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Anticancer Effect of Bovine Lactoferrin on Human Esophagus Cancer Cell Line

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Abstract

Background: Lactoferrin (Lf) is a glycoprotein, a member of the transferrin family. From ten known mechanisms of anti-cancer chemoperotecive compounds, Lf alone, has six of these functions and inhibits cancer. In this study, the effect of lactoferrin purified from bovine colostrum was studied as an anti-cancer agent on esophageal cancer cell line.

Materials and Methods: Bovine colostrum were collected immediately after giving birth. At first, the fat, casein, and some of the milk proteins were removed. Then, lactoferrin was purified using CM-Sephadex-C50 cation exchange chromatography by FPLC system. Purified lactoferrin with 80 kDa molecular weight and 2mg/ml concentration was obtained. Esophageal cancer cell line KYSE-30 and normal cell line HEK were cultured. After appropriate confluency, different concentrations of Lf were added to KYSE-30 and HEK for 20 h and its anti-cancer effect was evaluated by MTT and flow cytometric methods. The maximum concentration inhibitory effect was studied at different times using MTT method.

Results: MTT test determined that 500 µg/ml of lactoferrin reduced cell viability in esophageal cancer cell lines KYSE by 53% and 80% after 20 and 62 hours, respectively, but had no effect on normal cells. Also, flow cytometric analysis determined that lactoferrin was able to induce apoptosis in KYSE-30 cell line.

Conclusion: The isolated lactoferrin from bovine milk showed inhibitory effect on esophageal cancer cell line whereas; it did not have any significant effect on normal cells.

Keywords: Anticancer effect; Bovine lactoferrin; Esophageal cancer cell line; Flow cytometry

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Introduction

A good candidate for cancer preventive agent is proteins found in milk (1). Lactoferrin (Lf) known for its inhibitory action on cell proliferation, as well as for its anti-inflammatory and antioxidant abilities, has been described to have anti-carcinogenic properties in several *in vivo* and *in vitro* studies (2, 3). Lf is an 80-kDa iron-binding glycoproteins (4, 5) and a member of the transferrin family that secretions, such as colostrum, tears and exocrine granules of neutrophils, and the secondary strongly binds two ferric ions (6,7). Also, it is produced by mucosal epithelial cells in various mammalian species including humans, cows, goats, horses, dogs, rodents and fish (8). It has several biological functions including iron homeostasis, cellular growth and differentiation, host defense against microbial infection, anti-inflammatory activity and cancer protection (9, 10). Lactoferrin is particularly abundant in colostrum (approximately 10 mg/ml) and also in mammalian epithelial cell secretions such as tears, saliva and seminal fluid in various amounts) 0.01 to 2 mg/ml (11).

It has been demonstrated that more than 60% of administrated bovine Lf survived passage through the adult human stomach and entered the small intestine in an intact form (12). Bovine Lf protein and partial Lf are likely to exert various physiological effects in the digestive tract. Moreover, subcutaneously administration of Lf inhibited the growth of implanted solid tumors and exerted preventive effects on metastasis (13). Dietary supplementation with bovine Lf (bLf), derived from bovine milk (14), could inhibit the development of azoxymethane (AOM)-induced aberrant crypt foci (ACF) as precursor lesions of tumor development, as well as carcinomas in the rat colon, without any toxic effects on major organs (15, 16). Esophageal cancer is the third most common gastrointestinal cancer and the sixth leading cause of cancer-related death worldwide. The possibility of an association have been proposed between esophageal cancer and genetic factors since high incidence of the disease exist in some parts of the world (17). Chemotherapy drugs are widely used in cancer treatment, but have the disadvantage of nonspecific toxicity because these factors, all rapidly dividing cells, target without any discrimination between normal and malignant cells (18). Lf was shown to reduce the growth and metastasis of solid tumors (13) and bLF had chemopreventive effects in the esophagus and lung in addition to the colon (19). Therefore, bLf is considered to be a good protein for a chemopreventive agent in human cancer development. In current study, we treated human esophagus cancer cell line (KYSE-30) with different concentrations of bLf and investigated the anticancer properties and apoptosis induction of bLf by MTT test and flow cytometry.

Materials and Methods

Isolation of Lactoferrin from cow's milk

Bovine colostrum were collected within 72 hours after delivery of eight cows in sterile containers.Then centrifuged in 1000 xg for 20 min at 4 °C until the cream was isolated. In order to isolate the caseins, the pH of skim milk was reduced to 4.6 using HCl 2N for 30 min at 40 °C.

Casein precipitates was separated by centrifugation in 12000×g for 20 min at 4 °C. The acid whey was neutralized by NaOH and its pH reached to 6.8. For isolation of globulins from whey, 267 g/l of ammonium sulfate was slowly added to the acid whey then centrifuged in 1000 xg for 20 min at 4 °C. The precipitate of globulins were discarded, and 250 g/l of ammonium sulfate was added to the remaining supernatant. Lf was separated by centrifugation using previous conditions. Dialysis was done in order to separate extra salt from the protein using dialysis bags with Cut-off 10 kDa in 20 mM phosphate buffer with speed of 200 rpm/min for 24 h at 4 °C. The protein was powdered in freeze dryer (CHRIST) for 24 h.

Purification using FPLC cation exchange column of CM-Sephadex-C50

Lf is a cationic protein with an isoelectric pH greater than 9 that helps to isolate Lf from other proteins found in milk. High isoelectric pH of Lf causes a positive charge in neutral pH in protein that can be purified using cation exchange (20). So cation exchange column at neutral pH, is the conventional method for isolation of Lf from whey derived from milk. For purification of Lf, FPLC (LP models Biorad America) was used. For this purpose, sample of powdered protein was solved in 20 mM phosphate buffer and then was taken on CM-Sephadex-C50 column. Lf protein was eluted in gradiant of 0.4 to 0.5 M NaCl in speed of 0.75 ml per minute under pressure 5 MPa. The Lf purity was checked by SDS-PAGE electrophoresis (BioRad) and the concentration of the protein was determined using the Bradford method.

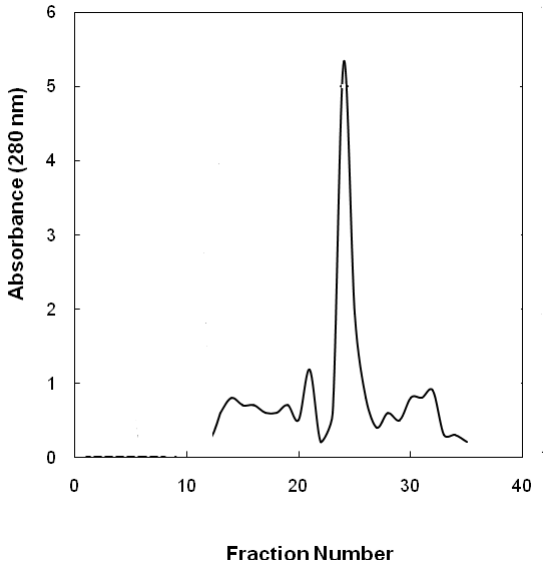
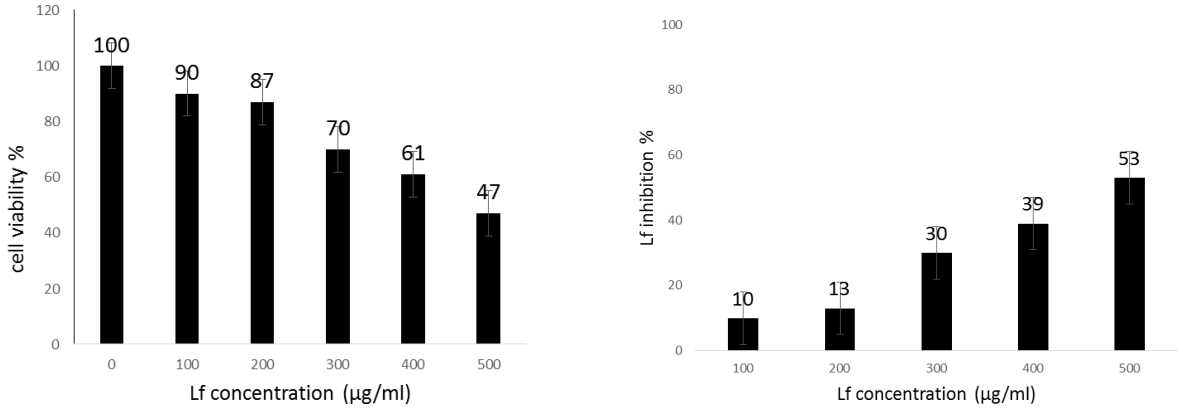


Figure 1. Curve of bovine colostrum Lf purification using cation exchange column.

Cell culture

KYSE-30 esophageal squamous cell carcinoma and HEK human epithelial cells like normal cells were purchased form Pasteur institute (Iran). KYSE-30 was cultured in 45% RPMI1640, 45% Ham's F12 plus 10% FBS and HEK normal cells were cultured in 90% DMEM plus 10% FBS. Incubation was done in 5% CO2 incubator (Memert, Germany) at 37 °C.



A **B**

Figure 2. Lf effect (concentrations of 0, 100, 200, 300, 400, 500 µg/ml) on KYSE-30 cell survival at intervals of 20 hours. (A) Percentage of cell viability (B) Percentage of lactoferrin inhibition. Values are shown as mean ± SD.

MTT Assay

In order to assess the toxicity of Lf, MTT assay was used. MTT Reagent (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) is a yellow tetrazolium salt that is absorbedby active and live cells mitochondria. Due to the activity of dehydrogenase enzymes, it produces purple formazan crystals that are dissolved in a suitable solvent. The amount of color produced was measured by a spectrophotometer at a wavelength 490 nm (18).

Up to15×10^3 cells in complete medium were cultured in each well of 96 well plates. Cells incubated at 37 ° C and 5% CO2 for 24 h. Different concentrations of Lf (0, 100, 200, 300, 400, and 500 µg/ml) were added to each well in triplicates. Incubation continued for 20 hours. Also, the experiment was conducted using 500 µg/ml concentration at different intervals (20, 42 and 62 hours). 50 ml of MTT dye solution (Sigma) was added to each well and every one hour the cells were observed by microscope until the formation of purple crystals (maximum 3-4 hours). After the formation of crystals, the supernatant was carefully removed and 150 ml of acidic isopropanol was added to each well. The cells were kept in a dark place for 30 min. Absorption of purple color formation was read at 490 nm using Eliza reader (ELX800).

Assessment of cell death by propidium iodide (PI) staining and flow cytometry

The cells were cultured in 6 wells plate in complete medium containing 10% FBS and incubated for 24 h at 37 °C and 5% CO2. Lactoferrin with 500 µg/ml concentrations were added to separate wells in triplicate and three wells without lactoferrin were considered as control. Incubation was continued for 20 hours. The cells were separated using 2 ml EDTA and trypsin and centrifuged at 2000 g for 5 min and they were washed again with 2 ml of PBS then centrifuged. The cells were fixed with 2 ml of cold ethanol at 4 °C overnight then wash twice with PBS and centrifuged at 2000 rpm for 5 min. 1 mL of PI dye solution (Sigma) was added and treated with 50 µl of 100 µg/ml RNase A (Fermentas) to ensure that only DNA is stained, then incubated in the dark at room temperature for 30 minutes. Finally, dead cells were analyzed using flow cytometry (Partec).

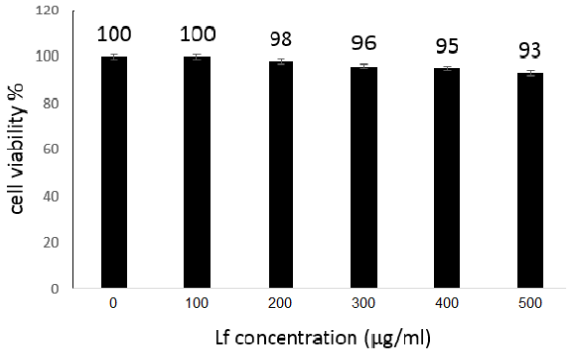


Figure 3. Effects of Lf (concentrations of 0, 100, 200, 300, 400, 500 µg/ml) on the survival of HEK cells in 20 hour intervals. Values are shown as mean ± SD.

Statistical analysis

The results of cytotoxicity were analyzed applying one way ANOVA and T-test in Graphpad Prism 5.0, and SPSS (SPSS, Chicago, IL, USA), respectively. A *P*-value ≤0.05 was considered significant and data were shown as mean ± standard deviation (SD).

Results

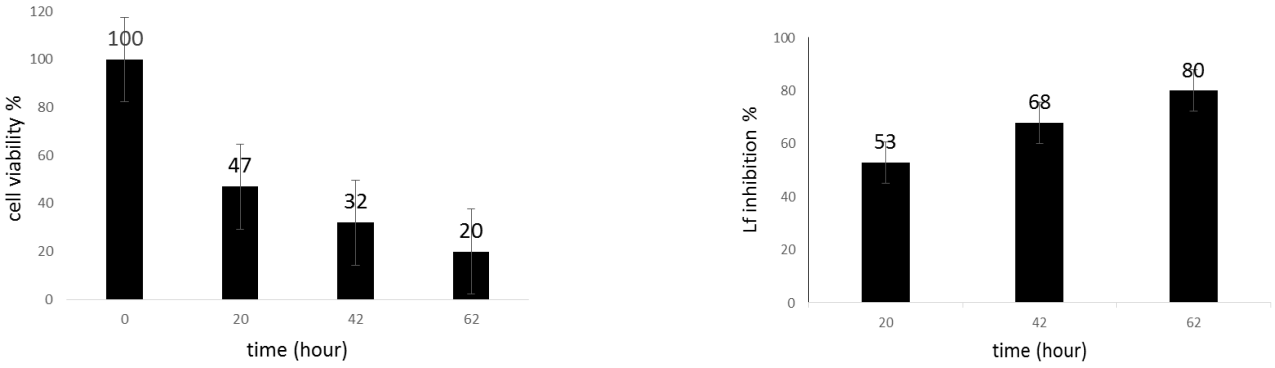
The curve of protein elution in cation exchange chromatography consists of long peak belonging to Lf eluted in 40-50% salt concentration (Figure1). The

purity of the protein was confirmed by 10% SDS-PAGE gel electrophoresis in our previous study (21). Lf concentration was 2 mg/ml which was determined by Bradford method.

Cell viability using MTT Assay

MTT assay was done after growth of the cells up to 70% confluency. Different concentrations of Lf including: 0, 100, 200, 300, 400 and 500 µg/ml were considered for treatment of the cells. During 20 hours of treatment with lactoferrin, the lowest cell viability was observed at 500 µg/ml concentrations (47%), equivalent to 53% of the inhibitory effect of lactoferrin (Figure 2).

The effect of Lactoferrin on normal HEK cells were also studied. The inhibitory effect was not observed at different concentrations and survival rate was up to 93 - 100% (Figure 3). The experiment was performed using 500 µg/ml concentration at different intervals (20, 42, and 62 hours). After these hours, the cell survival rates were 42%, 32 %, and 20%, respectively, (and 53%, 68% and 80% inhibition rate, respectively) (Figure 4).

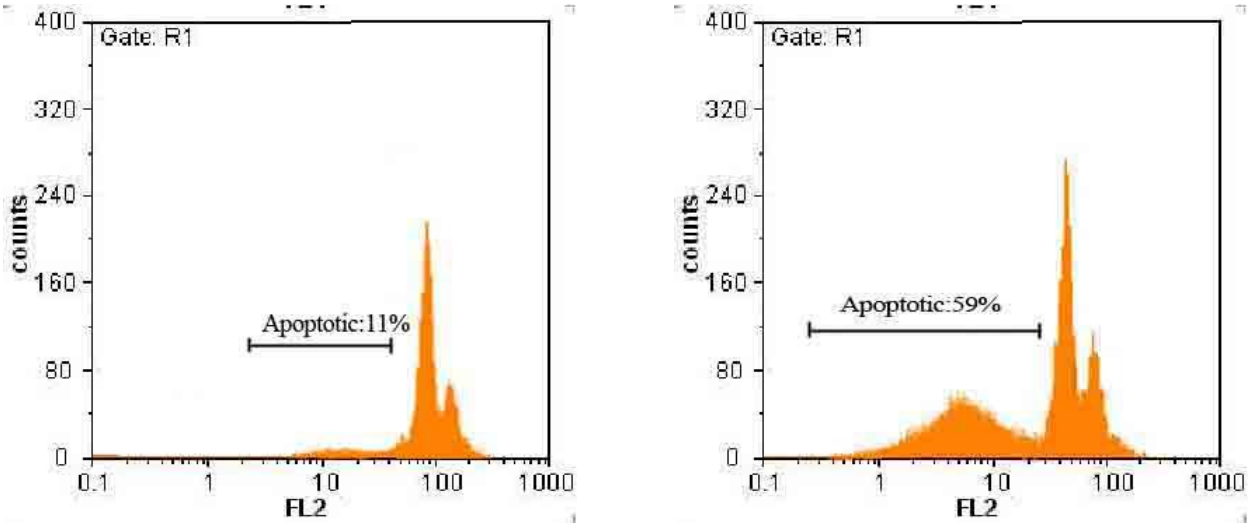


A **B**

Figure 4. Lactoferrin effect (500 µg/ml) on survival of KYSE-30 cell line in intervals 20, 42, 62 hours. (A) Percentage of cell viability (B) Percentage of lactoferrin inhibition. Values are shown as mean ± SD.

Flow cytometry analysis using propidium iodide staining

The results of the propidium iodide staining for detecting death of cells by flow cytometry is shown in Figure 5. The KYSE-30 cells treated with 500 µg/ml lactoferrin for 20 h and the cells without Lf considered as control.



A **B**

Figure 5. Diagram of percentage of KYSE-30 apoptosis. A) Without lactoferrin; B) The cells treated with lactoferrin at concentration of 500 µg/ml.

The apoptotic cells, among other typical features, are characterized by DNA fragmentation and consequently loss of nuclear DNA content. Using a fluorochrome, such as PI, that is capable of binding and labeling DNA makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis, and subsequent identification of hypodiploid cells. Analysis of DNA stained by PI and analysis using flow cytometry showed the presence of a broad peak of hypodiploid particles characteristic of apoptotic cells and the sharp diploid DNA peak of normal cells. Debris and residuals of necrotic cells can be easily gated-off. A precise calibration of the flow cytometer is necessary to properly gate out debris. The rate of apoptosis in treated cells with lactoferrin was 59% and in non-treated samples was 11%, resulting the effect of lactoferrin on apoptosis.

Discussion

A likely area of research, is clinical trials with cancer preventive proteins in milk such as lactoferrin (22). Today, infant formula containing bovine Lf are sold in Japan, Korea and Indonesia. There are other products containing bovine Lf including yogurt, full-fat milk, oral supplements, animal food, infant food and cosmetics. Lf production were about 79 tons by a single company in 2003, which indicates the importance of this protein (23). Lf purification process using diverse column chromatography including affinity chromatography and ion exchange is applicable that only in affinity can be purified in a single step and in other cases, purification need gel filtration column in next step. Although using affinity chromatography, Lf can be purified in a single step but the disadvantage of this method is inactivation of protein and needed to reactivate protein by activation methods. (24). The results of our purification method with respect to single-step was comparable with affinity chromatography. The efficiency of purification was 90%. In this study the amount of Lf concentration derived from one liter of bovine colostrum was about 2 mg/ml. However, others reported 0.1 to 0.4 mg/ml concentration (25).

The purified lactoferrin, showed that it was cytotoxic to human esophageal cancer cells, but had no adverse effect on viability of human normal epithelial cells.

To the best of our knowledge this research was the first to study different concentrations of lactoferrin on cancerous KYSE-30 cell lines. Duarte et al. investigated the effect of 12.5 μg/ml bLf on human breast cancer cell lines like T47D and HS578T, and showed 54% and 47% cell viability reduction after 48 hours, respectively. The antitumor effect of 50 μg/ml lactoferrin was studied in gastric cancer SGC-7901 cells for 24 and 48 h and inhibition of proliferation detected (26). Zemann et al. found that lactoferrin inhibited proliferation of human HeLa epithelial cancer cell line. Lactoferrin-induced growth inhibition at 50 and 500 μg/ml concentrations after three days and resulted in a very substantial reduction of growth by about 75% after six days of treatment.

In earlier stages of the research low concentrations of 0, 12.5, 25, 50 and 75 μg/ml lactoferrin treatment on KYSE-30 were used for 20 hours of incubation but significant changes were not observed. After that the higher concentrations of Lf like, 100, 200, 300, 400, and 500 μg/ml were examined. Cell viability levels decreased with increasing the concentration, and at 500 μg/ml Lf, the inhibition of growth was the greatest. Propidium iodide flow cytometric method (PI) has been widely used to assess apoptosis in various experimental models. PI is a red fluorescent dye which binds to the DNA of dead cells. In this case, normal cells are not stained. This method for different types of cells, including tumor adherent cell lines is applied with some modifications in the basic method. PI is a fluorogenic compound that binds stoichiometrically to nucleic acids (27) so that fluorescence emission is proportional to the DNA content of a cell. When apoptotic cells are stained with PI and analyzed with a flow cytometer, they display a broad hypodiploid (sub-G1) peak, which can be easily discriminated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channels. This method has some advantages. It allows a fast, reliable and reproducible estimate of apoptosis, and coincident analysis of cell cycle parameters of surviving cells (28). A fast and simple method for measuring apoptosis of mouse thymocytes stained with PI by flow cytometric was previously published (29). Xiang and Liu investigated the effect of deguelin on cell cycle of Raji cells by PI flow cytometry (30). Ormored et al. examined apoptosis in IL-3-dependent haemopoietic cells by PI flow cytometry (26). Apoptotic cells had a sub-G1 peak. According to previous studies and PI staining benefits, we determined apoptosis after treatment of Lf using this method.

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Author contributions

MF and RA contributed in the study conception, design, critical revision and finalizing the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest in this article.

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