Inhibition of Tumor Growth by Recombinant Adenovirus Containing Human Lactoferrin through Inducing Tumor Cell Apoptosis in Mice Bearing EMT6 Breast Cancer

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(Received June 18, 2010/Revised October 1, 2010/Accepted October 12, 2010)

Human lactoferrin (hLTF), an 80-kDa iron-binding glycoprotein, has antitumor activity. In this study, a recombinant adenovirus containing the human lactoferrin cDNA (ad-rhLTF) was constructed and its effect on tumor growth was investigated in mice bearing EMT6 breast cancer. Ad-rhLTF was injected seven times within 14 days into the tumor site at two concentrations (106 and 5 × 108 pfu/mL) in mice bearing EMT6 breast cancer. Injected ad-rhLTF had considerable cytotoxicity on mice breast cancer, and significantly reducing the weight of tumor produced and increasing the tumor inhibition rate up to 52.64%. The presence of apoptotic cells was confirmed using TUNEL staining and flow cytometry assays. At the same time, RT-PCR and Western blot analyses demonstrated that ad-rhLTF also decreased expression of Bcl-2 and increased Bax and caspase 3 expressions. Therefore, we conclude that ad-rhLTF inhibits tumor growth by inducing tumor cell apoptosis in mice with breast cancer by triggering the mitochondrial-dependent pathway and activation of caspase 3. The results indicate that ad-rhLTF might be a promising drug for breast cancer gene therapy.

Key words: Lactoferrin, Adenovirus vector, Gene therapy, Breast cancer, Apoptosis, Pathway

INTRODUCTION

Lactoferrin (LTF), an iron-binding glycoprotein, is mainly present in mammalian milk colostrum. It is also found in exocrine secretions of mammals and is released from neutrophil granules during inflammation (Artym et al., 2003). LTF contains 703 amino acids and has a molecular weight of 80 kilodaltons (González-Chávez et al., 2009). The primary functions of LTF are to improve immunological responses (Zimecki et al., 2007), iron transport, storage and chelation (Ward and Conneely, 2004).

LTF also exhibits many useful biologic functional activities that have been used in antibacterial, antivirus, antioxidant and immunoregulatory roles (Shimazaki, 2000; Conneely, 2001; Mulder et al., 2008). LTF also has antitumor effects and inhibits the proliferation of different tumor cells including esophageal carcinoma, oral cancer, lung cancer, liver cancer, colon carcinoma, and bladder cancer (Ward et al., 2002; Tsuda et al., 2002; Mader et al., 2005). However, the pharmacologic mechanisms of LTF on breast cancer are unknown.

Commercial LTF is mainly bovine LTF that is extracted from whey proteins in bovine milk. The prohibitive cost has restricted therapeutic development efforts utilizing bovine LTF. The production of recombinant human LTF (rhLTF) provides a method for production of large volumes at low cost (Cerven et al., 2008; Tutykhina et al., 2009). Previously, we...
reported on the construction of adenoviral vectors containing human LTF cDNA (ad-rhLTF) and a green fluorescent protein (GFP)-reported gene fusion, and described the abundant expression of human LTF in the milk of goats and rabbits (Han et al., 2007, 2008).

In this study, we repeatedly injected purified and titrated ad-rhLTF into the tumor sites of EMT6 bearing mice to explore the therapeutic potential of ad-rhLTF for breast cancer, unravel the possible mechanisms responsible for the antitumor activity of ad-rhLTF and to provide scientific experimental evidence for tumor gene therapy of ad-rhLTF.

MATERIALS AND METHODS

Drugs and chemicals
Human embryonic kidney (HEK) 293 and mouse breast cancer EMT6 cell lines were obtained from the Cancer Institute of the Chinese Academy of Medical Sciences. Prodim iodide, ribonuclease (RNase) and rabbit anti mouse LTF (L3262) monoclonal antibody were obtained from Sigma-Aldrich. The terminal deoxyribonucleotide transferase-mediated nick-end labeling assays (TUNEL) kit was purchased from KeyGEN Bio. Trizol was purchased from Gibco. Reverse transcriptase cDNA synthesis kit was purchased from Takara Bio. Bcl-2 (C21), Bax (B-9) and caspase 3(E-8) monoclonal antibodies were obtained from Santa Cruz Biotechnology. Horseradish peroxidase-labeled rabbit antigoat IgG antibody and actin polyclonal antibody were purchased from Biosynthesis Bio. The enhanced chemiluminescence kit was purchased from Amersham Pharmacia Biotech. All other chemicals used were of analytical reagent grade.

Preparation of viral stock
HEK 293 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS) and incubated at 37°C in an atmosphere of 5% CO₂. One hundred microliters of ad-rhLTF suspension was added to HEK 293 cells grown to 80% confluence (Wang et al., 2010). After 48 h, the titer of the adenovirus stock was determined using GFP on semi-confluent 293 cells. The 293 cells were collected and the virus was by four cycles of freezing in liquid nitrogen and melting at 37°C, diluting the virus stock concentration based on the plaque-forming units (pfu)/mL with normal saline to the desired concentration, and storing at −70°C until use.

Animals
Fifty female 6-week-old Kunming mice were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences. Animal experiments were conducted in accordance with the NIH Guide for the care and use of laboratory animals (NIH Publication No. 80-23; revised 1978 and the number approved by Administrated-Committee of Laboratory Animals was 062310). The mice were randomly divided into five groups (n = 10 per group). One group was used for the preparation of EMT6 tumor cells. The other four groups were used for different administrations of drugs (see below). Animals were housed in plastic cages with free access to food and water and maintained on a regulated environment (20 ± 2°C).

Prepared EMT6 tumor cells
The EMT6 breast cancer cell line (5 × 10⁶/mL EMT6 breast cancer cells) was injected into the right forelimb in one group of mice (0.2 mL/mouse). When each tumor had grown to 1 cm in size, it was removed and suspended in normal saline to a concentration of 5 × 10⁶/mL under aseptic conditions.

Animal model and drug treatment
Four groups of mice were all injected with 0.2 mL/mouse of 5 × 10⁶/mL EMT6 breast cancer cells in the skin under the right forelimb. The select drug was administered when each tumor had attained a size of 0.3-0.5 cm. One group was injected with 100 µL of normal saline in the tumor site as the control group. One group was given the standard antitumor reference drug cyclophosphamide (CTX; 25 mg/kg body weight, i.p. daily for 14 days); this group was the positive control group (CTX group). The other two groups were injected with 100 µL ad-rhLTF in tumor sites at a dosage of 10⁸ pfu/mL or 5 × 10⁸ pfu/mL (ad-rhLTF groups). All of the injections were given once every two days for a total of seven times in 14 days. After 14 days, all mice were weighed and killed, and each tumor was removed and weighed. According to the mean weight of tumor, the rate of tumor inhibition was calculated as follows: rate of inhibition (%) = [(mean tumor weight of control group − mean tumor weight of treated group) / mean tumor weight of control group] × 100.

Morphologic analysis of tumor tissues
Tumor specimens collected from the control group, CTX group and the 5 × 10⁸ pfu/mL ad-rhLTF group were fixed in 10% (v/v) neutral formalin solution, dehydrated through a graded ethanol series and embedded in paraffin. Tissue serial sections 4 µm in thickness were stained with hematoxylin and eosin and then examined under the light microscope.