Cytotoxicity and Apoptosis Induction of Bovine Alpha-lactalbumin-oleic Acid Complex in Human Breast Cancer Cells

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Received July 2, 2014; Accepted October 3, 2014

Breast cancer is the mostly commonly diagnosed cancer in women worldwide. Using food-based approach to breast cancer chemoprevention has attracted a great deal of attention in recent years. In the present study, α-lactalbumin was purified from fresh bovine milk and the complex with oleic acid (BLA-OA Complex) was prepared by chromatographic method. The cytotoxic and apoptotic activities of BLA-OA complex on human ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells was investigated by morphological observation, MTS assay, LDH release, DAPI staining, DNA ladder assay and cell cycle analysis. The results showed that BLA-OA complex inhibits cells growth and induces apoptosis in both human ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cell lines. The findings of this study are positive for cow milk industry and human breast cancer chemoprevention.

Keywords: bovine alpha-lactalbumin-oleic acid, cytotoxicity, apoptosis, human breast cancer cells

Introduction

Breast cancer is a leading cause of morbidity and mortality in women, in developed and increasingly also developing countries (Siegel et al., 2013; Jemal et al., 2011). Although many active cytotoxic agents are used in the treatment of breast cancer, their efficiency is limited because of drug resistance (Dean et al., 2005; Gonzalez-Angulo et al., 2007). Therefore, the need for development of novel therapeutic or chemopreventive agents active against breast cancer remains an important goal. In recent years, using “food-based” approach to cancer chemoprevention has attracted a great deal of attention (Ramos 2008; Stan et al., 2008; Zhong et al., 2013).

Breast cancer is a clinically heterogeneous disease. Approximately 60 – 70% of breast cancers express estrogen receptors (ER) and/or progesterone receptors (PR) and approximately 20 – 30% of breast cancers have amplified HER-2 and express high levels of the HER-2 protein. The remaining 15 – 20% of breast cancers is in a so-called receptor-negative or triple-negative category, as defined by absent expression of these three proteins (Shao and Brown, 2004; Cleator et al., 2007). The triple-negative breast cancer patients are typically treated with a combination of surgery, radiation therapy and chemotherapy. Current treatments for ER/PR-positive and HER2-positive breast cancers are not working (Cleator et al., 2007). Therefore, scientists are still researching targeted treatments for triple-negative breast cancer.

In the present study, MCF-7 and MDA-MB-231 cell lines were employed as the cell model. MCF-7 cells are highly hormone-dependent in growth and known to form well-differentiated tumors...
in a xenograft animal experimental model. MDA-MB-231 cells lack the expression of either estrogen receptor (ER) or progesterone receptor, which implies totally hormone-independent and poorly-differentiated tumors in vivo (Bando et al., 2003).

α-lactalbumin, is a major whey protein (123 amino acids, 14 KDa) present in the milk of most mammals and accounts for 28% of the total protein in human milk. In a 1995 study, it was noted that multimeric α-lactalbumin, a compound isolated from a fraction of human milk called casein, induced what appeared to be apoptosis in human lung carcinoma cells, pneumocococcus bacteria, and other pathogens, while leaving healthy, differentiated cells unaffected (Håkansson, et al., 1995). The active component responsible for the tumoricidal activity was finally elucidated in 2000 and found to be a complex of α-lactalbumin and oleic acid (Svensson, et al., 2000), termed HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells).

Endogenous human α-lactalbumin is complexed with a calcium ion and serves as a cofactor in lactose synthesis, but has no tumoricidal properties. It must be partially unfolded to allow for release of the calcium ion and replacement with an oleic acid molecule. The partially folded conformation is essential to the cytotoxicity of HAMLET, as mutagenesis studies have shown that completely unfolded α-lactalbumin does not retain the functional properties of HAMLET (Gustafsson et al., 2005; Pettersson-Kastberg, et al., 2009). The oleic acid is necessary for stabilizing this molecule in this partially unfolded state.

The use of the α-lactalbumin-oleic acid complex for cancer therapy appears to be quite promising, considering the positive results obtained in the treatment of glioblastomas (Fischer et al., 2004), skin papillomas (Gustafsson et al., 2004), and bladder cancer (Mossberg et al., 2007; Mossberg et al., 2010). However, most previous studies have been performed using human milk protein. Some reports have shown that the bovine counterpart of HAMLET, bovine alpha-Lactalbumin-oleic acid complex (BLA-OA complex) shows similar tumoricidal activity to the HAMLET (Rammer et al., 2010; Zhang et al., 2010). The possibility to prepare the cytotoxic protein-lipid complexes using bovine milk is of great importance for the putative future use of such compounds in clinics, because it will circumvent the problem of limited availability of human milk for large-scale production.

The aim of present study was to prepare the BLA-OA complex and investigate whether or not it could in vitro induce cytotoxic activity and apoptosis in ER-positive (MCF-7) and ER-negative (MDA-MB-231) human breast carcinoma cells, regardless of their ER status.

**Materials and Methods**

**Preparation of BLA-OA complex** The BLA-OA complex was prepared by a modification of the method described by Brinkmann et al. (2011). Firstly, α-lactalbumin (α-LA) was purified from fresh bovine milk. In brief, α-LA was purified by subjecting the whey fraction of bovine milk to two consecutive DEAE-Sepharose fast flow columns in the presence or absence of Ca\(^{2+}\) on the first and second column, respectively. The α-LA containing fractions were identified by SDS/PAGE followed by western blotting using affinity purified anti-α-LA serum antibody. The identification of α-LA was verified by N-terminal amino acid sequencing of the α-LA-containing fractions, performed by automatic Edman degradation assays. The α-LA-containing fractions were pooled, lyophilized and stored at −80°C until use. Secondly, LA-OA complex was prepared from the purified α-LA. In brief, the purified bovine α-LA was dissolved in EDTA containing buffer (10 mm Tris, 0.08 mm EDTA, pH 8.5) to deplete the calcium, then was loaded onto a DEAE-Sepharose fast flow column conditioned with OA. Unbinding OA was removed from the column by washing with high salt buffer (1 m NaCl, 10 mm Tris, pH 8.5). After removal of excess α-LA with a low salt buffer (0.2 m NaCl, 10 mm Tris, pH 8.5), the α-LA:OA complex was eluted using the high salt buffer the same as above. The eluate was dialyzed against PBS (138 mm NaCl, 2.7 mm KCl, 8 mm NaHPO\(_4\), 1.5 mm KH\(_2\)PO\(_4\), pH 7.4). The samples were lyophilized in a freeze-drier and kept at −80°C until use.

**Cell culture** MCF-7 and MDA-MB-231 cells were cultured in phenol red-free high-calcium (1.05 mM CaCl\(_2\)) Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (DMEM/F12, Atlanta Biologicals) supplemented with 5% fetal bovine serum (FBS), and antibiotic-antimycotic (100 unit/mL penicillin G sodium, 100 mg/mL amphotericin B) (Gibco Cell Culture™).

**Cell morphology** MCF-7 and MDA-MB-231 cells (1×10\(^4\)) in DMEM/F12 medium containing 5% FBS were seeded in a 6-well plate. After a 24h culture, the medium was replaced with DMEM/F12 supplemented with dextran-coated charcoal (DCC) (dextran T-70, Pharmacia; activated charcoal, Sigma)-treated 5% FBS. The cells were treated with 200 µg/mL BLA-OA complex, and photomicrographs were obtained following treatment of 0 and 24 h using an Olympus M021 microscope with a digital camera.

**Cell proliferation assay (MTS assay)** A total volume of 100 µL medium containing 4,000 breast cancer cells was seeded in each well of a 96-well plate in DMEM/F12 medium and incubated in 37°C for 24 h. The following day, the medium was replaced by 100 µL DMEM/F12 supplemented with 0.2% BSA and incubated in 37°C for another 24h. Freeze-dried BLA-OA, dissolved oleic acid and α-lactalbumin were respectively reconstituted in DMEM/F12 medium at a concentration of 5 mg mL\(^{-1}\) and filtered with 0.1 µm sterile filters. Aliquots were made and stored at −80°C. For each assay, a new aliquot was used. The cells were treated with 25, 50, 100, 200, 400 µg/mL BLA-OA complex for 48h. The proliferation of breast cancers was measured by adding 20 µL fresh mixture of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) (20:1) solution (Promega) to each well. After incubation at 37°C for one to three hours, optical density values
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were measured by a kinetic microplate reader (Molecular Devices) at 490 nm wavelength and the cell growth was compared.

Lactate dehydrogenase (LDH) release assay (Cytotoxicity) At the end of treatment, cell culture medium was collected and briefly centrifuged. The supernatants were transferred into wells in 96-well plates. Equal amounts of lactate dehydrogenase assay substrate, enzyme and dye solution were mixed. A half volume of the above mixture was added to one volume of medium supernatant. After incubating at room temperature for 30 min, the reaction was terminated by the addition of 1/10 volume of 1N HCl to each well. Spectrophotometrical absorbance was measured at a wavelength of 490 nm and reference wavelength of 690 nm.

DAPI staining Cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The cells were washed twice with PBS and analysed under a fluorescence microscope.

DNA fragmentation analysis DNA gel electrophoresis was used to determine the presence of internucleosomal DNA cleavage. Briefly, the cells were treated with 100, 200 µg/mL of BLA-OA complex for 24 h, which was followed by adding 400 µL of a lysis buffer (0.5% Triton X-100 in 10 mM EDTA, and 10 mM Tris-HCl, pH 8.0) and incubating the cells for 30 min on ice. The cells were then separated by centrifugation for 30 min at 12,000 × g. An equal amount of phenol solution (phenol: chloroform: isoamylalcohol =25:24:1) was added to the supernatant and centrifuged for 1 min at 12,000 × g. The phenol extraction procedure was repeated three times. After adding 0.1 volume of 3 M sodium acetate, the DNA was precipitated with 2.5 volumes of cold ethanol overnight at −70°C. The mixture was centrifuged at 12,000 × g for 10 min, and then 700 µL of 70% ethanol was added to the pellet, which was shaken and centrifuged for 1 min at 12,000 × g. The pellet was treated with 30 µg/mL RNase A at 37°C for 4 h. The DNA purity and concentration were determined by electrophoresis on 1% agarose gel containing 0.5 mg/mL ethidium bromide (EtBr; Sigma). Images of the gel were obtained using a UV transilluminator and the Fujifilm LAS-3000 image system (Fujifilm Medical Systems, Inc.).

Flow cytometry analysis Cells were plated at a density of 5 × 10⁴ cells in a 35 mm dish. After 48 h of exposure to BLA-OA complex, cells were collected by trypsinisation, washed with cold PBS, then resuspended in 50 µg/mL PI containing 0.1% sodium citrate with 0.1% Triton X-100 for 20 min at 4°C. Cells were then analysed by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems). Fluorescence intensity was determined using a FACScan flow cytometer and analysed by CellQuest software (Becton Dickinson).

Results Cell growth inhibition and morphological changes by BLA-OA complex treatment.

The MCF-7 and MDA-MB-231 cell lines were treated with 25, 50, 100, 200, 400 µg/mL BLA-OA complex and its components for 48 h to determine whether BLA-OA complex, oleic acid or α-lactalbumin caused a decrease in the number of cells. Fig. 1 shows that the BLA-OA complex treatment inhibited cell growth in a dose-dependent manner, as determined by the MTS assay. At 48 h, the maximum effect was observed with 400 µg/mL BLA-OA complex, which inhibited the proliferation of MCF-7 and MDA-MB-231 cells by 93.7% and 95.6%, respectively, while the native BLA or OA alone did not appear to have a significant effect on the cell growth (Fig. 1). Cell morphology was evaluated under a microscope after treatment with 200 µg/mL BLA-OA complex for 24 h. Both cell lines in the treatment group were shrunken and round in shape with condensed nuclei. In addition, cell-to-cell contact was lost. The percentage of abnormal cells was much larger in the 200 µg/mL BLA-OA complex-treated group compared with the vehicle-treated group (Fig. 2). These results suggest that the anti-proliferative effect of the BLA-OA complex treatment was associated with nuclear and cytosol shrinkage, which were determined to be apoptotic events (Dini et al., 1996).

Induced LDH release by the BLA-OA complex on breast cancer cells The morphological observations were confirmed by following lactate dehydrogenase (LDH) release which was used as an indicator of cytotoxicity. LDH is present in cytoplasm dominantly as a high molecular weight protein (140 kDa) and is released only when cell damage occurs. MCF-7 and MDA-MB-231 cells were cultured with a range of concentration of BLA-OA complex (25 – 400.0 µg/mL) for 24 h, and release of LDH was measured. Results indicated that increase of LDH release of MCF-7 and MDA-MB-231 cells took on concentration characteristic (Fig. 3).

Induction of DNA fragmentation by BLA-OA complex on breast cancer cells In order to confirm whether the growth inhibitory effect of BLA-OA complex was due to apoptosis, breast cancer cells were treated with BLA-OA complex for 24 h, and the nuclear morphological changes were analyzed using DAPI staining (Choi et al., 2001). As shown in Fig. 4, nuclei with condensed chromatin and apoptotic bodies, typical characteristics of apoptosis, were observed in MCF-7 and MDA-MB-231 breast cancer cells incubated with BLA-OA complex, respectively. The number of apoptotic cells increased as the BLA-OA complex concentration increased while the purified bovine α-lactalbumin or oleic acid alone failed to induce the change (data not shown).

Because DNA fragmentation is another characteristic of apoptosis (Parrish et al., 2001), DNA fragmentation assay was carried out by Agarose gel electrophoresis assay. After a 24 h treatment, the vehicle control had no low molecular weight DNA, whereas MCF-7 and MDA-MB-231 cells treated with 50 and 100 µg/mL BLA-OA complex showed distinct ladder pattern which was the evidence of DNA fragmentation, a hallmark of apoptosis,
Apoptosis was triggered by BLA-OA complex on breast cancer cells. The apoptotic morphological change described above was also confirmed with a flow cytometric analysis, and the sub-G1 fraction was used as a measure of the apoptotic cells. After the breast cancer cells were treated with BLA-OA complex at designated concentrations (0, 25, 50, 100, 200 and 400 μg/mL) for 48h, they were harvested and stained with propidium iodide, and the cell populations of each phase were counted by flow cytometry. As shown in Fig. 6, the sub-G1 population, which indicated apoptotic cells, increased in dose dependent manner after exposure to BLA-OA complex for 48h. Although the G1 population decreased along with an increase of sub-G1, the other portion of non-apoptotic cells did not show a significant change. These results suggested that BLA-OA complex can induce apoptosis in breast cancer cells.

Discussion
Breast cancer is a heterogeneous group of diseases. Approximately 60 – 70% of breast cancers express estrogen receptors (ER) and/or progesterone receptors (PR) and...
approximately 20 – 30% of breast cancers have amplified HER-2 and express high levels of the HER-2 protein (Brenton et al., 2005; Rahman et al., 2009). Molecularly targeted therapies that inhibit the estrogen pathway or that target amplified HER-2 are effective in the treatment of breast cancer in patients whose tumors express these targets. However, in approximately 15 – 20% of patients with breast cancer, the tumors do not express ER or PR and do not have amplification of HER-2 (Brenton et al., 2005). These tumors are called triple-negative breast cancer and patients with these tumors have a poor prognosis (Brenton et al., 2005; Rahman et al., 2009). There is no clinically validated, molecularly targeted therapy for these patients and they can be treated only with chemotherapy.

Thus, identification of novel, molecularly targeted therapies for triple-negative breast cancer would be of great benefit. In addition, a “food-based” approach to cancer chemoprevention is emerging as an attractive additional strategy for this disease control.

In the last few years, a remarkable apoptotic-like activity toward cultured cancer cells of a complex between the calcium-free form of human α-LA and oleic acid has been described (Mok et al., 2007). The complex could either be isolated from human milk (Håkansson et al., 1995) or formed on a diethylaminoethyl trisacryl column equilibrated with OA (Svensson et al., 2000). The complex was named human alpha-lactalbumin made lethal to tumor cells (HAMLET). When tested against several different cell types, HAMLET was reported by Svanborg et al. (2003) to show strongest activity against tumor cells, whereas mature differentiated
cells were not affected. Accordingly, HAMLET appears to be a promising anti-cancer candidate. It is of great significance to be able to prepare the protein-lipid complex using bovine milk because it will allow large-scale production, circumventing the problem of limited availability of human milk. However, little information about the complex from bovine material has been presented.

In the present study, the BLA was purified from fresh bovine milk and the complex with oleic acid (BLA-OA complex) was prepared by a two-step chromatographic method. The cytotoxic and apoptotic activity of BLA-OA complex on human ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells was investigated. We demonstrated that BLA-OA complex is able to inhibit the growth of these cancer cell lines, while each of pure components (bovine α-lactalbumin and oleic acid) is inactive in the cell proliferation assay.

Induction of apoptosis is a useful approach in cancer therapies. Apoptosis, a major process of programmed cell death, plays an important role in maintaining cellular homeostasis (Elmore, 2007; Lopez-Beltran et al., 2007). Apoptosis or apoptosis-like cell death

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**Fig. 6.** Cell cycle analysis of MCF-7 (A) and MDA-MB-231 (B) breast cancer cells treated with BLA-OA complex by flow cytometry. The percentage of each phase analyzed by Cell Quest software.
has previously been proposed as the major cytotoxic effector mechanism of HAMLET (Hallgren et al., 2006, 2008). Previous studies have shown that the protein-lipid complex rapidly enters cancer cells and accumulates in the endolysosomal compartment, where it induces a lysosomal death pathway through leakage of lysosomal cathepsins into the cytosol (Rammer et al., 2010). In our study, BLA-OA induced cell death was observed after several minutes. This rapid response, indicated as LDH release, could suggest that permeabilization of the plasma membrane may play a role in the induction of cell death, at least at high concentrations.

Furthermore, in apoptotic cells, several cellular and molecular biological features, such as cell shrinkage, DNA fragmentations, and activation of the caspase cascade, are exhibited (Germain et al., 1999). In present study, we also observed cell shrinkage and DNA fragmentation, as determined by morphological observation, DAPI staining and DNA ladder assay, respectively. Regulation of the cancer cell cycle is one strategy in the development of anticancer drugs (Carnero, 2002). In addition, cell cycle analyses of the BLA-OA complex-treated breast cancer cells showed significant cell arrest at sub-G1-phase in a dose-dependent manner, which indicating apoptosis.

In conclusion, we demonstrate that BLA-OA complex inhibits both human ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells growth and induces apoptosis. Although further study on the effects of BLA-OA on breast cancer is required, the findings of this study are positive for cow milk industry and human breast cancer chemoprevention.

Acknowledgements This research project is supported by the grants from the Natural Science Foundation of Guangdong Province (No: S2012040006790), National Natural Science Foundation of China (No: 31201424) and the start-up fund from GD OU (E12319).

References


