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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)

and 0.2 ml incomplete Freund's adjuvant was used for the rest 5 boosters.

Particle size analyzer (Malvern, British) was used to study the morphological features and mean diameter of liposomes. Free liposomes and free peptide were separated through medium-pressure silica gel column chromatography. The quality of free bFGF was determined by BCA Protein Assay Kit (Pierce Biotechnology). The encapsulation efficiency was calculated as (1 − free bFGF/total bFGF) × 100%.

### 2.4. Immunization and tumor models

C57 mice were randomly divided into 5 groups (5 mice for each group) and injected in both quadriceps of hind legs with liposomal vaccines containing mixture of bFGF and monophosphoryl lipid A (MLB), bFGF alone (LB), liposomes alone (L) and 0.005 mol/l PBS (PBS), respectively, once every 2 weeks for six times. For the positive control group (FB), mice were vaccinated with the mixture of bFGF peptide and complete Freund's adjuvant for the first immunization and then with the mixture of bFGF peptide and incomplete Freund's adjuvant for the rest 5 boosters, at the same time as other groups.

### 2.5. Determination of antibody titer

Blood was collected from the tail vein of mice before each vaccination as well as when mice were sacrificed. Sera were obtained through centrifugation at 4000 × *g*, 4 °C, for 10 min. Then anti-bFGF-specific antibody titer was measured via ELISA. Antigen bFGF was dissolved in carbonate buffer (50 nM, pH 9.6) (bFGF concentration was 20 μg/ml, 100 μl/well). 96-Well plates were coated with recombinant bFGF. Then, plates were incubated overnight at 4 °C and blocked with 5% nonfat dry milk and 0.05% Tween 20 in PBS (PBS/T), 1 h at 37 °C. Plates were washed 3 times with PBS/T, and incubated with serial dilutions of anti-sera from vaccinated animals (L, LB, FB, MLB group) in PBS/T for 2 h at 37 °C. Naive mouse serum was used as negative control. Plates were washed 5 times with PBS/T and then incubated with HRP labeled secondary antibody Protein A (20 μg/ml, 100 μl/well), 1 h at 37 °C. Plates were washed again, and wells were incubated with TMB developer (Nanjing Jiancheng Biotechnology, China) for 20 min. Then O.D. value was read at 405 nm by the Spectramax M5 Microtiter Plate Luminometer (Molecular Devices, USA).

Each plate also contained some wells without antigen that treated with serum and secondary antibody. Briefly, naive mouse sera (the titer was regarded as 1) and anti-sera of mice treated with empty cationic liposomes had equivalent reactivity values compared with that in empty wells. Antibody titer was defined as the reciprocal of the serum dilution giving an O.D. reading of two standard deviations above the mean O.D. for normal mouse serum.

### 2.6. Experimental pulmonary metastasis models and histological analysis

One week after the sixth immunization, C57 mice (5 groups, group size = 5) were injected with $1 \times 10^5$ LL/2 cells in tail veins. 3 Weeks after the challenge, mice were sacrificed and lungs were obtained. Wet lungs were weighed and then were fixed with 10% buffer formalin. Tissues were embedded in paraffin through routine ways and stained with hematoxylin and eosin (H&E).

### 2.7. In vitro spleen cell responses

The cytokine IFN-γ and IL-4 in supernatant of cultured splenocytes, indicating Th1 and Th2 immune response, respectively, was measured through ELISA method (Khajuria et al., 2007; Mosmann and Coffman, 1989). In order to investigate the type of the major immune response this vaccine mediated, cytokines secreted by mice splenocytes was measured *in vitro* by ELISA (Jaafari et al., 2005). Mice from each group were sacrificed at 2 weeks after the last booster, spleens were aseptically removed quickly and then tissues were triturated and homogenized to a single-cell suspension. The ammonium chloride was used to disrupt erythrocytes. The splenocytes were washed and suspended in complete medium (RPMI 1640) twice and seeded at $1 \times 10^6$/ml in 96-well plates and then incubated at 37 °C in 5% $CO_2$ until they were stable. The spleen cells were stimulated with either bFGF (10 and 20 μg/ml) or Con A (10 and 20 μg/ml) or medium alone at 37 °C in 5% $CO_2$ incubator (3 repeated wells). Suspensions were collected and supernatants were obtained by centrifugation. The concentration of IL-4 and IFN-γ were determined using ELISA method according to the manufacturer's instructions (Sigma Chemical Co, Inc., St. Louis, MO), and the OD values were measured at 450 nm by the Spectramax M5 Microtiter Plate Luminometer (Molecular Devices, USA). This experiment was repeated twice using splenocytes from different mice immunized in the same way.

### 2.8. Statistical analysis

SPSS 16.0 was used for statistical analysis. Data of five experimental groups (group size = 5) was calculated via this software. The statistical significance of results in all of the experiments was determined by Student's *t*-test and ANOVA. The findings were regarded as significant if $P < 0.05$.

## 3. Results

### 3.1. Characterization of recombinant bFGF

After two-step chromatography, the purity of recombinant bFGF can be achieved up to 99%. The Western blotting results confirmed the characterization of recombinant bFGF and the purity of recombinant bFGF was observed through SDS-PAGE (Fig. 1).

### 3.2. Characterization of liposomes

Liposomes in this research were small unilamellar vesicles morphologically, heterogeneous in size with mean diameters 148.7 ± 77.1, 173.5 ± 82.9 and 100.2 ± 71.6 nm for the LB, MLB and L, group, respectively. Zeta electric potential of LB and MLB was measured between +40 and +60 mV. The encapsulated efficiency of LB and MLB was about 50%. To facilitate following investigation, the concentration of bFGF was adjusted to 20 μg/100 μl after purification and calculation of encapsulation efficiency.

### 3.3. Antibody titer analysis

bFGF peptide was injected at the dose of 20 μg each time and the final dose amounted to 120 μg. The ELISA result confirmed that the vaccinated mice including LB, MLB and FB groups were able to elicit potent immunoreactivity to bFGF (Fig. 2). In contrast, antibody was not detected in L and PBS group (their data were too low to be shown). The bFGF-specific immune response could be induced by neither 0.005 mol/l PBS nor equivalent empty liposomes as the LB and MLB group. MLB vaccine significantly increased the antibody titer of IgG, compared with the control group ($P < 0.01$). Furthermore, antibody titer of MLB vaccine was statistical higher than that in LB group ($P < 0.05$). FB vaccine also induced effective immune response nevertheless the antibody titer was lower than MLB group ($P < 0.05$). This important result indicated that MLB vaccine induced a more effective immune response.

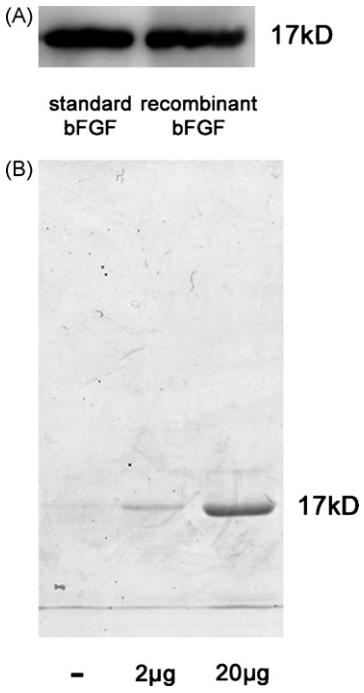


**Fig. 1.** Characterization of recombinant bFGF peptide and SDS-PAGE. (A) Characteristics of recombinant bFGF was confirmed by Western blot. (B) Purity of recombinant bFGF peptide was analyzed via SDS-PAGE. From left to right, the lanes were blank with loading buffer alone, 2 μg bFGF and 20 μg bFGF, respectively.

### 3.4. Inhibition of pulmonary metastasis

As can be seen in Fig. 3A, tumors grew progressively in nonimmunized mice. Tumor metastasis was partly inhibited in LB group. There was a significant inhibition of tumor growth in MLB group and FB group. Meanwhile, lung weight of each group also showed evident differences (Fig. 3B). The lung weights of vaccinated groups were obviously lower than that of PBS group and L group. The lung weight in MLB group showed the lowest compared with the LB group and FB group ($P < 0.05$). These results indicated that liposomes containing MPLA was even more effective than Freund's adjuvant as a role of immunological adjuvant.

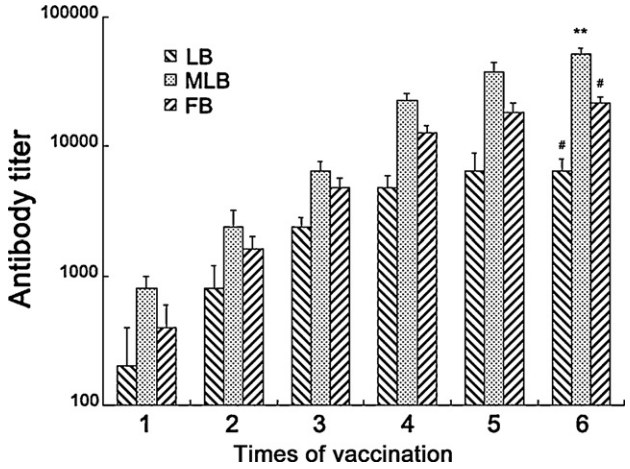


**Fig. 2.** Titer of total bFGF-special IgG created by LB, MLB and FB vaccine. Mice were vaccinated once every 2 weeks for six times in total. Sera were collected before each vaccination as well as when mice were sacrificed and IgG was analyzed by ELISA. Titers in PBS and L group were not shown because they were much lower than 100. MLB vaccine significantly increased the IgG titer, compared with PBS group and L group ($^{**}P < 0.01$). Meanwhile, it also elicited a greater humoral immunity, compared with LB group and FB group ($^{\#}P < 0.05$). Column, mean titer. Bar, S.E.M. Each column represents the mean ± S.E.M ($n = 5$).

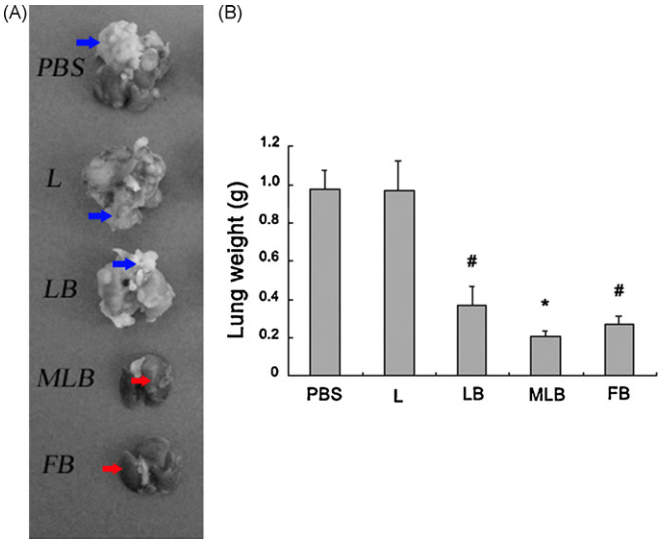


**Fig. 3.** Lung tissue and lung weight from each group 3 weeks after challenge with LL/2 tumor cells. (A) Lung tissues. The rows of lungs from bottom to top were lungs from FB, MLB, LB, L and PBS group mice, respectively. Tumor pulmonary metastasis was significantly repressed in FB group and MLB group. Meanwhile, no protection against tumor metastasis could be observed in control groups L and PBS group. (B) Lung weights 3 weeks after challenge. The lung weight of MLB group was significantly low as a result of tumor metastasis inhibition, compared with the control groups ($P < 0.05$). Furthermore, and the lung weight from MLB group was obviously lower than that of LB group ($P < 0.05$) and even lower than that of FB group in statistical ($P < 0.05$). Column, mean lung weight. Bar, SD. Each column represents the mean ± SD ($n = 5$). Blue arrows show tumor tissue; red arrows show normal lung tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

According to H&E staining results (Fig. 4), most microscopic areas were covered with tumors in PBS group and L group. Obvious tumor cells could also be detected in LB group but fewer than those in PBS and L group. In contrast, only few tumor cells could be observed and major pulmonary alveoli were in normal condition in MLB group and FB group. Apparently, tumor growth was effectively inhibited by these bFGF vaccines containing liposomes plus MPLA and Freund's adjuvant.

### 3.5. In vitro cytokine production by splenocytes

The measurement of IFN-γ and IL-4 level was repeated twice and results came out similar. The levels of IFN-γ in the MLB, FB and LB group were significantly higher than the other two groups (stimulated with either 10 or 20 μg/ml bFGF) (Fig. 5). IFN-γ level was evidently higher in MLB group compared with control groups ($P < 0.05$) and LB group ($P < 0.05$). However the level of IL-4 was not statistically different among all groups. The up-regulated IFN-γ production revealed that this vaccine preferentially induced Th1 type immune response.

## 4. Discussion

Several observations have been made in present study concerning the immunization with liposomal vaccine containing bFGF peptide and MPLA, antitumor immunity, and autoimmune response. The present study has demonstrated that cationic liposomes encapsulated human bFGF as vaccine can effectively induce antitumor immunity. Specific anti-bFGF antibody in sera was identified. IgG was substantially increased in response to xenogeneic bFGF vaccine. Tumor growth was significantly suppressed in the experimental pulmonary metastasis model. In addition, our find-

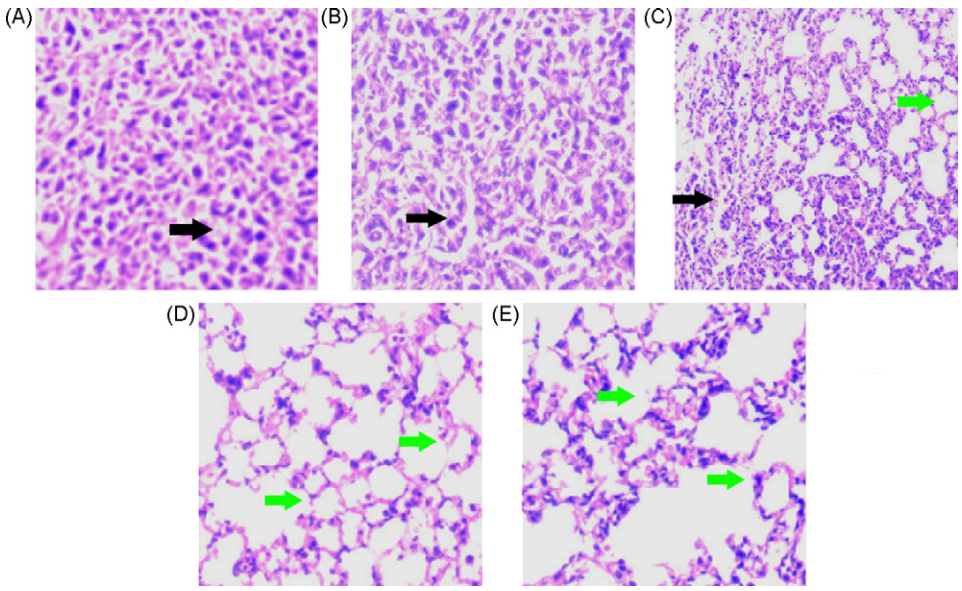


**Fig. 4.** H&E staining of lung tissues (400× magnification). (A) PBS group; (B) L group; (C) LB group; (D) MLB group; (E) FB group. Tumor cells were observed in large areas of lungs in PBS group and L group. Tumor growth in LB group was obviously inhibited. Moreover, lung alveoli were well protected and few tumor cells could be observed in MLB and FB group. The results suggest mice in these two groups had better antitumor activity. Black arrows show tumor cells; green arrows show normal lung architecture. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ings suggested that this bFGF vaccine mediated preferentially a more potent Th1 type immunity response related with cellular immunity. The humoral immunity and cellular immunity may be responsible for the antitumor activity by the vaccination of bFGF vaccine. Furthermore, our observations revealed that MPLA could potently enhance the bFGF vaccine efficacy by comparing results from vaccination with MLB vaccine and LB vaccine. The bFGF vaccine containing MPLA exhibited even better in inducing autoantibody and inhibiting tumor metastasis than the bFGF vaccine using the Freund's adjuvant. Taken together, these findings suggested that this xenogeneic human bFGF vaccine successfully induced both humoral and cellular immune response that may contribute to the antitumor immune activity against tumor lung metastasis.

As described in Fan et al.'s review (Fan et al., 2006), excessive angiogenesis occurred in cancer when some kind of angiogenesis stimulators abnormally derived from diseased cells, such as vascular endothelial growth factor, basic fibroblast growth factor and hepatocyte growth factor, overwhelmed the effects of natural angiogenesis inhibitors (e.g. angiostatin, endostatin and thrombospondin). Then, tumor cells escape into the circulation through new vessels and metastasize to other organs. Therefore it has been a novel and feasible strategy to treat or prevent cancer by inhibiting the angiogenesis procedure. Many previous studies discovered that angiogenesis exhibited an important role in tumor metastasis and made it successful to suppress tumor progression via antiangiogenesis therapy (Ren et al., 2007; Chandru et al., 2007; Fang et al., 2007; De Palma et al., 2007). In the present study, we chose basic fibroblast growth factor, the indispensable stimulator in angiogenesis course, as the target, since it was significantly over-excreted by carcinoma cell during tumor metastases formulation (Nyberg et

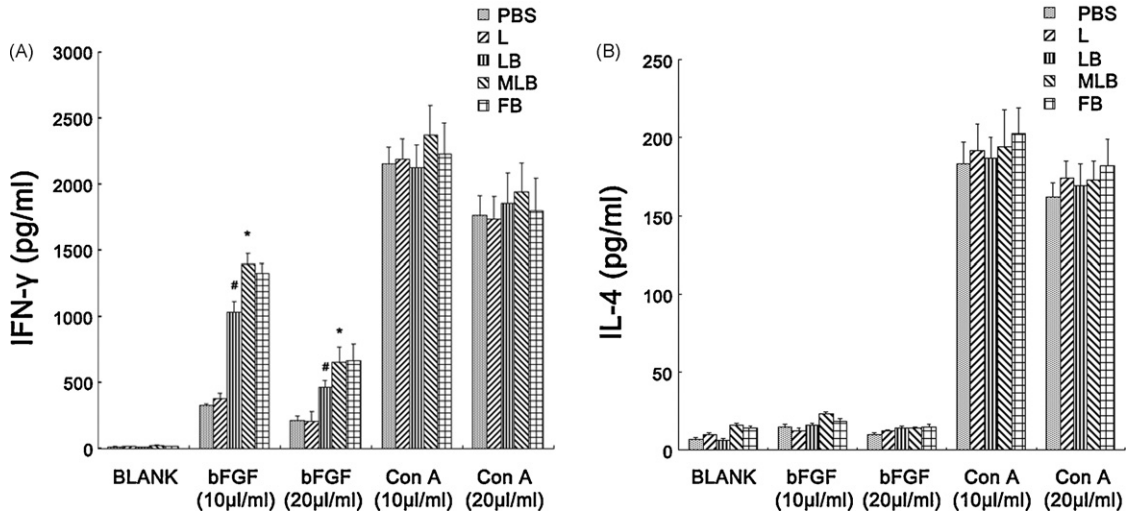


**Fig. 5.** Levels of IFN-γ (A) and IL-4 (B) were assessed by ELISA. The splenocytes cultured *in vitro* were stimulated with bFGF (10 and 20 μg/ml), concanavalin A (10 and 20 μg/ml, positive control) and normal saline, respectively. Columns with different marks represented levels of cytokine production in different groups, as shown in the figure. There are no significant differences in the level of IL-4, however, IFN-γ level of MLB group increased significantly when splenocytes were stimulated by recombinant bFGF ($^{*}P < 0.05$, compared with PBS group or L group; $^{\#}P < 0.05$, compared with LB group). Column, mean. Bar, SD. Each column represents the mean ± SD ($n = 5$), different columns represent different groups as shown in the figure.

al., 2005). Besides, FGF family has been well known to be conserved in the evolution procedure. High similarity between human bFGF and murine bFGF peptide provided a possibility of producing a vaccine based on xenogeneic immunogen to elicit cross-reactivity. In our study, the vaccine based on human bFGF successfully broke the immunity tolerance against bFGF and induced bFGF-specific antibody in mice and the subsequent antitumor activity was effective. Moreover, we could estimate that it will be interesting to produce a vaccine for human therapy with murine bFGF or human bFGF. Maybe a vaccine based on human bFGF could induce even more efficient antitumor activity *in vivo* due to higher similarity between immunogen and self-molecule.

Immunogenicity, with which antigens stimulate spontaneous immune response, is an important part to consider when an efficient vaccine strategy is planned. Liposome vehicle has been proved as an effective way to avoid rapidly clearance and short circulation time and to significantly prolong the plasma residence time of drugs. Thus it has been widely used in vaccine production for it enhances immunogenicity of antigens including recombinant peptides (Wang et al., 2007; Lee et al., 2002; Tardi et al., 2000; Emerson et al., 2000; Boman et al., 1995). MPLA, a well known adjuvant, increased efficacy of several liposomal vaccines based on protein antigens (McElrath, 1995; Harris et al., 1999; Samuel et al., 1998; Gordon et al., 1995; Alonso et al., 2004). In current study, vaccine based on bFGF peptide plus MPLA (MLB) encapsulated in liposomes also efficiently increased antibody titer and the humoral immunity was quite stable during the vaccination period. The result demonstrated that cationic liposomes and MPLA were supposed to be feasible to enhance immunogenicity of recombinant bFGF and it also suggested a potential application for other recombinant peptides. The effect from MPLA was too important to ignore. Liposomal bFGF + MPLA vaccine increased the endpoint of antibody titer, compared with liposomal bFGF vaccine without MPLA. Moreover, the MLB vaccine could induce even more active immune response compared with FB vaccine containing Freund's adjuvant. Freund's adjuvant was considered an effective adjuvant to use in the vaccine production; unfortunately, it had been proved toxicity to human in clinical trials. Our findings suggested another potential adjuvant for vaccine because liposomes have been well known for its characteristics of being safer and more friendly to people. By the way, we did not detect any toxicity of liposomes by examining mice receiving empty liposomes.

We immunized mice and gave them a subsequent challenge with LL/2 tumor cells in order to examine the antitumor activity from vaccines. The challenge results showed tumor growth in each group were apparently different. Tumor tissues grew progressively in nonimmunized mice and made lungs heavier than normal lungs. Normal structure of lung alveoli was badly destroyed and the host was deprived of normal respiration. In contrast, the metastasis in MLB group was obviously inhibited and the lungs were well protected. It suggested that the vaccine aiming at inhibiting the angiogenesis stimulator bFGF provided an effective prevention against tumor metastasis. Our findings support the previous study about enhancing antitumor activity with a vaccine based on VEGF peptide (Wang et al., 2007) and prove that blocking angiogenesis stimulator via immunotherapy method is feasible with regard to angiogenesis-dependent cancer therapy. Early in 2003, a humanized monoclonal antibody against VEGF-A, bevacizumab, was first used in clinical trials to inhibit tumor angiogenesis and prolong survival time in patients with metastatic colorectal cancer (Kabbinavar et al., 2003). The present study might provide a novel strategy of antiangiogenesis therapy for future cancer immunotherapy.

IFN-γ and IL-4 were the indicative cytokines of Th1 and Th2 immune response, respectively. In Mazumdar et al.'s research, the liposomal vaccine stimulated the Th1 immune response preferentially (Mazumdar et al., 2004). Our findings were similar to his. IFN-γ, the indicating cytokine in Th1 immune response, was significantly up-regulated by the vaccines. In contrast, IL-4, regarding the Th2 immune response, retained in a low level. Th1 immune response, correlated with cellular immunity, plays an important role in tumor immunity. It was reported that IL-18 could enhance Th1 immunity and protection against tumor (Marshall et al., 2006). In this research, the liposomal vaccine induced a more potent Th1 immunity preferentially and potentialized the cellular immunity, which was partially responsible for the whole antitumor activity.

In summary, the findings in the present study may provide a new vaccine strategy for preventing tumors through inducting the autoimmune response against bFGF by the immunization with bFGF vaccine, and may be of importance to the further exploration of the role of the breaking of immune tolerance to self-proteins through the cross-reaction between xenogeneic homologous and self-molecules in cancer therapy. Our study may also suggest a way to make a new adjuvant to substitute Freund's adjuvant and it may be possible to be adopted in future clinical trials.

## Acknowledgement

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