The effects of human milk proteins on the proliferation of normal, cancer and cancer stem like cells

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Abstract: Human breast milk (HBM) provides neonates with indispensable nutrition. The present study evaluated the anti-cancer activity of diluted and pasteurized early HBM (<6 weeks' lactation) on human breast cancer cell lines. The cell lines MCF7 and MDA-MB231 were exposed to 1% HBM from the 1st, 3rd, and 6th weeks of lactation and exhibited reduced proliferation rates. As controls, breast cell lines (293T and MCF-10A), breast cancer cell lines (MCF-7 and MDA-MB-231), and CD133<sup>+</sup>CXCR4<sup>+</sup>ALDH1<sup>+</sup> patient-derived human cancer stem-like cells (KU-CSLCs) were treated with prominent milk proteins β-casein, κ-casein, and lactoferrin at varying doses (10, 50, and 100 μg) for 24 or 48 hrs. The impact of these proteins on cell proliferation was investigated. Breast cancer cell lines treated with κ-casein and lactoferrin exhibited significantly reduced viability, in both a dose- and time-dependent manner. Interestingly, κ-casein selectively impacted only cancer (but not normal breast) cell lines, particularly the more malignant cell line. However, β-casein-exposed human breast cancer cell lines exhibited a significantly higher proliferation rate. Thus, κ-casein and lactoferrin appear to exert selective anti-cancer activities. Further studies are warranted to determine the mechanisms underlying κ-casein- and lactoferrin-mediated cancer cell-selective cytotoxic effects.

Key words: human breast milk, beta-casein, kappa-casein, lactoferrin, Cell-proliferation, breast cancer cell

1. Introduction

Human breast milk (HBM) possesses a wide range of beneficial micro- and macro-molecules in biological functions. The HBM exhibits antimicrobial effects, boost the immune reaction, enhances the intestinal function, and supports for the developmental process. Human breast milk is highly recommended for at least six months and can be extended for two years in combination with other balancing foods. The potential of breast milk to ameliorate the threat of the allergic disorders, infection- and inflammation-related diseases...
in infants is reported.\textsuperscript{5,6} Moreover, the antioxidant property of breast milk is vital for the protection of newly born from the endogenous as well as, the environmental oxidative stress and its consequences, such as tissue pathological changes and toxicity.\textsuperscript{7,8} Recently, several studies reported that selected specific components of HBM showed anti-cancer effects.\textsuperscript{1,2} Moreover, HBM feeding was protective against the development of the maternal breast cancer.\textsuperscript{9} The risk of breast cancer was significantly reduced by 4.3 \% for each year in HBM feeding groups compared to other groups.\textsuperscript{2} HBM was reported continuously to have anti-tumor effects.\textsuperscript{1,2} Interestingly, camel's milk was recently reported to trigger apoptosis of human breast cancer cells.\textsuperscript{4,5} Up to date, pasteurized HBM was not tested for anti-cancer effects and the exact reason of the cancer preventive effects of HBM feeding was not known yet. Therefore, we hypothesized that pasteurized HBM and some protein components of HBM abundant in the early lactation stages would have anti-proliferative effects of various cancer cell lines. In our previous experiments of proteomic analysis, interestingly we found that β-casein, κ-casein, and lactoferrin showed significantly different abundance in the human milk of different lactation stages.\textsuperscript{7} Therefore, we want to select these three major components of HBM and test its anti-proliferative effects against various cell lines including cancer and cancer-like stem cell.

2. Methods and Materials

2.1. Human milk samples

HBM samples were obtained at 1\textsuperscript{st}, 3\textsuperscript{rd}, and 6\textsuperscript{th} week after delivery from three healthy mothers. Inclusion criteria were the milks donated from non-smoking healthy mothers, from mothers who delivered their baby vaginally with normal serologic tests for hepatitis B virus, syphilis and human immunodeficiency virus, and from mothers who followed the nutritional instruction for breast feeding mothers.\textsuperscript{6} After sample collections, pasteurization was employed at 65.5 °C for 30 min (Holder’s pasteurization) and all of these milks were found to be sterile by microbiologic tests. The milk samples were stored in the plastic containers at -80 °C until use.

2.2. Cell culture and proliferation for evaluation of the effects of diluted human milk

Human breast cancer cell lines (MCF7 and MDA-MB231) are purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were maintained using high glucose-Dulbecco's modified Eagle medium (DMEM) or Roswell Park Memorial Institute medium 1640 (Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 10 \% fetal bovine serum, and 1 \% penicillin and streptomycin. Cells were maintained at 37 °C in a humidified incubator supplied with 5 \% CO\textsubscript{2}. Cell viability was measured as follows: 1) cells seeding (2 × 10\textsuperscript{4} cells) in each well of multi-well plates. 2) cells trypsinizing and counting by a hemocytometer after the certain incubation time.

2.3. The effect of breast milk on the cell viabilities of breast cancers cells lines

We attempt to study the impact of breast milk samples at different lactation periods on the proliferation of human breast cancer cells. Due to the limitation of its absolute volume and comparison under the same concentration, we diluted breast milk samples with the culture media to give the final concentration of 1 \% (v/v). For the evaluation of cell proliferation, seeding of 2 × 10\textsuperscript{6} cells/well in 9-well dishes was performed. After treatment of diluted breast milk samples for 24 and 48 hours to human breast cancer cells, cells were trypsinized and then counted using a hemocytometer.

2.4. Cell culture and proliferation for evaluation of the effects of the purified proteins

Normal human breast cell lines (293T and MCF-10A) and human breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). In addition, Patient-derived CD133\textsuperscript{high}/CXCR4\textsuperscript{high}/ALDH1\textsuperscript{high} human cancer stem like cells (KU-CSLCs), were obtained from breast tumor tissue of chemotherapy-exposed patients at the Breast Cancer Center,
Konkuk University Hospital, Seoul. The KU-CSLCs were malignant and metastatic cancer cells. Three sets of the cell lines for each group were maintained using high glucose-Dulbecco's modified Eagle medium (DMEM) or Roswell Park Memorial Institute medium 1640 (RPMI 1640) (Sigma-Aldrich, Saint Louis, MO, USA), which was supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. The culture of the spontaneously immortalized breast epithelial cell line, MCF-10A was employed using DMEM/F12 medium (Gibco-BRL) supplemented with 5% horse serum (Gibco-BRL), 1% P/S, 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 10 μg/mL insulin (Invitrogen, Carlsbad, CA, USA), 0.5 μg/mL hydrocortisone (Sigma-Aldrich), 0.1 μg/mL cholera enterotoxin (Sigma-Aldrich), 2 mM l-glutamine (Sigma-Aldrich), and 0.5 μg/mL amphotericin B (Sigma-Aldrich). Cells maintenance was carried out at 37 °C in a humidified incubator supplied with 5% CO₂.

2.5. The effect of the purified proteins on the cell viabilities of breast cancers cell lines

In order to study the effect of several purified proteins from different lactation periods, we had to choose the representative one. Based on our previous experiments of proteomic analysis, β-casein, κ-casein, and lactoferrin showed significantly different abundance in the human milk of different lactation stages. Therefore, we want to select these three major components of HBM and test its anti-proliferative effects against various cell lines including cancer and patient-derived KU-CSLCs stem cells. These cell lines were treated with 10, 50, and 100 μg of β-casein, κ-casein, and lactoferrin at 24 and 48 hrs of incubation periods. After seeding of 2 × 10⁴ cells into each well, the cell viabilities were calculated and compared with percentage of control cells.

2.6. Statistical analysis

The comparisons of cell viabilities between different cell lines, different lactation period groups and different incubation period groups were performed with Kruskal-Wallis test and Mann-Whitney U test using GraphPad Prism 6 software for Windows (GraphPad Software Inc., La Jolla, CA, USA). For the post-hoc analysis of Kruskal-Wallis test, Dunn's multiple comparison test was performed.

Our study was approved by the Institutional Review Board of Konkuk University: KU-7001355-201511-HR-093 and KU-7001355-201712-E-060.

3. Results

3.1. The effects of 1% diluted human milk

We monitored the cell proliferation by cell counting. Due to the limitation of its absolute volume and the comparison under the same concentration, we diluted breast milk samples with the culture media to give the final concentration of 1% (v/v). Human breast

Fig. 1. The cell viabilities of control group and breast cancer cell lines after 24 and 48 hours of incubation periods.

*, Regardless of the incubation periods (24 and 48 hours), MCF7 cell lines showed lowest cell viabilities than the control group in all of 1st, 3rd, and 6th week milks treatment groups in the Kruskal-Wallis test followed by Dunn’s multiple comparison analysis (P < 0.05). **, The cell viabilities of MDA-MB231 (24 hours of incubation period) and MCF7 (48 hours of incubation period) were lower when treated with 6th week milk than 3rd milk.
cancer cell lines exposed to 1% HBM showed overall decreased cell viabilities compared with the control group (Fig. 1). Regardless of the incubation period (24 and 48 hours), the cell viabilities of control groups were significantly different when treated with all of 1st, 3rd, and 6th week milks ($p = 0.024$) from 31.0 to 72.1 percentage. Moreover, MCF7 breast cancer cell lines showed lower cell viabilities than the control group in all of 1st, 3rd, and 6th week milks treatment groups in the post-hoc test ($p < 0.050$, Fig. 1). However, other cell lines failed to show significantly different cell viabilities compared with control group. Interestingly, the cell viabilities of MDA-MB231 (24 hours of incubation period) and MCF7 (48 hours of incubation period) were lower when treated with 6th week milk than 3rd week milk.

* Fig. 2. The cell viabilities of control group and breast cancer cell lines after 24 and 48 hours of incubation periods.
* Regardless of the incubation periods (24 and 48 hours), MCF7 cell lines showed lowest cell viabilities than the control group in the all of 1st, 3rd, and 6th week milks treatment groups in the Kruskal-Wallis test followed by Dunn’s multiple comparison analysis ($p < 0.05$).
* The cell viabilities of MDA-MB231 (24 hours of incubation period) and MCF7 (48 hours of incubation period) were lower when treated with 6th week milk than 3rd milk.

Vol. 31, No. 6, 2018
3.2. The effects of β-casein, κ-casein, and lactoferrin on various cell proliferations

When we treated the purified components of HBM, such as β-casein, κ-casein, and lactoferrin, there were different changes on its cell viability comparing to control and other groups including cancer cell groups and KU-CSLCs. Fig. 2 demonstrates the difference of effects. The changes were more significant when incubated for 48 hrs than 24 hrs, and with higher doses of the proteins. The comparisons between the effects of study proteins on different cell types are shown in Fig. 3. The changes were more significant when incubated longer, and with higher doses.

The β-casein was found to enhance cellular proliferations of the most of cell lines. Interestingly, the cellular viability was further higher in the cancer cell groups (MCF-7, MDA-MB-231, and KU-CSLCs) than control cell groups (293T and MCF-10A). On the contrary, κ-casein, and lactoferrin were found to decrease cancer cell viabilities mostly. The effect was more remarkable in longer incubated group (48 hrs). The anti-proliferative effect of these purified proteins to normal cells was not significant (293T and MCF-10A). However, when the κ-casein, and lactoferrin was applied, the cell viabilities of cancer

Fig. 3. The cell viabilities of control group and breast cancer cell lines after treatment with β-casein, κ-casein, and lactoferrin according to the different cell types and the incubation periods (24 and 48 hours). For the groups showed statistically significant differences in the Kruskal-Wallis test (P < 0.05), the post-hoc test with Dunn’s multiple comparison analysis was preformed (*P < 0.050, and **P < 0.010). 293T and MCF-10A, Normal human breast cell lines; MCF-7 and MDA-MB-231, human breast cancer cell lines; KU-CSLCs, patient-derived CD133\textsuperscript{high}/CXCR4\textsuperscript{high}/ALDH1\textsuperscript{high} human cancer stem like cells.
The effects of human milk proteins on the proliferation of normal, cancer and cancer stem like cells

Vol. 31, No. 6, 2018

Cell groups significantly decreased (MCF-7, MDA-MB-231, and KU-CSLCs) at a concentration-dependent manner. In the Fig. 3, the effect of cell proliferation were compared and illustrated among the different cell types. The changes were more significant when incubated for 48 hrs, and with higher doses of these proteins. The β-casein was found to enhance the cellular proliferations of most of the cell lines. Even, the cellular viability was further higher in the cancer cell groups (MCF-7, MDA-MB-231, and KU-CSLCs) than control cell groups (293T and MCF-10A).

On the contrary, κ-casein, and lactoferrin were found to decrease cell viabilities of the most of cell lines. The effect was more remarkable in longer incubated group (48 hrs). However the anti-proliferative effect of these proteins to cancer cells was not significant in control cells (293T and MCF-10A). The κ-casein, and lactoferrin significantly decreased the cell viabilities of cancer cell groups (MCF-7, MDA-MB-231, and KU-CSLCs). Interestingly, 10 μg and 100 μg of κ-casein when exposed for 24 hrs and 48 hrs, respectively was found to suppress cancer cell proliferations selectively, especially for KU-CSLCs that is the more malignant cells compared with control groups in post-hoc tests (p < 0.010).

4. Discussion

In the present study, diluted HBM treatment was found to decrease cell viability of breast cancer cells compared with control group. The results correspond with recent previous similar studies worked with camel’s milk. The camel’s milk was reported to suppress human hepatoma (hepG2) and breast cancer (MCF7 and BT-474) cell lines. They issued that the effect of the milk is triggering apoptosis pathway of cancer cells.

As most diluted HBM of early lactation stages showed anti-cancer effects, we selected β-casein, κ-casein, and lactoferrin as the candidate proteins by literature reviews. Several authors reported that β-casein, κ-casein, and lactoferrin were relatively abundant in the HBM of early lactation stages in a previous proteomic study. In the present study, κ-casein and lactoferrin were found to decrease cell viability of breast cancer cells compared with control groups. There were several reports that the exogenous treatment with lactoferrin and its derivatives could inhibit the growth of tumors and could reduce the susceptibility to various cancers. However, consistent outcomes of the anticancer effects were not reported and mainly the colorectal, lung, prostate, and bladder cancer models were studied. Only small numbers of studies were performed on the effects of the lactoferrin to the breast cancer cells and they suggested that lactoferrin increased apoptosis and decreased migration of cancer cells. Lactoferrin is also known to have a variety of biological functions, including DNA synthesis, immune responses, iron transport, antimicrobial action, RNase activity, transcriptional activating functions, and receptor-mediated lipid uptake, any of which could play a role in the development and regulation of tumors such as breast cancer and cancers of the female reproductive tract.

The numbers of reports also supported that whole casein and α-casein showed anti-cancer effects in small intestinal and colorectal cancer cells. Even though we did not study on the effects of α-casein to the aforementioned cell lines, Kampa et al. reported that the derivatives of human α-casein could inhibit human breast cancer cell lines (T47D) in a dose dependent manner. Later, the role of signal transducer and activator of transcription 1 (STAT1) signaling activation was suggested for the suppression of breast cancer growth and metastasis by α-casein. Interestingly, the whole casein was reported to increase prostate cancer cell growth and α-casein was found to promote the proliferation of cancer cells, but breast cancer cells were not affected by the treatment of both of whole caseins and α-casein. Because we found the selective anti-cancer effects of κ-casein with only minimal effects on the control cells, if they evaluated the effects of other casein subtypes (α-, β-, and κ-casein), the proliferation of breast cancer cells might be affected. In addition, more malignant breast cancer cells (KU-CSLCs)
were found to be affected more largely compared with other cancer cell types. There was a report that the lactaptin, proteolytic fragment of κ-casein, suppressed the growth of breast cancer cells (MDA-MB231).23

On the contrary, β-casein was found to enhance cellular proliferations of breast cancer cells, as well as control cells. It seemed to have pro-cancerous effects. According to our results, β-casein might have pro-cancerous effects and κ-casein might have anti-cancer effects. However, there were not enough reports supporting the issue. Oliverira et al. reported that beta-casein-derived peptides stimulated colon cancer cell invasion and motility. Moreover, the level of β-casein-like peptide was higher in the breast nipple aspirates of breast cancer patients. These evidences might support the pro-cancerous effect of β-casein.

To our knowledge, this is the first report that diluted HBM has cytotoxic effects to breast cancer cells and that κ-casein would have cytotoxic effects selectively to breast cancer cells, especially to more malignant cells. Moreover, slightly weaker anti-cancer effect of lactoferrin was found as well. However, the mechanisms how the κ-casein inhibited the proliferation of cancer cells with minimal effects to normal cells was not clear as one of the major limitations of the present study. Further studies are warranted to find out the mechanisms how κ-casein showed cytotoxic effects selectively to breast cancer cells, especially to more malignant cells.

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References


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Vol. 31, No. 6, 2018