Abnormal apoptosis of stomach cancer cell SGC-7901 and regulation of Akt signaling way induced by bovine lactoferrin

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ABSTRACT

Lactoferrin, a protein from bovine milk belonging to the transferring family proteins, contains 2 bound Fe^{3+} ions. Recent research has revealed that lactoferrin exhibits not only antimicrobial activity by its high affinity for Fe^{3+} but also remarkable anticancer capacity in cancer cell lines. Meanwhile, increasing evidence suggests that aberrant activation of Akt is involved in both normal cells and human cancers and that inhibition of Akt signaling pathway might be a promising strategy for cancer treatment. In the present study, we investigated the effect of the antitumor induced by exposing stomach cancer cell SGC-7901 to lactoferrin for 24 and 48 h. The cell viability was assessed by 3-(4,5)-dimethylthiazolyl-3,5-diphenyltetrazoliumromide (MTT) assay and apoptosis was quantified by propidium iodide uptake and Annexin V-fluorescein isothiocyanate fluorescent probe label through flow cytometry. Our investigation indicates that inhibitory ratio of 50 μM lactoferrin for proliferation of stomach cancer cell SGC-7901 is much higher than 12.5 and 25 μM, and for the extended treatment time, the concentration of 50 μM has more efficiency than 100 μM lactoferrin. To elucidate a mechanism involved in its antitumor effect, we studied the Akt cell signaling pathway of SGC-7901 while treated by 50 μM of lactoferrin after 0, 24, and 48 h, particularly Akt phosphorylation of 2 individual residues, Ser473 and Thr308, Akt/glycogen synthase kinase-3β, forkhead in human rhabdomyosarcoma, and nuclear factor-κB proteins, respectively, activated by Western blot. The expressions of Akt, phosphorylated Akt Ser473, phosphorylated Akt Thr308, phosphorylated nuclear factor-κB p65 Ser536, and Bcl-2 significantly decreased; however, the expressions of phosphorylated glycogen synthase kinase-3β Ser9, phosphorylated forkhead in human rhabdomyosarcoma Ser256, and phosphorylated caspase-9 Ser196 increased in response to lactoferrin treatment in SGC-7901. These results suggest that lactoferrin inhibits Akt activation and modulates its downstream proteins phosphorylation in apoptosis of SGC-7901 human stomach cancer cells.

Key words: stomach cancer, lactoferrin, apoptosis, Akt signaling pathway

INTRODUCTION

Stomach cancer is one of the most serious malignant tumors. The treatment of stomach cancer is varied (e.g., radiotherapy, chemotherapy and medication). However, radiotherapy and chemotherapy are harmful to humans, though they are very effective because they pay attention only to the focus of infection. Drug therapy includes chemical drugs and natural medicines. Now, it has been verified that many natural medicines have effects on stomach cancer, and people accredit them gradually (Tsubono et al., 2001; Yoo et al., 2005) because they are harmless. Lactoferrin is just one natural medicine and is readily available (Levay and Viljoen, 1995).

The lactoferrin from bovine milk is a protein that has the function of transferring iron. Lactoferrin has many physiological functions such as supplementation of human nutrition (Steijins and van Hooijdonk, 2000), antibiosis (Farnaud and Evans, 2003), antivirus (Dapsanse et al., 2001; Beljaars et al., 2004; Wakabayashi et al., 2004; Waarts et al., 2005), guard of host and regulation of immunity, promotion of iron absorption (Suzuki et al., 2001), inhibition of growth and transfusion to cancer (Varadhachary et al., 2004; Xiao et al., 2004), and effect on the production of free radical. Now we find many physiological functions of lactoferrin, but research of inhibition of cancer at the molecular level is very scarce. Although many scientists confirm the inhibition of cancer by lactoferrin, the concrete mechanism is not clear. The manifestation of that inhibition is that lactoferrin can induce stomach cancer apoptosis.

Apoptosis was first discovered as a programmed cell death. A series of extracellular or intracellular events is involved in multicellular organisms during apoptosis, such as regulation of body growth and control of cell death by certain genes. Later studies found that the mechanism of apoptosis is very different than cell...
death. It is a process controlled by a diverse range of cell signals. Many factors may help regulate apoptosis. Heat, radiation, medicines, and other extracellular signals can bind nuclear receptors to trigger the release of intracellular apoptotic signals; some genes may originate intracellular apoptosis, such as c-myc, Bcl family, caspase, and Akt (Bussink et al., 2008), genes related to cell phagocytosis (families of Caenorhabditis elegans cell death protein), antigen encoding genes to apoptosis (families of tumor necrosis factor/Fas), death receptor families in the surface of cell, and systems of cell signaling pathways. During apoptosis, Akt acts as an antiapoptotic signaling molecule (Dudek et al., 1997) and it plays a critical role in the function of cancer cells by promoting the cell survival of damaged or modulating downstream signaling family to induce apoptosis indirectly.

Akt, a Ser/Thr kinase, is also called phosphorylated kinase B. The phosphorylation of 2 individual residues, Ser473 and Thr308, is the basis of activation of Akt (Pitt and Chen., 2008). Once activated, Akt can regulate growth factor-mediated cell survival by phosphorylating and deactivating proapoptotic proteins, such as caspase family, forkhead box class O family, Bcl-2 family, and glycogen synthase kinase-3β (GSK3β). Akt also modulates cell survival by stimulating prosurvival proteins such as nuclear factor κb (NF-κb). Overall, activation of Akt facilitates growth factor-mediated cell survival not only by phosphorylating and deactivating proapoptotic proteins, but also linking to other downstream mediators that facilitate the evasion from apoptosis or support tumor angiogenesis.

In this experiment, we investigated whether lactoferrin plays a role in inducing apoptosis of stomach cancer cell SGC-7901. This allowed the discovery of the mechanism of regulation of Akt signaling way through the process of apoptosis induced by lactoferrin, which may help to further elucidate the function of lactoferrin in anticarcinoma.

MATERIALS AND METHODS

Main Reagents and Equipment

Stomach cancer cell SGC-7901, belonging to epithelial cell, was derived from a fragment of a metastatic tumor lymph node of a 56-yr-old female patient with a histologically proven diagnosis of adenocarcinoma of the stomach, and was obtained from Harbin Medical University (Harbin, China). The CO₂ incubator was from Heal Force Bio-Meditech Holdings Ltd. (Hong Kong, China). The lactoferrin of bovine milk was purchased from Force Bio-Meditech Holdings Ltd. (Hong Kong, China). Bovine serum was obtained from NQBB (Hong Kong, China), and dimethyl sulfoxide and chemicals for SDS-PAGE (i.e., acrylamide, N,N-dimethylacetamide, glycine) were purchased from Sigma (St. Louis, MO). Annexin V-propidium iodide kit was from Byotime (Shanghai, China). Phosphorylated kit, phosphorylated NF-κb p65 Ser536, phosphorylated caspase-9 Ser196, and phosphorylated forkhead in human rhabdomyosarcoma (FKHR) Ser256 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated inhibitor was purchased from Calbiochem (Schwalbach, Germany).

Cell Culture

Cells were cultured in RPMI-1640 medium supplemented with 10% bovine serum in a humidified incubator with CO₂ concentration of 5% at 37°C. Cells in logarithmic growth phase were used for further experiments.

Cell Viability Assay

The viability of the cells was assessed by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Briefly, stomach cancer cells in logarithmic growth phase were collected and 1 × 10⁵ cells/well were dispensed within 96-well culture plates in 100-μL volumes. The cells were first incubated in bovine serum free from RPMI-1640 medium until its adherence. Then, different concentrations of lactoferrin (12.5, 25, 50, and 100 μM) were put in different wells. Each of the concentrations was regarded as a treated group; there was no lactoferrin in the control group. Each of the treated or control groups contained 3 parallel wells and the wells were then incubated continuously for 4 h. The mixture liquid in each well was discarded, then 150 μL of dimethyl sulfoxide was added to each well and incubated for 30 min. After incubation, 96-well plates were determined for absorbance at 570 nm using an ELISA plate reader.

Apoptosis Detection by Flow Cytometry

To assess apoptosis, cells were first incubated in RPMI-1640 medium and allowed to adhere overnight. Then, 50 μM lactoferrin was used to treat the cells for

Reference:
Dudek et al., 1997; Pitt and Chen., 2008.
24 or 48 h. The culture media with different concentrations (12.5, 25, 50, and 100 μM) of lactoferrin were regarded as treated groups. There was no lactoferrin in the control group. Both adherent and floating cells were harvested after treatment. All the processes for Annexin V-propidium iodide method were followed per the instructions provided by the manufacturer.

**Western Blot Analysis**

The cells were first incubated in RPMI-1640 medium until adherence. Then, 40 mg/mL concentration of lactoferrin was used to treat the cells for 24 and 48 h. The culture media with different concentrations (12.5, 25, 50, and 100 μM) of lactoferrin were regarded as treated groups. There was no lactoferrin in the control group. For Western blotting, cells were washed with ice-cold PBS twice and lysed for 30 min at 4°C, then debris was removed by centrifugation for 15 min at 15,000 × g at 4°C, and equivalent amounts of protein were separated by 15% SDS gel and transferred onto nitrocellulose membrane. The membranes were first stained to confirm uniform transfer of all samples and then blocked in 5% milk for 1 h at room temperature. The membranes were incubated overnight with the primary antibodies (diluted 1:500, vol/vol, for antiphosphorylated NF-κb p65 Ser536, antiphosphorylated caspase-9 Ser196, and antiphosphorylated FKHR Ser256; diluted 1:300, vol/vol, for anti-Akt, antiphosphorylated Akt Ser473, antiphosphorylated Akt Thr308, and anti-Bcl-2) at 4°C in 5% BSA, and finally incubated with respective peroxidase conjugated secondary antibodies (1:1,500, vol/vol) for 1 h at room temperature. Blots were developed using a super-enhanced chemiluminescence detection kit, and the protein bands were visualized after exposure of the membranes to X-ray film (Kodak, Rochester, NY).

**RESULTS**

**Effects of Lactoferrin on Growth Inhibition of Stomach Cancer Cells**

To investigate the cell growth inhibition effects of lactoferrin on stomach cancer cells, cells were treated with various concentrations of lactoferrin for 24, 48 and 72 h and cell viability was determined using the MTT assay. As shown in Figure 1, lactoferrin (over 12.5 μM) had significant growth inhibition effects on stomach cancer cells in a dose- and time-dependent manner. Compared with the control group, the cell inhibitory ratio was 4.19 ± 1.65, 9.58 ± 2.38, 28.08 ± 4.68, and 38.52 ± 4.69 at 24 h; 4.88 ± 1.98, 19.88 ± 3.19, 40.12 ± 3.66, and 53.49 ± 6.42 at 48 h; and 6.23 ± 2.12, 25.44 ± 3.57, 57.43 ± 5.14, and 65.12 ± 5.78 at 72 h for 12.5, 25, 50, and 100 μM of lactoferrin, respectively.

**Flow Cytometry Analysis**

To show whether the growth inhibition induced by lactoferrin in stomach cancer cells was caused by induction of apoptosis, stomach cancer cells were stained with both fluorescein isothiocyanate-labeled annexin-V and propidium iodide, and then analyzed by flow cytometry. As shown in Figure 2, exposure to lactoferrin at 50 μM for 24 and 48 h significantly induced apoptosis in SGC-7901 cell line, and the rate of apoptosis was increased in a time-dependent manner.

**Expression of Apoptosis Genes**

To clarify whether the mechanism of lactoferrin-induced apoptosis involved affecting Akt signaling way in stomach cancer cells, the major proteins involved in Akt signal pathway were detected by Western blots. Compared with the control, SGC-7901 was treated separately with 50 μM lactoferrin for 24 and 48 h; Akt and its active forms, phosphorylated Akt Ser473, phosphorylated Akt Thr308 expression were shown (Figure 3). The expression of total Akt in SGC-7901 was unvaried with the increased concentration of lactoferrin and the prolonged time of treatment. However, the phosphorylated Akt Ser473 and phosphorylated Akt Thr308 levels were decreased with the increased concentration of lactoferrin and the extended time of treatment.
Because of the Akt phosphorylation observed after 24 and 48 h of lactoferrin treatment, we investigated the effect of this bovine bioactive protein on the downstream proteins, which can be activated by Akt (Figure 4). The result revealed that the expression of phosphorylated NF-κb p65 Ser536 was substantially reduced in a dose-and time-dependent manner. However, the amounts of phosphorylated GSK3β Ser9, phosphorylated FKHR Ser256, and phosphorylated caspase-9 Ser196 were accumulated in a dose-and time-dependent fashion.

As potential targets, the effect of lactoferrin on amount of Bcl-2 in SGC-7901 was also monitored (Figure 5). With the concentration increased from 0, 12.5, 25, 50 and 100 μM and the time of treatment extended from 24 to 48 h, lactoferrin significantly reduced the amount of Bcl-2 compared with control.

These findings identify that lactoferrin has ability of reducing Akt activation and the downstream proteins expression, which are involved in stomach cancer SGC-7901 cell survival, such as phosphorylated NF-κb p65 Ser536. Meanwhile, lactoferrin affects SGC-7901 apoptosis by increasing expression of Bcl-2 and some proteins, which can be phosphorylated by the activated Akt and involved in apoptosis induction, such as phosphorylated GSK3β Ser9, phosphorylated FKHR Ser256, and phosphorylated caspase-9 Ser196.

**DISCUSSION**

The intake of dairy products has been reported play a role in cancer prevention (Parodi, 2007). Several clinical or in vitro studies have shown inverse association between cancer risk and higher consumption of some dairy products, especially those containing whey proteins (Marshall, 2004). Results from prospective studies confirmed that whey protein can prevent and treat cancer. However, the mechanism of anticarcinoma is argued and is shown by either simulating immunity or increasing glutathione concentration in relevant tissue (Bounous, 2000). Some research even concluded that the iron-binding capacity of whey may also contribute to anticancer potential (Weinberg, 1996). Whey lactoferrin comprises a single polypeptide chain with 2 binding sites for ferric ions, and its functionality is related to the strong iron-bind properties, such as antimicrobrial, antiviral, and anticancer. However, the cancer prevention mechanism is still not clearly known. The effect of prevention and inducing apoptosis on human gastric adenocarcinoma SGC-7901 cells by bovine lactoferrin was determined in this article, and one of the cell signal pathways, Akt signaling pathways, was focused on in particular.

Gastric cancer is a serious public health problem. According to the recent assessment of global cancer incidence, gastric cancer is the fourth most common cancer worldwide (Crew and Neugut, 2006). Therefore, development of new therapeutic agents for stomach cancer is a top priority. Carcinogenesis process is the result of disturbed balance between cell division and growth; meanwhile, it also contains programmed cell death, such as apoptosis. In recent years, it has been shown that the proteins involved in phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathways are often activated or deactivated during human malignant cell growth, differentiation, and development (Fresno Vara et al., 2004). As a main downstream kinase of PI3K, phosphorylated/activated Akt plays a central role in some of the fundamental processes, including cell
proliferation, cell death, cell motility, and revascularization. Full activation of Akt requires phosphorylation of 2 individual residues, Thr308 and Ser473. In many models, Akt facilitates growth factor-mediated cell survival by phosphorylation and deactivating proapoptotic proteins, such as Bcl-2, caspase-9; the forkhead box class O family of forkhead transcription factors, such as FKHR; and the glycogen synthase kinase-3β (GSK-3β) (Cardone et al., 1998; Drosten et al., 2004; Min et al., 2007; Reagan-Shaw and Ahmad, 2007; Nakamura and Sakamoto, 2008; Siddiqui et al., 2008). Meanwhile, activation of Akt is also linked to other downstream mediators that facilitate the evasion from apoptosis, or support tumor angiogenesis, such as NF-κB. Overall, the evidence suggest that the PI3K/Akt signaling pathway is implicated in a wide range of biological and pathophysiologic responses, such as inhibition of cell apoptosis and stimulation of cell proliferation.

Apoptosis is the most common and direct form of cell death, involving a series of steps, and acts as a physiological suicide mechanism to preserve tissue homeostasis through proper cell turnover. Growth inhibition of stomach cancer cell SGC-7901 can be a result of apoptosis induced by lactoferrin, according to analysis of MTT assay and fluorescein isothiocyanate-propidium iodide flow cytometry. In our research, the dosages of lactoferrin we choose were 12.5, 25, 50, and 100 μM. Our data showed that the inhibition rate increased with the increased concentration of lactoferrin and the extended time of treatment. The concentration of lactoferrin at 50 μM had more inhibitory efficiency on stomach cancer cell SGC-7901 proliferation than did other concentrations. It has been reported that lactoferrin has ability of downregulation of G1 cyclin-dependent kinases during growth arrest of head and neck cancer cells, and the concentration of lactoferrin was only 10 μM (Xiao et al., 2004). The concentration of lactoferrin treated on SGC-7901 in our research was 5 times greater than that treated on head and neck cancer cells. It may be because of the multidrug resistance of gastric cancer, which is a major cause of poor sensitivity to chemotherapy (Shigemitsu et al., 2001; Zhang and Fan, 2007). The response rate of stomach cancer to each anticancer agent is approximately 20% (Alexander et al., 1997), which means that much higher dosage is required for stomach cancer cell SGC-7901 treatment.

To further study the mechanistic of lactoferrin-induced antitumor effects in stomach cancer cell line SGC-7901, we examined the activation of Akt and the relative proteins expression. Akt signaling pathway itself widely participates in cell growth, cell splitting, and cell apoptosis and can activate or inactivate many genes that can affect apoptosis. We now know that activation of both phosphorylated Akt Ser473 and phosphorylated Akt Thr308 is necessary to activate Akt completely; it is the vital step to bring Akt into full play. After activation, Akt can activate or inactivate the following genes that affect apoptosis. We demonstrated that the addition of lactoferrin did not affect total Akt of SGC-7901; however, it blocked the Akt survival pathway by inhibiting the Akt phosphorylation of 2 individual residues, Thr308 and Ser473. Furthermore, several downstream factors of Akt signaling pathway seems also have been affected by lactoferrin. It has been previously reported that Ser196 of caspase-9 (Michael et al., 1998), Ser9 of GSK3β (Young et al., 2007), and Ser256 of FKHR (Shannon and Nihal, 2007; Tomohiro and Kazuichi, 2008) are phosphorylated by the activated Akt, and their encouraging function to apoptosis...
is inhibited. Ser536 of NF-κb p65 (Siddiqui et al., 2008) is phosphorylated by activated Akt, and its inhibiting function to apoptosis is encouraged. In addition, the inhibiting function of Bcl-2 protein to apoptosis is also encouraged by activated Akt. Through our experiment we found that lactoferrin had the function of apoptosis of stomach cancer cell SGC-7901 and also changed the expressions of such intracellular genes. We can see through induction of lactoferrin to stomach cancer cells that the expressions of phosphorylated GSK3β Ser9, phosphorylated FKHR Ser256, and phosphorylated caspase-9 Ser196 are increased with the increased concentration of lactoferrin and the extended time of treatment, and the expressions of Akt, phosphorylated Akt Ser473, phosphorylated Akt Thr308, phosphorylated NF-κb p65 Ser536, and Bcl-2 are decreased with the increased concentration of lactoferrin and the extended time of treatment. The lactoferrin inhibition effect on Akt activation, Akt GSK-3, Akt FKHR, and the relative cascade signaling pathway has not yet been reported by others.

CONCLUSIONS

Lactoferrin induced apoptosis of stomach cancer cells through Akt signaling pathway, which includes many genes that can affect apoptosis. Understanding the mechanism of the possible inhibitory effect of bovine lactoferrin on proliferation of gastric adenocarcinoma cells could provide us a meaningful way by which people can prevent stomach cancer through daily diet.

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