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Research Paper

Recombination adenovirus-mediated human lactoferrin cDNA inhibits the growth of human MCF-7 breast cancer cells

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Keywords
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Abstract

Objectives Human lactoferrin, an 80 kDa iron-binding glycoprotein, has anti-tumour effects. We have explored the potential therapeutic role of re-expressing human lactoferrin gene product in human breast cancer.

Methods A recombinant adenovirus expressing the human lactoferrin cDNA (ad-hLTF) was constructed and used to infect breast cancer cells.

Key findings Seventy-two hours after infection, ad-hLTF had considerable cytotoxicity on MCF-7 cells. A time-course study showed that ad-hLTF infection of MCF-7 cells at 100 plaque-forming units per cell increased the number of cells in G_0/G_1 phase and appeared markedly at Sub-G_1 apoptotic peak. The presence of apoptotic cells was confirmed using Annexin V-fluoresecein isothiocyanate apoptosis detection by flow cytometry. Ad-hLTF also resulted in a decrease of Bcl-2 protein and an increase in Bax protein.

Conclusions Ad-hLTF plays an important role in the induction of cell cycle arrest and apoptosis in MCF-7 cells. The results demonstrated that ad-hLTF could have potential benefits in the treatment of breast cancer.

Introduction

Lactoferrin, an 80 kDa iron-binding glycoprotein, is mainly present in mammalian milk colostrums. It is also found in exocrine secretions of mammals and is released from neutrophil granules during inflammation.[1] The primary functions of lactoferrin include improving immunological responses, as well as iron transport, storage and chelation.[2,3] It exhibits many useful biological functional activities that have been used in antibacterial, antivirus, antioxidant and immunoregulation.[4–6] It has been reported that lactoferrin has an antitumour effect and inhibits the proliferation of different tumour cells. Similar preventative effects of bovine lactoferrin on carcinogenesis in other organs have been demonstrated with no side effects, even after long-term administration.[7–9] Thus, lactoferrin could be considered a good candidate as a chemo-preventive agent for human cancers.

Apoptosis is essential not only for development and maintenance of tissue homeostasis but also for the elimination of damaged cells, such as cells damaged by microbial infections and cells found in neoplasms.[10,11] Many anticancer drugs may induce tumour cell apoptosis, and induction of apoptosis is an obvious strategy for cancer therapy. The Bcl-2 family of proteins are important intracellular modulators of apoptosis and can be divided into two groups based on their ability to generate either anti-apoptotic (Bcl-2, Bcl-xL) or pro-apoptotic (Bax, Bak, Bid, Bad) signals. The ratio of anti- to pro-apoptotic molecules such as Bcl-2/Bax determines the response to death signals and leads to cytochrome C release from mitochondria, which triggers the final execution of cell death by the caspase cascade.[12–14] It was reported that bovine lactoferricin selectively induced apoptosis in rat colon mucosa, and human leukaemia and carcinoma cell lines by regulating apoptosis-related-genes Bcl-2 and Bax expression.[15] The activity of caspases-8, -3 and -9, and Bcl-2/Bax proteins were evaluated as the predictive indicator for chemotherapy response in advanced breast cancer.[9,16–18] It is not known if similar apoptotic molecular mechanisms mediate the chemotherapeutic effects of lactoferrin in breast cancer cells. In this study, we have investigated the effects of lactoferrin on Bcl-2 /Bax apoptosis pathway.

The commercial use of lactoferrin has not been developed due to the prohibitive cost of lactoferrin. The production of recombinant human lactoferrin provides a method for production of large volumes with low cost.[19,20] Breast cancer cells express a high number of adenovirus receptors ($6–9 \times 10^3$/cell), rendering these cells ideal as targets for recombinant adenovirus-mediated gene transfer.[21] To the end, we constructed adenoviral vectors expressing the human lactoferrin cDNA and green fluorescent protein (GFP) gene (ad-hLTF) as a reporter gene and studied its therapeutic potential for human breast cancer. The purpose of this research was to unravel the possible mechanisms responsible for the antitumour activity of ad-hLTF.

Materials and Methods

Cell culture

Human breast cancer cell line MCF-7 and human embryonic kidney (HEK) cells transformed with adenovirus type 5 (HEK/293) were obtained from the Cancer Institute of Chinese Academy of Medical Sciences (Beijing, China). All the cell lines were cultured in improved minimal essential medium containing 10% fetal bovine serum and incubated at 37°C in atmosphere with 5% CO_2, as described previously.[22,23]

Construction of adenoviral vectors and infection procedure

A recombinant adenovirus containing the human lactoferrin cDNA and GFP gene, designated ad-hLTF, was constructed by homologous recombination using methods described previously.[22,23] Adenoviral plaques were screened for the presence of lactoferrin sequences by polymerase chain reaction. Adenovirus containing human lactoferrin cDNA and GFP gene was propagated in HEK/293 cells, purified by the commercial purification kits (Qiagen, Shanghai, China), titrated, and stored at −70°C. The control adenovirus had an identical backbone vector to ad-hLTF, but only contained green fluorescent protein gene (ad-GFP), and was constructed simultaneously and used in this study.

Infection with ad-hLTF and the control virus was conducted by incubating the cells with different multiplicity of infection (MOI) of the virus, expressed in plaque-forming units (pfu) per cell, in serum-free medium for 2 h, followed by the addition of medium containing 10% serum and further incubation for the required time at 37°C.

Western blot analysis

Cells plated in 9-cm dishes (1×10^6 cells/dish) were infected with various concentrations of ad-hLTF (0, 1, 10 or 100 pfu/cell) or ad-GFP (100 pfu/cell) for the indicated times. The whole cells were washed with ice-cold phosphate buffered saline (PBS) solution and lysed in 300 μl cell lysis buffer for 30 min, and centrifuged at 12 000*g* for 15 min at 4°C. The supernatant was collected, and protein concentrations were determined according to the Bradford method.[22,23] Samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis after they were boiled for 5 min, and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) by a semi-dry transfer method. The membrane was blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, washed three times with TBST, and incubated with TBST containing 5% nonfat dried milk and primary antibody (lactoferrin: L3262, Sigma Chemical, St Louis, MO, USA; Bcl-2: C21, Bax: B-9, Santa Cruz Bio Inc., Santa Cruz, CA, USA) for 2 h at room temperature. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature under constant agitation. Proteins were visualized using an Enhanced Chemiluminescence (ECL) kit (Amersham, Shanghai, China) and exposed to X-ray film. Actin was used as an internal control for all Western blotting. The intensity of protein bands was quantified by using LabWork 3.0 UVP software (UVP, Upland, CA, USA).

Cell viability

The cells were dispensed in 96-well flat bottom microtitre plates with 100 μl at a density of 5×10^4 cells/well. After 24-h incubation at 37°C, cells were exposed to increasing concentrations of adenovirus vectors (0–10000 pfu/cell) and incubated for the required time at 37°C. Then 20 μl 3-(4,5)-dimethylthiahiazo(-2-yl)-3,5-di-phenytetrazoliumromnide (MTT) (5.0 mg/ml) was added to the medium and incubated for an additional 4 h at 37°C, the medium was then removed. The formazan crystals in the cells were solubilized with a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (pH 4.7). The level of MTT formazan was determined by measuring its absorbance at the wavelength of 490 nm with a Multiskan MK3 reader (Thermo Electron Corporation, San Jose, CA, USA). The rate of cell growth inhibition was calculated using the following formula:

$$\text{rate of cell growth inhibition} = (C\text{-}T)/C \times 100$$

Where C is the average optical density (OD) value of uninfected cell and T is the average OD value of infected cell with ad-hLTF or ad-GFP. Regressive curve was determined as the rate of MCF-7 cell growth inhibition to adenoviral vectors acted concentration. The value of the dose at which the cell growth rate was inhibited by 50% (IC50) was calculated.

Cell cycle analysis

Cells were plated in 9-cm dishes (1×10^6 cells/dish) one day before infection and exposed to various concentrations

(1, 10, 100 pfu/cell) of ad-hLTF or ad-GFP for 72 h. Cells were harvested, washed three times with PBS, fixed with 70% ethanol for 30 min at 4°C, and stained with propidium iodide (PI) for cell cycle analysis. DNA content and cell cycle were measured using a flow cytometer; 10000 cells were counted, per sample. The data was analysed using CellQuest software (Becton Dickinson, San Jose, CA, USA).

Apoptosis analysis

Cells were infected with various doses of ad-hLTF (1, 10 or 100 pfu/cell) or ad-GFP (100 pfu/cell) for 72 h. Apoptotic number was detected using an Annexin V-fluoresecein isothiocyanate (FITC) Apoptosis Detection Kit (KeyGEN, Nanjing, JS, China) according to the manufacturer's instructions. The percentages of apoptosis were measured at 488 nm by flow cytometry. The results were analysed with CellQuest software (Becton Dickinson).

Statistical analysis

All values were expressed as means ± SD One-way analysis of variance and Duncan's multiple range tests were used for determining differences between groups, and $P < 0.05$ was regarded as statistically significant.

Results

Adenovirus-mediated human lactoferrin expression in human breast cancer MCF-7 cells

The expression of human lactoferrin was examined by Western blotting, following the infection of MCF-7 human breast cancer cells with ad-hLTF at various MOIs ranging from 1 to 100 pfu/cell. Western blot analysis demonstrated that the level of ad-hLTF increased substantially 48 h after infection with ad-hLTF in the MCF-7 cell line in a dose-dependent manner, with 100 pfu/cell being the most effective concentration for exogenous lactoferrin expression (Figure 1).

Inhibition effects of ad-hLTF on MCF-7 breast cancer cells

The ability of ad-hLTF to induce cytotoxicity in human breast cancer cells was assessed using the MTT staining procedure. seventy two hours after infection with ad-hLTF, MCF-7 cells showed a dramatic increase in cell inhibition of growth and practically almost 80% of the infected cells died at an MOI of 10 000 pfu/cell, whereas nearly 80% of cells retained viability after infection with the control virus at the same concentration ($P < 0.05$). The IC50 value for ad-hLTF was approximately 100 pfu/cell in MCF-7 cells, while that for the control virus was over 10 000 pfu/cell (Figure 2).

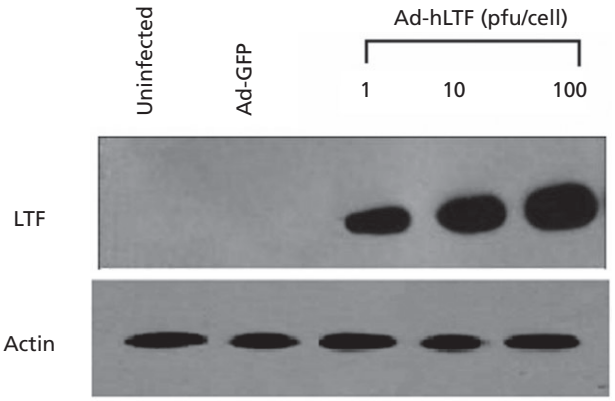

Figure 1 Expression of recombinant adenovirus containing human lactoferrin cDNA and green fluorescent protein gene (ad-hLTF) in human breast cancer cells. Cells plated in 9-cm dishes (1×10^6 cell/dish) were infected with ad-hLTF (1, 10 or 100 pfu/cell), control adenovirus only containing green fluorescent protein gene (ad-GFP; 100 pfu/cell) or were uninfected. After 48-h incubation, whole cell lysates were prepared and subjected to Western blot analysis.

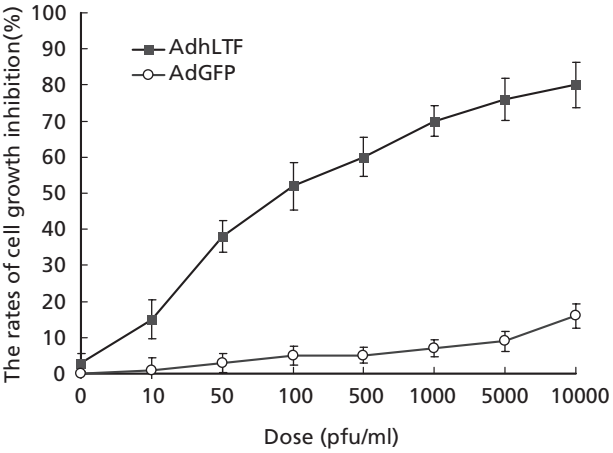

Figure 2 Cytotoxicity of recombinant adenovirus containing human lactoferrin cDNA and green fluorescent protein gene (ad-hLTF) in MCF-7 breast cancer cells. MCF-7 cells plated in 96-well plates (1000 cells/well) were infected with different concentrations of ad-hLTF, control adenovirus only containing green fluorescent protein gene (ad-GFP; 100 pfu/cell) or were uninfected. Seventy two hours after infection, the rates of cell growth inhibition were analysed by the MTT assay. Values shown are means ± SD from three independent determinations.

Ad-hLTF-induced cell cycle arrest and apoptosis in breast cancer cells

To examine the mechanisms of cytotoxicity mediated by overexpression of hLTF, we determined whether ad-hLTF induced cell cycle arrest and apoptosis in breast cell lines. MCF-7 cells were infected with ad-hLTF at an MOI of 100 pfu/cell and harvested for cell cycle analysis by flow

cytometry. A time-course study between one and three days showed that ad-hLTF infection of these cells at 100 pfu/cell increased the number of cells in G_0/G_1 phase and decreased that in S and G_2/M phases. Figure 3 is a representative experiment with cells infected with ad-hLTF or ad-GFP for 72 h. Lactoferrin over-expression appeared to inhibit the cell cycle at the G_1/S-phase transition. However, no arrest of the infected cells in G_2/M phase was observed. The number of cells in the Sub-G_1 (< 2N ploidy) population was assessed further and found to be markedly increased at 72 h after infection with ad-hLTF (100 pfu/cell), while ad-GFP infection had little effect on the number of Sub-G_1 cells, indicating that ad-hLTF-infected cells might have become apoptotic (Figure 3). We further confirmed the presence of apoptotic cells in ad-hLTF-infected MCF-7 cells by using the Annexin V–FITC Apoptosis Detection Kit. The results showed that MCF-7 cells infected with ad-hLTF at 100 pfu/cell but not ad-GFP displayed a significant apoptosis, and the apoptotic rates reached to 31.92% (Figure 4).

Effect of the ad-hLTF on the expression of Bcl-2 and Bax

To investigate the mechanisms of apoptosis, Bcl-2 and Bax apoptosis-related proteins were detected in MCF-7 infected cells. The expression of Bcl-2 was reduced while the expression of Bax was increased (Figure 5a and b). The downregulation of Bcl-2 and upregulation of Bax led to a decrease in the ratio of Bcl-2/Bax (Figure 5c). However, the expression of the above apoptosis related genes were not significantly changed in the ad-GFP infected cells compared with the uninfected cells (Figure 5b).

Discussion

In this study, the effects of ad-hLTF on human breast cancer MCF-7 cells have been investigated. The results showed that treatment of MCF-7 cells with ad-hLTF led to the predicted increase in human lactoferrin levels. Meanwhile, Bcl-2 and Bax proteins were elevated in ad-hLTF-infected MCF-7 cells. As a result of ad-hLTF infection, cell cycle arrest and apoptosis were observed in a significant proportion of the target cell population. Our findings were consistent with previous studies using bovine lactoferrin, which showed G_1 arrest, caspase-3 activation, poly ADP ribose polymerase cleavage and DNA fragmentation in oral, head and neck and colon cancers.[17,24,25]

It has been reported that human breast cancer cells MCF-7 have lost caspase-3 gene activity, owing to a 47-bp deletion within exon 3 of the caspase-3 gene.[26,27] Caspase-3 is an executioner caspase of the apoptosis pathway, so it is interesting to observe whether apoptosis can occur in the absence of active caspase-3. Recently, Deng *et al.*[21] reported that caspase-3 was not necessarily essential for the morphological changes associated with apoptosis in MCF-7 cells. In our

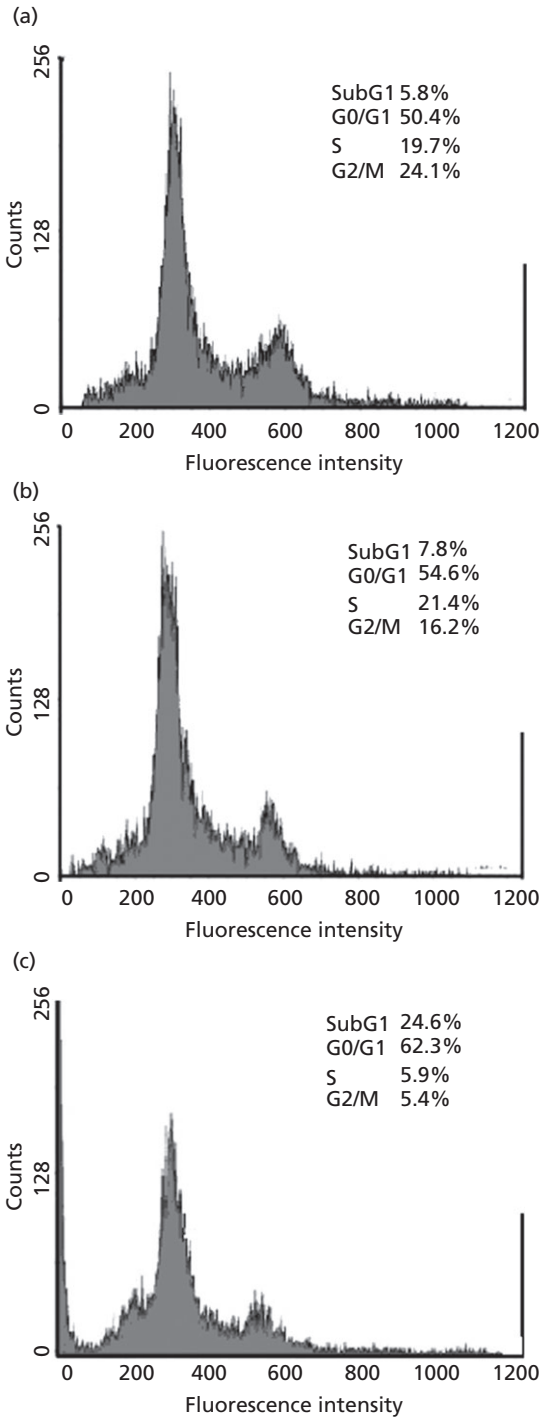

Figure 3 Cell cycle analysis of recombinant adenovirus containing human lactoferrin cDNA and green fluorescent protein gene (ad-hLTF) or control adenovirus only containing green fluorescent protein gene (ad-GFP) infected MCF-7 breast cancer cells. Cells plated in 9-cm dishes (1×10^6 cells /dish) were (a) uninfected, or infected with (b) 100 pfu/cell ad-GFP or (c) 100 pfu/cell ad-hLTF. Seventy two hours after infection, cells were stained with propidium iodide and subjected to flow cytometry analysis. The percentage of sub G_1 population was obtained using CellQuest software.

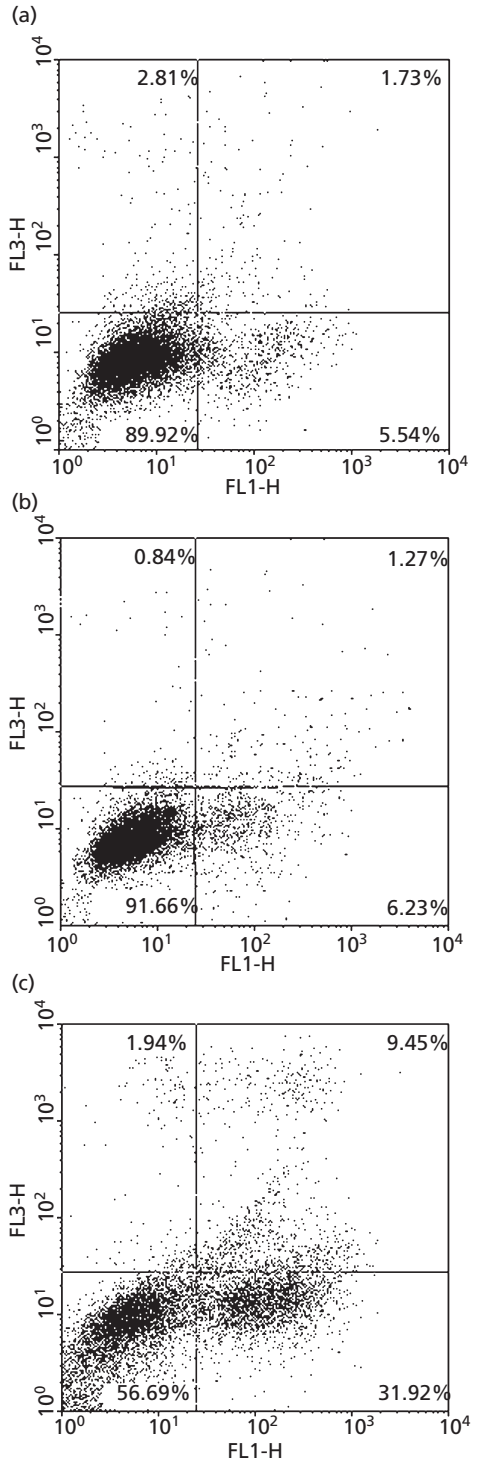

Figure 4 Induction of apoptosis in MCF-7 breast cancer cells. Cells, plated in 9 cm dishes were (a) uninfected, or infected with (b) 100 pfu/cell ad-GFP or (c) 100 pfu/cell ad-hLTF for 72 h, harvested and analysed by Annexin V-FITC-based flow cytometry as described in Materials and Methods. Ad-GFP, control adenovirus only containing green fluorescent protein gene; ad-hLTF, recombinant adenovirus containing human lactoferrin cDNA and green fluorescent protein gene.

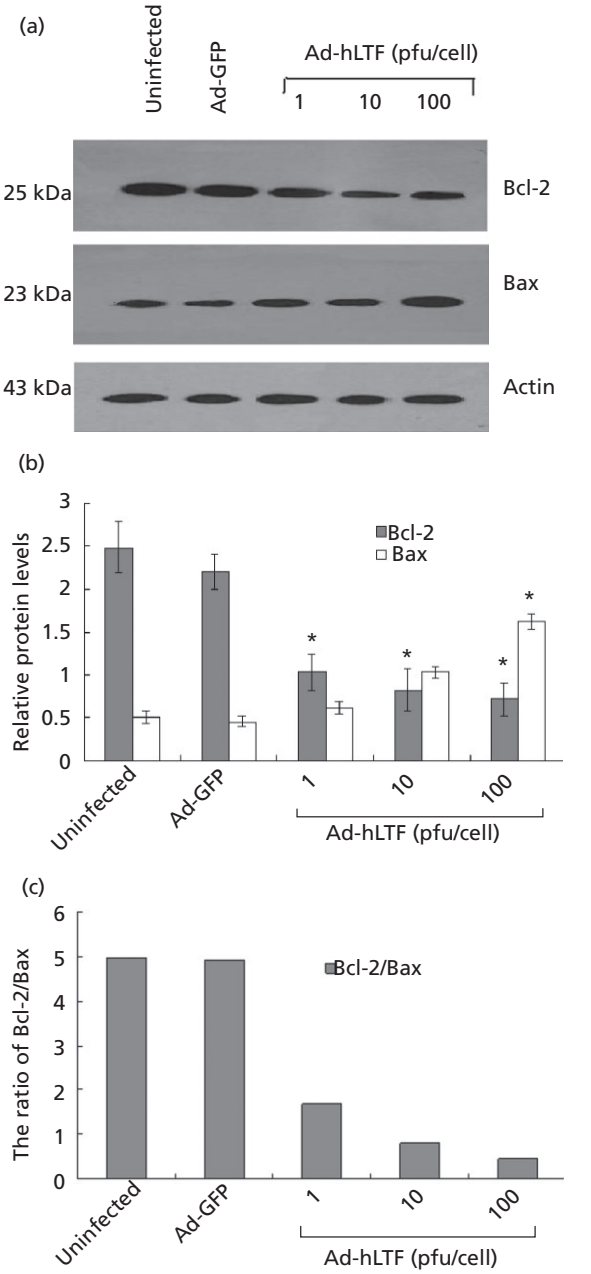

Figure 5 Expression of Bcl-2 and Bax proteins in MCF-7 breast cancer cells. Cells plated in 9 cm dishes were infected with various concentrations of ad-hLTF (1, 10 or 100 pfu/cell), ad-GFP (100 pfu/cell) or uninfected, respectively. After incubation for 72 h, whole cell lysates were prepared and subjected to Western blot analysis on SDS-PAGE gel and immunoblotted with the antibody against Bcl-2, Bax or actin, respectively. (a) Western blot analysis of Bcl-2 and Bax expression. β-Actin was used as a control to ensure equal loading. Data shown is representative of three independent experiments. (b) The intensity of Bcl-2 and Bax bands was quantified and was shown as relative expression level after normalized by β-actin ($n = 3$, means $\pm$ SD). $^*P < 0.05$ vs uninfected cells. (c) The ratio of Bcl-2/Bax. Ad-GFP, control adenovirus only containing green fluorescent protein gene; ad-hLTF, recombinant adenovirus containing human lactoferrin cDNA and green fluorescent protein gene.

study, the results showed that ad-hLTF-infected MCF-7 cells exhibited significant characteristics of apoptosis based on the Annexin V-FITC/PI apoptosis assay. Thus, giving further support of the notion that apoptosis can occur in the absence of active caspase-3.

Although the detailed signalling pathways that trigger apoptosis are incomplete, the mitochondrial pathway is thought to play a major role in response to cancer treatments and is mediated by the Bcl-2 family of proteins which are often over-expressed in many tumour cells.[18,28,29] Many researchers have shown that bovine lactoferricin selectively induced apoptosis in human leukaemia and carcinoma cell lines by downregulating the expression of Bcl-2 and regulating the activity of caspase-8, -3, -9; induced cell death involving apoptosis by regulation of apoptosis related-gene Bcl-2 and Bax expression in rat colon mucosa and human breast cancer cell lines.[15–18] Those results suggested that one of the apoptotic mechanisms induced by ad-hLTF was to trigger the mitochondrial-dependent pathway. In this study, the results showed that the treatment with ad-hLTF decreased the expression of Bcl-2 and increased the expression of Bax protein levels. The upregulation of Bax expression and the reduction of Bcl-2 expression in the treated cells led to a decrease in the ratio of Bcl-2/Bax, which might be responsible for the drug-induced apoptotic processes and which has been found to be associated with a better prognosis.[30,31]

Adenovirus vectors offer several advantages over other vector systems. Adenovirus vectors are highly efficient for in-vivo gene delivery and direct in-situ tumour transduction, and so they have been extensively used in experimental studies and, in some instances, in the clinical gene therapy of a variety of cancers in recent years. Furthermore, the combination of recombinant adenovirus vector technology with chemotherapy seems to be promising.

This study has been the first investigation into the effects of ad-hLTF on human breast cancer cells and has demonstrated that recombinant human lactoferrin, mediated by adenovirus vectors, had similar biological activity to natural lactoferrin, with more economical and efficient procurement than natural lactoferrin. It should greatly facilitate the future research of human lactoferrin in the fields of cancer therapy and biotechnology.

Conclusions

Ad-hLTF resulted in the growth inhibition of tumour cells by inducing apoptosis, and the mechanism likely occurred through triggering the mitochondrial-dependent pathway. This suggested that ad-hLTF could have potential benefits in treating human breast cancer.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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