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A novel liposomal vaccine improves humoral immunity and prevents tumor pulmonary metastasis in mice

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ABSTRACT

Basic fibroblast growth factor (bFGF) is an important stimulator of angiogenesis involving in neovascularization progression. The aim of this study is to evaluate whether a liposomal vaccine (MLB) based on xenogeneic human bFGF plus monophosphoryl lipid A (MPLA) could effectively induce cross-reaction immunity in mice and increase antitumor activity. Sera of mice were analyzed and IgG antibody titer in MLB group was obviously higher than other groups including the mice immunized with liposomal bFGF vaccine, bFGF plus Freund's adjuvant, empty liposome and PBS. Furthermore, tumor metastasis was significantly inhibited in MLB group, compared with L and PBS group. The IFN-γ production of cultured splenocytes *in vitro* was evidently up-regulated meanwhile IL-4 production sustained in a low level, revealing that this vaccine stimulated Th1 immunity response preferentially. Taken together, these findings suggested that this novel bFGF vaccine could effectively induce humoral immunity through cross-reaction, mediate Th1 immune response preferentially and enhance antitumor activity *in vivo*.

1. Introduction

Basic fibroblast growth factor (bFGF) is a member of a large family of structurally related proteins involving in several physiology such as the cell growth, differentiation, migration and survival of a wide variety of cell types (Bikfalvi et al., 1997). bFGF affects the growth and development of neovascularity, which is a driving forth of numerous cancer and related diseases (Bikfalvi et al., 1997). Previous study revealed that bFGF played an important role as an angiogenesis stimulator in the corneal eye pocket (Gaudric et al., 1992). Miyake et al. (1996) reported that injecting high expression bFGF gene into renal carcinoma cell lines resulted in increased neovascularization and metastatic potential *in vivo*.

Angiogenesis, a basic process that microvessel sprouts the size of capillary, involving in a few physiological conditions including embryonic development, reproduction and wound healing, is also a considerable factor for tumor growth and metastasis formulation (Folkman, 2007). Numerous pathologies such as ocular neovascular disease, chronic inflammatory diseases and cancer, were gravely driven by uncontrolled angiogenesis directly or indirectly (Bikfalvi, 2006). Antiangiogenesis is considered as a novel method to treat cancer or prevent tumor by restraining or destroying tumor vessels (Garkavtsev et al., 2004; Marx, 2000, 2003; Marshall, 1998; Lee et al., 2002). In fact, angiogenesis procedure should be completed when all the steps occur, in which bFGF is a dispensable factor (Nyberg et al., 2005; Eskens and Verweij, 2006; Zheng et al., 2007; Chen et al., 2005). These findings also suggested antiangiotherapy by inhibiting bFGF would be a feasible approach to decrease tumor metastasis. Besides, it has been reported that similarity between infectious agents and self-protein mainly contributed to trigger mechanism of autoimmune diseases induced by parasites, bacteria and virus (Karlsen and Dyrbert, 1998). Thus, it is suggested that the exogenous protein of high similarity to autologous protein might induce autoimmune response. It is well known that some genes such as bFGF and VEGF are highly conserved during the evolutionary process. And further studies illustrated that immunization in a cross-reaction with a xenogeneic homologous protein or a xenogeneic homologous gene effectively inhibited tumor growth via inducing autoantibody against self-molecules, such as VEGF, VEGF-R and EGF-R (Liu et al., 2003; Wei et al., 2001; Lu et al., 2003). The specific autoantibody is the possible reason for the antitumor activity. According to these previous studies, it is a potential attempt to produce a protein vaccine based on human bFGF as a model antigen to break the immunity tolerance against bFGF in a cross-reaction

Abbreviations: bFGF, basic fibroblast growth factor; MPLA, monophosphoryl lipid A; MLB, liposomal bFGF and MPLA; L, liposomes; LB, liposomal bFGF; FB, bFGF and the Freund's adjuvant; PBS, phosphate buffered saline; LL/2, Lewis lung carcinoma cells; IL-4, interleukin-4; IFN-γ, interferon gamma; H&E, hematoxylin and eosin; IgG, immunoglobulin G; DOTAP, 1,2-dioleoyl-3-trimethylammoniumpropane; DOPE, dioleoyl-l-phosphatidylethanolamine; Con A, concanavalin A; TG-1, an *E. coli* K-12 strain which has no *EcoK* restriction enzyme; TMB, tetramethylbenzidine; IPTG, isopropy-β-D-thiogalactoside.

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between the xenogeneic homologous protein and mice autologous bFGF and in turn further inhibit angiogenesis-dependent tumor growth.

bFGF contains 12 anti-parallel β-sheets organized into a trigonal pyramidal structure, and consists of a number of basic residues (pI 9.6). Synthetic peptide bFGF was doubted of its immunogenic efficacy, since a large number of synthetic peptide antigens had no or low immunogenicity (Gregoriadis, 1990). Liposomes are a proved drug delivery system to effectively encapsulate most materials such as drugs, DNAs, peptides and proteins. Liposomes also enhanced the immunogenicity of various antigens and are designed to increase cellular or humoral immune response (Alving, 1995). Other studies commented on its helpful advantages like biodegradability, non-toxicity, synthetic material and elicitation of both humoral immunity and cell mediated immunity (Kersten and Crommelin, 1995; Alving, 1991). The main mechanism by which liposomes enhance the immune responses to the carried antigens is ascribed to the capture of liposomes by macrophages (Singh and Bisen, 2006). Immunoadjuvanticity of liposomes has been supported by a lot of animal immunization studies (Gregoriadis, 1990; Kersten and Crommelin, 1995; Allison and Gregoriadis, 1974). Therefore, we planed to design a vaccine by conjugating basic peptide bFGF to cationic liposomes. Moreover, the formulation monophosphoryl lipid A (MPLA), which was reported as a potent adjuvant in HIV vaccine (McElrath, 1995), liposomal prostate cancer vaccine (Harris et al., 1999), liposomal breast cancer vaccine (Samuel et al., 1998) and combination malaria and hepatitis B surface antigen vaccine (Gordon et al., 1995; Alonso et al., 2004), was also used in this vaccine.

In this study, the aim was to examine whether this cationic lipids-encapsulated vaccine containing bFGF and MPLA could stimulate efficient humoral immunity and inhibit angiogenesis-dependent tumor pulmonary metastasis. The Freund's adjuvant, well verified as a potent immunoadjuvant but toxic in clinical experiments, was designed as the positive control (Huleatt et al., 2007). C57 mice were inoculated with liposomal bFGF vaccine and then challenged with Lewis lung carcinoma cell (Cui et al., 2006). Anti-bFGF antibody in sera and tumor metastasis in lungs was examined. This bFGF vaccine was able to elicit bFGF-specific antibody and induce effective immune protection in mice. Pulmonary metastasis was significantly suppressed as well owing to blocking angiogenetic factor bFGF. In addition, in *in vitro* splenocytes response experiment, IFN-γ production, which is related with Th1 type immunity response, was significantly up-regulated in the vaccinated groups.

2. Materials and methods

2.1. The preparation of recombinant peptide bFGF

Human basic fibroblast growth factor (bFGF) was prepared as previous description (Weich et al., 1990). bFGF cDNA was inserted into prokaryotic expression plasmid pQE30 (Qiagen, USA) to get 6× His and bFGF fusion protein. Recombinant bFGF was expressed in TG-1 as soluble form. The TG-1 cells were harvested after 12 h post-induction by 1 mM IPTG. The pellet were washed three times in ice-cold PB buffer (20 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl) and resuspended in 50 mM sodium phosphate buffer (pH 7.0). Cells were lysed by high pressure (APV 2000, Denmark). Cell debris and insoluble protein were removed by centrifugation for 30 min at 20,000 × *g* and 4 °C. The supernatant fraction containing soluble bFGF was dialyzed using dialysis membrane (Millipore, USA) against 20 mM sodium phosphate buffer (pH 6.0) and then applied to SP-sepharose column (Pharmacia, Sweden). After washed with 20 mM sodium phosphate buffer (pH 6.0) appropriately using 5 column volumes, bFGF fusion protein was eluted with elution buffer (20 mM sodium phosphate buffer, pH 6.0, 200 mM NaCl). The elution fraction was dialyzed against 20 mM sodium phosphate buffer (pH 7.0), 10 mM imidazole, and bFGF fusion protein was purified to homogeneity using Ni-chelating sepharose column (Pharmacia, Sweden). The bFGF protein was eluted by 20 mM sodium phosphate buffer (pH 7.0), 100 mM imidazole, and then dialyzed against 20 mM sodium phosphate buffer (pH 7.0), finally stored at −20 °C before use.

The characterization of recombinant bFGF was determined with anti-bFGF antibody (BioVision Inc., USA) by Western blotting and the purity of protein was evaluated by SDS-PAGE (Weich et al., 1990). The commercial bFGF peptide (BioVision Inc., USA) was used as control. The recombinant bFGF peptide was used in subsequent *in vitro* and *in vivo* experiments.

2.2. Cell lines and animals

The mouse Lewis lung carcinoma cell lines LL/2 was purchased from American Type Culture Collection, ATCC. LL/2 was maintained in DMEM medium (GIBICO). Cells were supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, at 37 °C, 95% relative humidity, under 5% CO_2.

Six- and eight-week-old C57BL/6N female mice were purchased from the West China Hospital Experimental Animal Center of Sichuan University. All animals used in the experiments were treated humanely in accordance with Institutional Animal Care and Use Committee guidelines.

2.3. Preparation of bFGF liposomes

Cationic liposomal vaccines with recombinant bFGF and MPLA (MLB) were prepared as previous reference (Jaafari et al., 2005). The lipid phase containing DOTAP, DOPE (1:2 molar ratio) (Avanti Polar Lipids Inc., Alabaster, AL) and monophosphoryl lipid A (0.25% quality ratio of all formulations) (Sigma Chemical Co, Inc., St. Louis, MO) was dissolved in chloroform: methanol (1:1, v/v) in a round-bottom flask. Thin lipid film was obtained on the flask's wall through removing solvent by rotary evaporation. The lipid film was dried at 5 mbar overnight to ensure all solvent removed. The round-bottom flask was filled with distilled water and the lipid film was hydrated and dispersed by ultrasonic at 45 °C (200 W, 10 min), then empty liposomes were obtained. The recombinant bFGF (2% quality ratio of all formulations) was dropwised to empty liposomes under the condition of magnetism and agitation, and the mixture was incubated for half an hour at 4 °C. Subsequently, the mixture was quick-freezed in liquid nitrogen and then incubated 1 h in thermostatic waterbath at 4 °C by cold trap. Then freezing thawing repeated six times. The resulting formulations were extruded repeatedly through 450 nm polycarbonate membranes (Millipore, USA) 10 times. The suspension containing liposomal bFGF was obtained through super high speed centrifugation (Beckman, USA), at 100,000 × *g* for 1 h at 4 °C, removing unencapsulated bFGF. Mannitol injection (Sichuan Kelun Pharmaceutical Co., LTD), as the role of the freeze-dry excipient, was added to the suspension at a ratio of 5% of all lipids. The suspension with mannitol was freeze-dried and then stored at 4 °C. The final quality ratio of liposomes:lipid A:bFGF was about 98:0.25:2. To prepare cationic liposomes containing only recombinant bFGF (LB), MPLA was omitted; meanwhile bFGF and MPLA were omitted for control liposomes (L). These vaccines were redissolved in 1 ml PBS for use. Positive control vaccine (FB) was prepared as follows: the mixture of recombinant bFGF peptide (dissolve in 0.8 ml PBS) and 0.2 ml complete Freund's adjuvant (Sigma Chemical Co, Inc., St. Louis, MO) was injected to mice for the first immunization; while the mixture of bFGF (dissolve in 0.8 ml PBS)