PBMCs).** PBMCs from three normal healthy donors [approved by the Institutional Review Board (IRB), Charles Drew University, Los Angeles, CA, USA] were separated over Ficoll-hypaque density gradient centrifugation. Cells (1x10⁶/ml) were cultured with or without PFT (5.0 mg/ml) for 3 days. Cells were examined for the percentage of apoptosis.

**Statistical analysis.** Using the Student’s t-test, we tested the significance of difference in the percent changes of apoptotic
cancer cells and PBMCs post-culture with PFT as compared to control untreated cells alone. The level of significance was set at $p<0.05$.

**Results**

**Percent apoptotic cancer cells by flow cytometry.** AGS and 4T1 cells were cultured with PFT at concentrations 0-5 mg/ml for 3 days, and the percent apoptotic cancer cells was determined by flow cytometry using 7-AAD dye. Fig. 1 shows that PFT induced apoptosis in AGS cancer cells in a dose-dependent manner. An increase in the percentage of apoptotic AGS cells was detected at lower concentrations of PFT (0.3 and 0.6 mg/ml). The percent apoptotic cells became significant at a concentration of 1.2 mg/ml (37.0%, $p<0.05$), with a further increase at 2.5 mg/ml (53.1%, $p<0.001$) and maximized at 5 mg/ml (66.3%, $p<0.0001$). Notably, PFT does not induce apoptosis in 4T1 cancer cells ≤5 mg/ml.

**Morphological analysis of apoptotic cancer cells by Giemsa staining.** Cancer cells were cultured with PFT (5.0 mg/ml) and the percentage/number of apoptotic cells among the non-adherent and monolayer adherent cancer cells was examined.

**a) Non-adherent apoptotic AGS cancer cells.** AGS cancer cells were cultured with PFT for 0.5 and 24 h and the supernatant containing non-adherent cells was collected and the number of apoptotic cancer cells was examined by trypan blue staining and hemocytometer. Data depicted in Fig. 2 show a significant increased level of apoptotic non-adherent cells at 0.5 h. The number of apoptotic cancer cells was further increased at 24 h, showing a 2-fold increase in comparison to the 0.5 h.

**b) Monolayer adherent apoptotic AGS cells.**

**i) Morphological characteristics.** We were able to identify the apoptotic AGS cancer cells in Giemsa-stained monolayer AGS cells grown on cover glass post-culture with PFT (5.0 mg/ml) for 24 h. PFT induces the common morphological characteristics of apoptosis including cell swelling, membrane blebbing, and chromatin condensation. Fig. 3A shows non-apoptotic adherent AGS cells. Note the absence of chromatin condensation and membrane blebbing. Cancer cells undergoing apoptosis begin with chromatin condensation, in which the nucleus shrinks to about half the size of the cell (Fig. 3B). This is followed by membrane blebbing (Fig. 3C) and nuclear fragmentation (Fig. 3D). Finally, nuclear fragments become encased in membrane vesicles (Fig. 3E), and subsequently these vesicles become detached from the cell. Note that spherical and ovular vesicles are completely detached and autonomous from the apoptotic cell (Fig. 3F). On the other hand, we observed absence of chromatin condensation and
membrane blebbing in 4T1 cancer cells post-treatment with PFT (Fig. 4).

ii) Percentage of monolayer apoptotic AGS cells. The percentage of adherent AGS cells having morphological characteristics of apoptosis was examined at 0.5 and 24 h post-culture of cancer cells with PFT. Fig. 5 shows that a significant percentage of apoptosis was detected as early as 0.5 h post-culture of PFT with AGS cells (p<0.01). The apoptotic effect of PFT was further increased and became highly significant at 24 h (p<0.001). The percent of apoptotic cancer cells post-treatment with PFT for 24 h was 2.4-fold of those treated at 0.5 h.

Mechanisms underlying the PFT effect

a) Mitochondrial membrane potential (MMP). The effect of PFT (2.5 mg/ml) on the MMP of AGS and 4T1 cells was examined by flow cytometry. Data in Fig. 6 show that treatment of AGS cancer cells with PFT for 3 days resulted in a significant decrease in mitochondrial potential as compared with control untreated cells (p=0.007). Fig. 6A shows a representative flow histogram and Fig. 6B shows a bar graph representing the mean ± SD of 3 different experiments. In contrast, data in Fig. 7 show that PFT had no effect on MMP of 4T1 cells.

b) Bcl2 expression. Bcl2 expression of AGS cells post-culture with PFT (2.5 mg/ml) for 3 days was examined. Flow cytometry studies show that treatment with PFT resulted in a
significant downregulation of the expression of Bcl2 of AGS cells (p=0.004) as compared to control untreated cancer cells. Fig. 8A shows a representative flow histogram and Fig. 8B shows a bar graph representing the mean ± SD of 3 different experiments.

Effect of PFT on human PBMC. The effect of PFT treatment on PBMCs with respect to changes on the percentage of apoptotic cells was examined. PBMCs were co-cultured with PFT (5.0 mg/ml) for 3 days and the percentage of apoptotic cells was examined by flow cytometry. Fig. 9 shows that treatment of PBMCs with PFT caused no significant change in the percentages of apoptotic PBMCs as compared with control untreated cells.

Discussion

Lactic acid bacteria (LAB) has been found in milk products for thousands of years and is associated with inhibiting the growth of spoilage agents. Recently, scientists have revealed the additional potential of these bacteria as anticancer agents. Several reports suggest that fermented milk products and/or the fermentative bacteria themselves may have chemoprotective effects against cancer without the toxic side effects of conventional therapeutic drugs. In the present study we used a novel kefir product, Probiotics Fermentation Technology PFT, in which *Lactobacillus kefiri* P-IF is the main constituent. There are several characteristics that may allow P-IF to act as a potent anticancer agent. These include the ability of...
P-IF to grow three-dimensionally due to carbohydrate chains found on its surface, while other *L. kefiri* strains grow in a lengthwise-dimensional pattern. Moreover, P-IF can utilize galactose as a carbon source and produce carbonic acid (19). Our recent study demonstrated that PFT induces apoptosis on human multidrug-resistant (MDR) myeloid leukemia (HL60/AR) cells (16). These results prompted us to examine the apoptotic effect of PFT on other types of cancer. Data revealed that PFT exerts a selective apoptotic effect on human AGS cancer cells and did not exhibit apoptotic effects on 4T1 cells.

LAB can induce cancer cell death through a mechanism that involves apoptosis. There are two major pathways of apoptosis that have been extensively described in the literature; these are the extrinsic and intrinsic pathways. The former is mediated by activation of death receptors and caspase 8, while the latter involves mitochondria and caspase 9 (20). The Bcl-2 family of proteins have been shown to play an important role in the mitochondrial pathway and in the maintenance of the MMP. In this study, treatment with PFT caused significant downregulation in the level of Bcl-2 of AGS cancer cells, this was associated with a decrease in the mitochondrial polarization of AGS cells. This may result in the release of pro-apoptotic molecules that cause the activation of caspases and eventually lead to apoptosis. Similar effects were noted on HL60/AR cells post-treatment with PFT (16).

Chemotherapy such as 5-fluorouracil (5-FU), cisplatin, and doxorubicin, which are often used for the treatment of gastric cancer, aim to initiate apoptosis in gastric cancer cells (6,7). The apoptotic effect of PFT was shown to be both dose- and time-dependent on AGS cells. Flow cytometry studies showed that PFT induces apoptosis which was detected at a concentration of 0.3 mg/ml and peaked at 66.3% at 5.0 mg/ml. Morphological examination of Giemsa stained cytospin preparation confirmed the apoptotic effect of PFT against AGS cells, where the characteristics of apoptotic cells such as cell swelling, membrane blebbing and chromatin condensation were clearly identified. Induction of apoptosis by PFT was detected at 0.5 h post-treatment and the percentage of apoptotic cells showed a 2-fold increase at 24 h. Earlier reports showed that other LAB agents such as L.lac CF induced DNA fragmentation and chromatin condensation against human stomach adenocarcinoma, SNU-1 (21).

The mitochondrial pathway appears to be the main route for the induction of apoptosis against gastric cancer cells by different types of probiotics such as *P. freudenreichii* (22), *L. paracasei* IMPC2.1, and *L. rhamnosus* GG (L.GG) (23). Similarly, induction of apoptosis via the mitochondrial pathway was also noted in colon cancer cells treated with probiotics including propionibacteria (24,25), *L. rhamnosus*, *Bifidobacterium lactis* (26), and *L. delbrueckii* (27). Furthermore, LAB induces the mitochondrial pathway
of apoptosis in myeloid leukemia as well. For example, PFT induces apoptosis on human MDR myeloid leukemia (HL60/AR) (16), as well as L. reuteri (14) and L. casei rhamnosus (13) on human monocytic leukemia-derived cells. Two alternative mechanisms can be proposed to account for the induction of apoptosis by LAB. First of all it is possible that LAB induces apoptosis by binding to Toll-like receptors (TLR) on cancer cells and triggers apoptosis. This hypothesis is based on the reports that show some but not all TLRs trigger apoptosis in cancer cells (28-30). Second, it is possible that phagocytosed LAB may induce apoptosis. This hypothesis is based on studies by us and others that show cancer cells phagocytose microorganism (31-33) and subsequently cancer cells undergo apoptosis (17,34,35). The above hypotheses are not mutually exclusive. The reason for the inability of PFT to induce apoptosis in 4T1 may be due to the lack of appropriate TLRs that bind to PFT or failure of 4T1 cells to induce apoptosis post-phagocytosis of PFT.

Several studies have shown PFT to be a non-toxic agent. In this study, we noted no significant change in the percentage of apoptotic human PBMCs that were treated with PFT (5.0 mg/ml) for 3 days. Additionally, in vivo studies have shown that PFT-treated mice had no change in body weight, and showed no macroscopic or histopathological abnormalities in different organs (36). These results suggest that PFT is a selective apoptotic inducer for gastric cancer, and is also a safe, non-toxic, potential therapy for the treatment of gastric cancer.

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