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Bovine lactoferrin inhibits lung cancer growth through suppression of both inflammation and expression of vascular endothelial growth factor

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ABSTRACT

Lung cancers are among the most common cancers in the world, and the search for effective and safe drugs for the chemoprevention and therapy of pulmonary cancer has become important. In this study, bovine lactoferrin (bLF) was used in both in vitro and in vivo approaches to investigate its activity against lung cancer. A human lung cancer cell line, A549, which expresses a high level of vascular endothelial growth factor (VEGF) under hypoxia, was used as an in vitro system for bLF treatment. A strain of transgenic mice carrying the human VEGF-A_{165} (hVEGF-A_{165}) gene, which induces pulmonary tumors, was used as an in vivo lung cancer therapy model. We found that bLF significantly decreased proliferation of A549 cells by decreasing the expression of VEGF protein in a dose-dependent manner. Furthermore, oral administration of bLF at 300 mg/kg of body weight 3 times a week for 1.5 mo to the transgenic mice overexpressing hVEGF-A_{165} significantly eliminated expression of hVEGF-A_{165} and suppressed the formation of tumors. Additionally, treatment with bLF significantly decreased the levels of proinflammatory cytokines, such as tumor necrosis factor-α, and antiinflammatory cytokines, such as IL-4 and IL-10. Levels of IL-6, which is both a proinflammatory and an antiinflammatory cytokine, were also reduced. Treatment with bLF decreased levels of tumor necrosis factor-α, IL-4, IL-6, and IL-10 cytokines, resulting in limited inflammation, which then restricted growth of the lung cancer. Our results revealed that bLF is an inhibitor of angiogenesis and blocks lung cell inflammation; as such, it has considerable potential for therapeutic use in the treatment of lung cancer.

Key words: bovine lactoferrin, pulmonary cancer, vascular endothelial growth factor (VEGF), transgenic mice

INTRODUCTION

For several decades, lung cancer has been the most common cancer worldwide (Li et al., 2010). Most new cases of lung cancer now occur in developing countries (55%) and it is still the most common type of cancer in men (1.1 million cases, 16.5% of the total), with high rates in central, eastern, and southern Europe, North America, and East Asia (Ferlay et al., 2010). Although the treatment of lung cancer has improved, the mortality rate of lung cancer patients remains high. To reduce these high rates of mortality, many researchers have focused on methods for tumor prevention in addition to more effective treatments (Lin et al., 2009). Recently, researchers have found natural food components or products of digestion that could mediate the process of angiogenesis and metastasis (Singh et al., 2006; Yang and Wang, 2010). Researchers have shown the anticancer potential of dietary proteins, peptides, and amino acids, which may be natural products of fermentation and enzymatic hydrolysis or products of gastrointestinal digestion (de Mejia and Dia, 2010). These compounds mediate apoptosis and angiogenesis, which are vital steps in controlling tumor metastasis.

Bovine lactoferrin (**bLF**), a siderophilic protein with 2 iron-binding sites, has a wide range of biological activities, including anticancer effects, antimicrobial effects, and improvement of immunomodulatory functions (de Mejia and Dia, 2010). Chemopreventive and cell growth inhibitory activities of bLF have been demonstrated in esophageal (Ushida et al., 1999), lung (Li et al., 2011), colon (Tsuda et al., 1998), bladder (Masuda et al., 2000), mammary (Yamada et al., 2008; Duarte et al., 2011), stomach (Xu et al., 2010), and tongue (Tanaka et al., 2000) cancers. The anticancer functions of bLF are thought to be exerted through its innate

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ability to bind iron (González-Chávez et al., 2009). The iron could accelerate oxidation, thereby disrupting nucleic acid structure. Other potential mechanisms of anticancer functions include induction of programmed cell death and regulation of cell cycle protein expression (Lönnerdal and Iyer, 1995; Rodrigues et al., 2009). However, the antiangiogenesis effects of bLF during tumor growth are poorly understood.

In the early stages of cancer, the unregulated proliferation of cancer cells leads to a deficiency of both nutrients and oxygen, causing significant cell death. Cell death triggers an inflammatory response, activates hypoxia-inducible factor 1α (HIF-1α), promotes the expression of the vascular endothelial growth factor (**VEGF**)-A mRNA, and causes angiogenesis. Of the VEGF family, VEGF-A is the one of most interest in human medicine for specialists and medical teams. Four isoforms of VEGF-A are known, including VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}; VEGF-A_{165}, the common type, primarily functions to promote angiogenesis. Secreted VEGF-A_{165} binds to the receptor VEGFR2 and activates a downstream signal that induces vasculogenesis (Ferrara, 2002). When cancer cells secrete a large amount of VEGF-A_{165}, vasculogenesis is induced to provide sufficient nutrients and oxygen to the tumor, thus increasing the tumor growth rate. Expression of VEGF-A_{165} is positively related to the growth and spread of cancer cells (Coussens and Werb, 2002). Therefore, the development of medicines that target VEGF-A_{165} is an important topic of study.

In this study, we used both in vitro and in vivo approaches to investigate the effects of VEGF expression in lung cancers treated with bLF, which was purified from bovine milk. Human lung cancer cells and an animal model of human (**h**)VEGF-A_{165}-induced lung tumor transgenic mice were applied to examine the protection mechanisms of bLF on human and mouse lung carcinomas.

MATERIALS AND METHODS

Cell Culture

Human lung adenocarcinoma cell lines A549 [American Type Culture Collection (**ATCC**), Manassas, VA; cat. no. CCL-185] and CL1–0 (provided by J. J. W. Chen, Institute of Biomedical Science, National Chung Hsing University, Taichung, Taiwan; Chen et al., 2001) and the human bronchial epithelial cell line Beas 2B (ATCC; cat. no. CRL-9609) were cultured in Dulbecco's modified Eagle's medium (**DMEM**) supplemented with 10% fetal bovine serum (**FBS**). Cells were incubated in a 5% CO_2 incubator at 37°C (Chen et al., 2008c).

Cell Viability Assay

To measure the cytotoxicity of bLF (Sigma-Aldrich, St. Louis, MO) on cell proliferation, A549, CL1–0, and Beas 2B cells (2×10^5 cells/well) were seeded into a 96-well plate in triplicate and incubated for 3 h to allow cell adherence. First, 200 μL of fresh medium containing various concentrations of bLF (15 to 0.9375 mg/mL) or H_2O (control) was added into the cultures and incubated at 37°C for 48 h. Following the removal of the medium from the wells, 100 μL of tetrazolium salt solution [1 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**MTT**) in 10 mL of DMEM] was added. After 4 h of incubation at 37°C, the medium was removed and 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured in an ELISA reader at 570 nm (Yen et al., 2009). The cell viability ratio (%) was calculated as follows: % viability = (absorbance of test sample/absorbance of control) × 100%.

Growth Curve and Doubling Time

The A549 cell line was used to generate a growth curve using a 24-well microtiter plate. A seeding density of 1,500 cells in a 1-mL volume of DMEM supplemented with 10% FBS was used per well. Growing cultures were trypsinized at d 1, 4, 7, 8, 9, and 10, and the number of live cells per well was determined in triplicate (Chen et al., 2010). The doubling time (in hours) was calculated as $h \times \ln(2)/\ln(c2/c1)$, where c1 and c2 are the cell concentrations at the beginning and the end of the chosen period of time.

Reverse-Transcription PCR Analysis

The A549 cells were seeded in a 10-cm dish at 5×10^6 cells/well. After 2 d, 10 mL of DMEM supplemented with 10% FBS or 0.1% FBS and containing various concentrations of bLF (100, 50, 25, 12.5, and 6.25 mg per 10-cm dish) was added to the cultures and incubated at 37°C for 48 h. The reverse-transcription PCR (**RT-PCR**) protocol was described previously (Hung et al., 2010; Tsai et al., 2010). Primers for *vegf* were 5′-CAGAAGCAG AATGTGACCATC-3′ (sense) and 5′-CTTCTGGTCGATGTCATGAGC-3′ (antisense). β-Actin was used as an internal control with the following primers: 5′-CCGTCTTCCCCTC-CATCGTGGG-3′ (sense) and 5′-CGCAGCTCATT-GTAGAAG GTGTGG-3′ (antisense). The amplified RT-PCR products were analyzed with 2% agarose gel electrophoresis containing ethidium bromide (Tung et al., 2011).