A novel combined conjugate vaccine: Enhanced immunogenicity of bFGF with CRM197 as a carrier protein

HAI-LONG ZHANG[1*], CHUANG YUAN[1*], DONG-MEI ZHANG[1], HUA-SHAN SHI[1],
MENG LI[1], ZI-CHAO LUO[2], YANG WAN[1], LIAN LU[1], SHUN-TAO LUO[1] and LI YANG[1]

[1]State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School,
Sichuan University; [2]School of Life Science, Sichuan University, Chengdu, Sichuan, P.R. China

Abstract. Tumor growth is partly dependent on tumor-associated angiogenesis, which is regulated by angiogenic growth factors. As the first angiogenic growth factor to be identified, basic fibroblast growth factor (bFGF) plays a major role in angiogensis and tumor growth and has been an effective target for anti-tumor therapy. However, due to its low immunogenicity, injection with bFGF alone cannot stimulate the body to produce a strong immune response. In this study, we investigated the role of CF (containing bFGF and CRM197) assisted by CpG and alum in enhancing antigen-specific immune response and suppressing the growth of murine colon carcinoma. The results revealed that compared to bFGF, CF could not stimulate NIH-3T3 fibroblast proliferation even at a concentration of 10 μg/ml *in vitro*. *In vivo*, the CF-CpG-alum produced a stronger antigen-specific immune response and inhibited tumor growth. The anti-tumor activity was associated with generating antigen-specific antibody, suppressing angiogenesis, promoting the apoptosis of tumor cells and inducing the mixed Th1 and Th2 responses. This indicates that CRM197 may be an innovative intramolecular adjuvant and provides a rational preservation for mouse CT26 colon carcinoma.

Correspondence to: Professor Li Yang, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Keyuan Road 4, Chengdu, Sichuan 610041, P.R. China
E-mail: yl.tracy73@gmail.com

*Contributed equally

Abbreviations: bFGF, basic fibroblast growth factor; CRM197, nontoxic mutant of diphtheria toxin; NIH-3T3, mouse embryonic fibroblast cell; CT26, mouse colon carcinoma cell; VEGF, vascular endothelial growth factor; TGF-α/β, transforming growth factor-α/β; TNF-α, tumor necrosis factor-α; PVDF, polyvinylidene difluoride; MVD, microvessel density; PBS, phosphate buffered saline

Introduction

Angiogenesis is the process of new vascular network formation from existing vessels (1). This process not only plays a key role in various physiological activities but also occurs in pathological conditions, such as embryonic development, wound healing, rheumatoid arthritis and diabetic retinopathy (1,2). Accordingly, tumor growth is partly dependent on angiogenesis or neovascularisation (3,4). Antiangiogenesis may therefore be an effective strategy of antitumor treatment through inhibiting tumor growth (5-8).

Tumor angiogenesis is a multi-step process, which involves endothelial and smooth muscle cell proliferation and migration, formation of new capillaries, and extracelluar matrix turnover (9,10). Certain studies have indicated that a host of diffusible factors produced by tumor cells mediate these processes (11,12). Since then, an increasing number of angiogenic cytokines have been identified, including basic fibroblast growth factor (bFGF), the vascular endothelial growth factor (VEGF) family, angiopoietins, interleukin-8, transforming growth factor-α and -β (TGF-α and -β) and tumor necrosis factor-α (TNF-α) (13-15).

Basic fibroblast growth factor (bFGF), one of the first angiogenic growth factors to be identified, is a single-chain soluble heparin-binding polypeptide (16,17). It induces the migration and proliferation of endothelial cells and smooth muscle cells and the formation of vascular tubes and networks. In addition, evidence that bFGF plays an important role in angiogenesis has been provided by numerous experiments (18-20). It interacts with signaling FGFRs, the extracellular matrix and certain free molecules, and influences the initial period of angiogenesis and tumor growth (15,21). Based on these characteristics, bFGF could be used as a target of tumor therapy. However, due to its short half-life *in vivo* and low immunogenicity, the biological effect of bFGF is limited and it cannot stimulate the body to produce a strong immune response alone (22). Therefore, a study was carried out targeting a specific immune response to the bFGF molecule for the endogenous control of tumor growth (23).

CRM197 is a nontoxic mutant of diphtheria toxin and is immunologically indistinguishable from the native molecule. CRM197 is not only capable of inhibiting ovarian cancer as a specific inhibitor of HB-EGF (24,25), but also of enhancing

immunogenicity with capsular polysaccharides (PSs) as a carrier protein to prevent infection (26). As a carrier protein, CRM197 induces a rapid increase in Th1- and Th2-secreting T cells, which produce heterogeneous cytokines to activate B cells and regulate the quantity of PS antibody (27,28).

CpG is a type of bacterial DNA or synthetic oligodeoxy-nucleotide containing immunostimulatory CpG motifs. CpG DNA can induce the majority of (>95%) B cells to proliferate and predominantly trigger mixed IgG1/IgG2a secretion (29). Moreover, CpG DNA also directly activates monocytes, macrophages and dendritic cells to secrete a host of cytokines. These cytokines stimulate NK cells to secrete IFN-γ, which promotes B cell activation and Ig secretion (30). Overall, CpG DNA modulates immune functions by activating lympho-cytes, and by modulating Th1/Th2 balance (31).

In this study, we describe that the fusion protein, CF, which conjugates bFGF with CRM197, together with CpG and alum as an adjuvant, enhances the immunogenicity of bFGF and inhibits tumor angiogenesis and growth.

Materials and methods

Cell and cell cultures. Murine colon carcinoma cell line (CT26), purchased from the American Type Culture Collection (ATCC), was cultured in RM1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS and 100 μg/ml of amikacin and maintained in a humidified chamber at 37°C in a 5% CO_2 atmosphere.

Construction of recombinant plasmid. According to the cDNA sequences of CRM197 and hbFGF (28bp-438bp) in GenBank, we designed the corresponding primers that contain the appro-priate restriction sites (CRM197, 5'-GCTCTAGAAATAATTT TGTTTAACTTTAAGAAGG-3' and 5'-GGAATTCGCTTTT GATTTCAAAAAACAGCGAC-3'; hbFGF, 5'-GGAATTCCC GCCTTGCCCGAGGATGGC-3' and 5'-CCCAAGCTTATC AGCTCTTAGCAGACATTGGAAG-3'). Then, the cDNA fragments coding the CRM197 or hbFGF genes were amplified by PCR and subsequently subcloned into the pET-32a(+) vector (Novagen). The expressed recombinant protein, CF, consisted of CRM197 and hbFGF (10aa-155aa) (Fig. 1).

Protein expression and purification. *Escherichia coli* cells [BL21 (DE3) strain] harboring pET32a-CF were grown in 4 l of LB medium at 37°C with the selection marker, ampicillin (100 μg/ml). When the OD600 of the culture reached 0.6-0.8, the isopropyl-b-D-thiogalactopyranoside was added into it with the final concentration of 0.1 mM and the culture was maintained at 30°C for 16 h. Approximately 20 g (wet weight) of cells were harvested by centrifugation at 4500 x g and 4°C for 30 min. After being washed with ice-cold buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0), the cells were resuspended in 200 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, pH 8.0). The suspension was broken with a high-pressure homogenizer at a pressure of 600 Bar and then centrifuged at 70,000 x g for 30 min at 4°C. The inclusion bodies were obtained and then washed twice with 100 ml of buffer A (20 mM Tris–HCl, pH 8.0, 4 M urea, 20 mM β-ME). Then, the inclusion bodies were solubilized with 50 ml of buffer B (20 mM Tris–HCl, pH 8.0, 8 M urea,
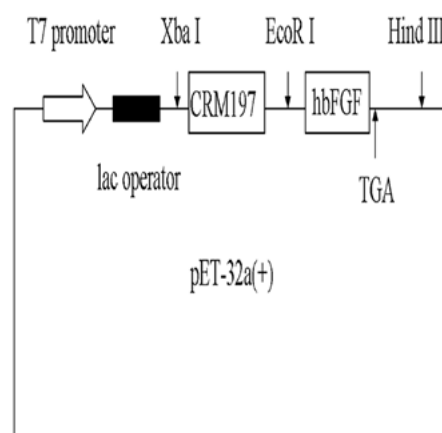

Figure 1. Construction of a vector for the expression of CF. The cDNA frage-ments coding the CRM197 or hbFGF genes were amplified by PCR and cloned into the pET-32a(+) vector. The fusion protein consisted of CRM197 and hbFGF (10aa-155aa).

20 mM β-ME) and subsequently centrifuged at 70,000 x g for 30 min at 4°C. The supernatant was slowly dropped into 1 l of refolding buffer, and then stirred continuously for 48 h. The refolding sample was concentrated by ultrafiltration and the buffer was transferred to buffer C (20 mM Tris-HCl, pH 8.0). The refolding solution was loaded onto an SP chelating sepharose (GE Healthcare Bio-science) column equilibrated with binding buffer (20 mM Tris-HCl, pH 8.0). The column was subsequently washed with the binding buffer and further washed with buffer D (20 mM Tris-HCl, 100 mM NaCl, pH 8.0). The absorbed proteins were eluted with the buffer E, in which the NaCl concentration was 300 mM. Eventually, the protein solution was dialyzed with 10 mM PBS.

Western blot analysis. Western blot analysis was used to confirm the characterization of CF. To start with, the purified CF was electrophoresed in 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA, USA). After being washed with PBST, the membrane was blocked with 5% skimmed milk powder at 4°C overnight. Then the blots were incubated with bFGF-immunized mouse serum or CRM197-immunized rabbit serum plus the secondary antibody, HRP-protein A (1:3000, Cabliochem, Darmstadt, Germany). Five washes (5 min per wash) were carried out after each incubation step. The protein bands were visualized via an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA).

Animal experiments. Female 8-week-old Balb/c mice were purchased from the West China Experimental Animal Center and housed at standard conditions: 22±2°C temperature, 55±5% relative humidity and pathogen-free cages. Mice were accommodated for 1 week prior to immunization.

Groups of mice (n=5) were immunized with CpG (20 μg/mouse) and alum mixed with equal volumes of various proteins: i) CRM197 (20 μg/mouse), ii) bFGF (20 μg/mouse), iii) CF (25 μg/mouse) in PBS, or iv) with PBS alone. Mice received a subcutaneous injection (100 μl) into both fold inguen once every two weeks (3 times in total), and the day of the first immunization was considered as

day 0. Each mouse was challenged subcutaneously with $3x10^5$ CT26 cells in the right flank on day 36. Following this, the tumors were palpable on day 44 and the tumor dimensions were measured with a vernier caliper every 3 days. The tumor volume was calculated by the standard formula (widh x width x length x 0.52). On day 62, the mice were sacrificed. Their organs (heart, liver, spleen, lung and kidney) and tumors were removed and fixed in 4% paraformaldehyde in PBS for histological analysis. All experiments involving mice were approved by the institute's Animal Care and Use Committee.

Detection of anti-bFGF antibodies and IgG subclasses in blood. Blood samples from immunized mice were obtained once every two weeks during the immunization period and stored at -80˚C. The anti-bFGF antibodies were measured in enzyme-linked immunosorbent assay (ELISA). Microwell plates (Dynatech Laboratories, Chantilly, VA, USA) were coated with 100 μl of 0.5 μg bFGF in 50 mM carbonate/bicarbonate buffer (pH = 9.6) at 4˚C overnight. The coated plates were washed three times with PBS-Tween wash buffer and then incubated with a 5% solution of non-fat dried milk in PBS-Tween for 1 h at 37˚C. After being washed five times with PBS-Tween, the plates were coated with 100 μl of plasma (10-time dilution): 1:10, 1:100, 1:1,000 and 1:10,000 for 1 h at 37˚C. After washing the plates five times with PBS-Tween, the assay was performed using peroxidase-conjugated affinipure goat anti-mouse IgG (ZSGB-BIO, Beijing, China) incubated for 1 h at 37˚C. After five washes with PBS-Tween, the substrate was developed with 3, 3, 5, 5-tetramethylbenzadine (Sigma-Aldrich). The colorimetric reaction was stopped with 0.5 M H_2SO_4 25 min later. The absorbances were read by an ELISA plate reader (Thermo LabSystems, Franklin, MA, USA) in 450 nm.

As mentioned above, the IgG subclasses were identified using HRP-conjugated anti-IgG1, anti-IgG2a and anti-IgG2b as secondary antibodies.

Histological analysis. Primary tumors were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and cut into 4-μm sections. The apoptotic cells within the tumor sections were evaluated by dead-end colorimetric terminal deoxynucleotidyl transferase-mediated dUTP Nick-End labeling (TUNEL) System (Promega, Madison, WI, USA) following the manufacturer's protocol. Apoptotic index was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field (5 high-power fields/slide).

The quantification of microvessel density (MVD) was assessed by the method of Weidner *et al* (32). The procedure of immunostaining for CD31 has previously been described in detail. The following antibodies and reagents were used: goat anti-mouse CD31 mAb (1:200, Santa Cruz Biotechnology, CA, USA), biotinylated polyclonal rabbit anti-goat (1:100, Santa Cruz Biotechnology), ABC Kit (Vector Laboratories, Peterborough, UK) and DAB substrate kit (ZSJQ-BIO, Beijing, China).

Toxicity observation. Drug toxicity indices including weight loss, ruffled fur, diarrhea, anorexia, skin ulcerations and toxic death were closely observed after the first immunization. After being fixed in 4% paraformaldehyde in PBS for more than 24 h, the tissues of heart, liver, spleen, lung, and kidney were embedded in paraffin and sliced. Sections were stained with hematoxylin and eosin and observed using the double blind method.

Statistical analysis. SPSS 16.0 was used for statistical analysis. The statistical analysis in each experiment was performed using one-way analysis of variance or the unpaired Student's t-test. The difference was regarded as significant if $P<0.05$.

Results

Expression and purification of the fusion protein. After transformation of the BL21 (DE3) bacterium, the recombind plasmid was induced with IPTG and expressed the fusion protein as an inclusion body (75 kDa). The fusion protein, which had been solubilized with a buffer (20 mM Tris-HCl, pH 8.0, 8 M urea, 20 mM β-ME) was refolded and purified with an SP chelating sepharose column (Fig. 2A). Meanwhile, the fusion protein was authenticated with Western blotting (Fig. 2B). Moreover, the proliferative bioactivity of CF was measured in NIH-3T3 cells. CF could not stimulate NIH-3T3 fibroblast proliferation even at a concentration of 10 μg/ml (Fig. 2C).

Detection of anti-bFGF antibodies and IgG subclasses in blood. The mice were immunized with CRM197-CpG-alum, bFGF-CpG-alum, CF-CpG-alum and PBS on days 0, 14 and 28. The blood of the mice was obtained on days 7, 21, 35 and 49, and the sera were used to analyze anti-bFGF antibodies by ELISA. Mice immunized with CRM197-CpG-alum and PBS did not dispay immunoreactivity to bFGF. However, the antibody did react against bFGF from days 7 to 49 in the mice from the other groups. The CF-CpG-alum group displayed a stronger immune response to bFGF from the first week ($P<0.05$) (Fig. 3A). We then analyzed the positive proportion of immunoreactivity to bFGF in each group. Immunoreactivity to bFGF was exhibited by 40% of mice immunized with CF-CpG-alum in the first week, whereas it was not observed in the bFGF-CpG-alum group. The latter achieved 20% positive proportion by the third week and 100% positive proportion by the seventh week, although the CF-CpG-alum group achieved 100% by the third week, with a lower bFGF content compared to the bFGF-CpG-alum group (Fig. 3B).

Finally, IgG subclasses in the blood were analyzed in the fifth week. The results showed that CF-CpG-alum could observably enhance the secretion of IgG1 and IgG2a in contrast to bFGF-CpG-alum (Fig. 3C).

Anti-tumor effect of CF-CpG-alum in vivo. A murine colon carcinoma model was established to examine the anti-tumor efficacy of CF-CpG-alum *in vivo*. Mice were challenged with CT26 cells following the final immunization with CRM197-CpG-alum, bFGF-CpG-alum, CF-CpG-alum or PBS. Alterations in tumor growth were monitored every 3 days (Fig. 4A). On day 26 after the CT26 cells were challenged, the
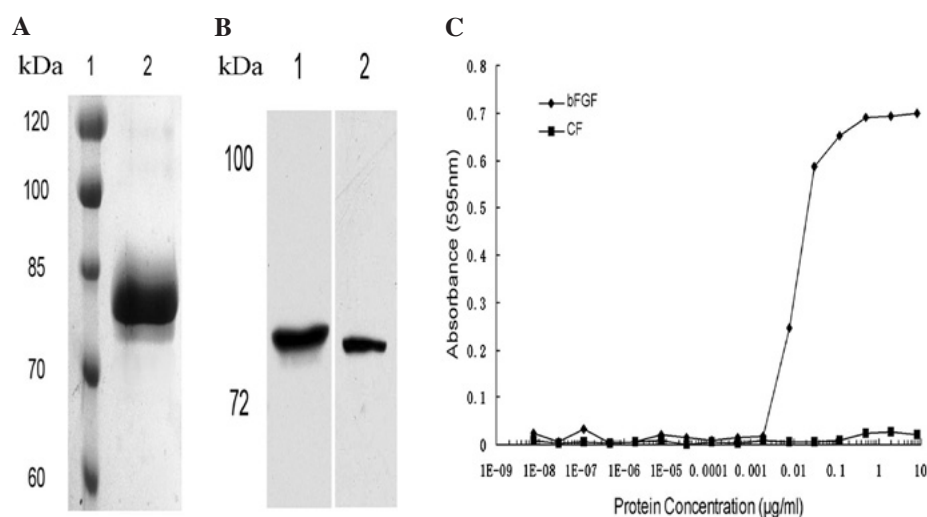
Figure 2. Expression, purification and verification of recombinant protein (CF). (A) SDS-PAGE analysis of the purification of CF expressed as a single band of 75 kDa (lane 2) compared with the marker (lane 1). (B) Western blot analysis of the characterization of CF using anti-CRM197 (lane 1) and anti-bFGF (lane 2). (C) Bioassay of CF compared with bFGF.
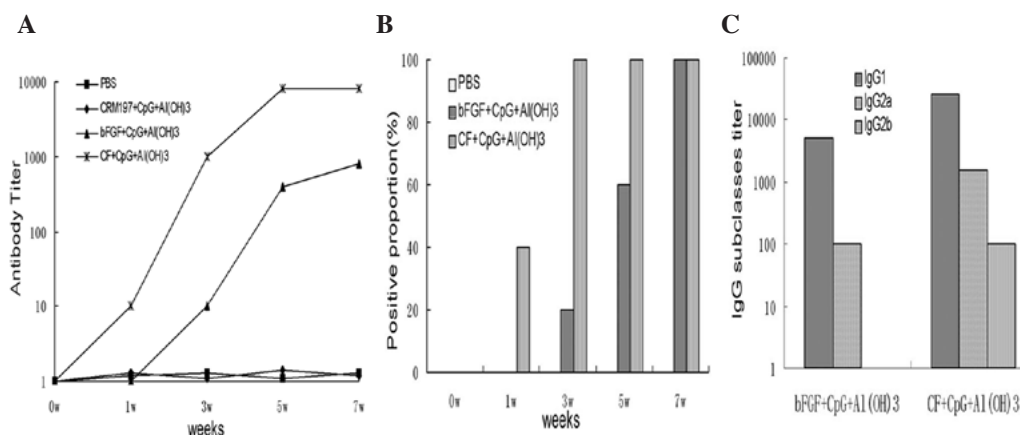

Figure 3. Detection of anti-bFGF antibodies, positive proportion and IgG subclasses. The mice were immunized with corresponding drugs on days 0, 14, and 28. The blood was subsequently obtained on days 7, 21, 35 and 49. Then, the sera were used to analyze (A) the anti-bFGF antibodies, (B) positive proportion and (C) IgG subclasses by ELISA.

mice were sacrificed and the tumor weights were measured (Fig. 4B). The CF-CpG-alum group exhibited effective inhibition of tumor growth, compared to the other groups (P<0.01). Furthermore, we observed that immunization with CF-CpG-alum resulted in average tumor weight reductions of 82.5%, 77.7% and 71.4% compared to PBS, CRM197-CpG-alum and bFGF-CpG-alum, respectively (P<0.01). These results indicate that CF-CpG-alum can effectively suppress tumor growth.

Increase of apoptosis and inhibition of tumor-induced angiogenesis in vivo. In order to estimate the apoptosis in tumor tissues, the tumor sections were stained with TUNEL reagent (Fig. 5A). Marked necrosis was observed in the CF-CpG-alum group (data not shown). Meanwhile, substantially more apoptotic cells were observed in the tumor sections of mice immunized with CF-CpG-alum than with PBS, CPM197-CpG-alum or bFGF-CpG-alum. The apoptotic index also revealed that the CF-CpG-alum displayed the highest indices

among all the groups (Fig. 5B). The MVDs of tumor sections were analyzed to determine whether the increased apoptosis was associated with anti-angiogenesis in mice immunized with CF-CpG-alum. Each tumor section was stained with anti-CD31 antibody (Fig. 6A). Tumors of the control groups (including PBS, CRM197-CpG-alum and bFGF-CpG-alum groups) exhibited larger microvessel counts than those of the CF-CpG-alum group (Fig. 6B). These results indicate that the decreased tumor volumes of the CF-CpG-alum group may be caused by increased apoptosis, which was associated with antiangiogenesis.

Toxicity. In order to evaluate the potential side-effects of the drugs, the toxicity assessment was performed. No marked differences were observed in the gross measures among the groups (data not shown). Moreover, no pathological changes were detected in the heart, liver, spleen, lung, or kidney (data not shown).
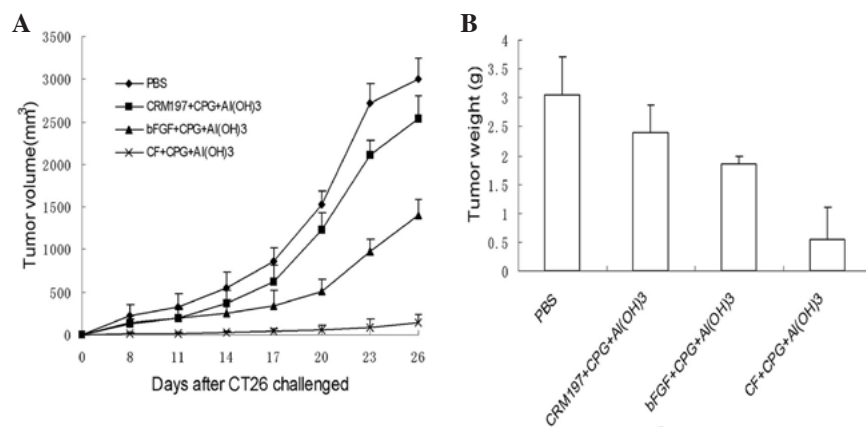
Figure 4. Anti-tumor efficacy of CRM197-CpG-alum, bFGF-CpG-alum and CF-CpG-alum. The mice immunized with corresponding complexes were challenged with 3x10^5 CT26 cells. (A) Tumor sizes on each mouse were monitored every 3 days and (B) tumor weights were measured.
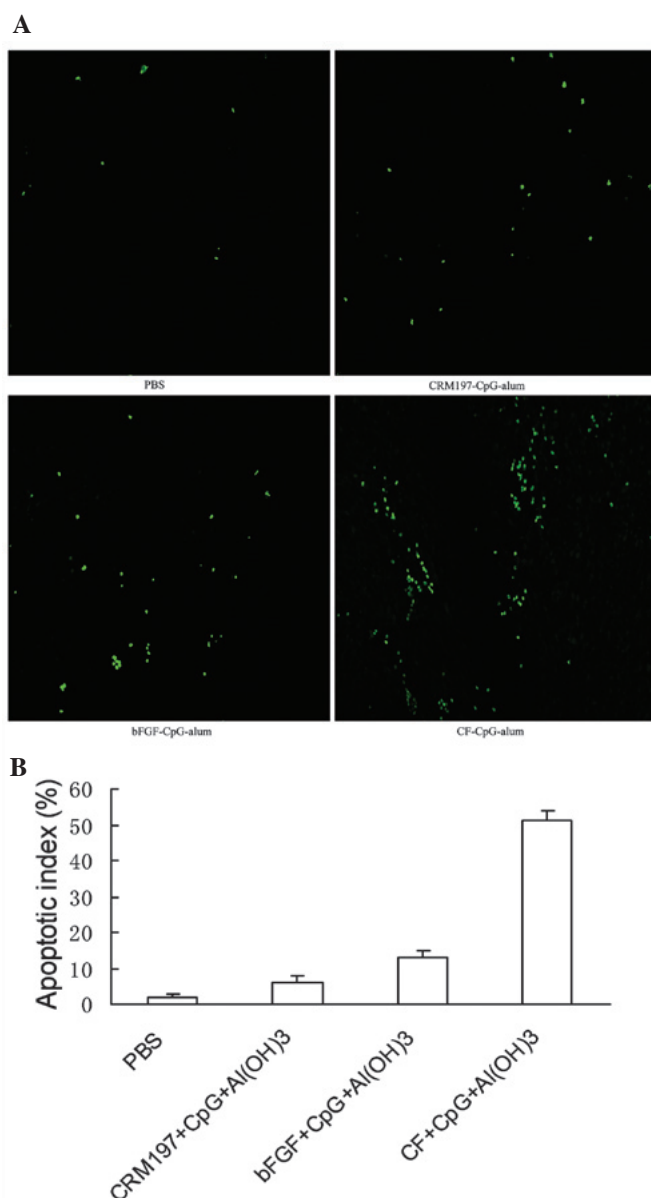

Figure 5. Detection of apoptosis by TUNEL staining of tumor tissues. On day 62, tumor tissues were sectioned and stained with FITC-dUTP. The percentage of apoptosis was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field. (A) TUNEL staining in each group (original magnification, x200). (B) Percentage of apoptosis in each group.
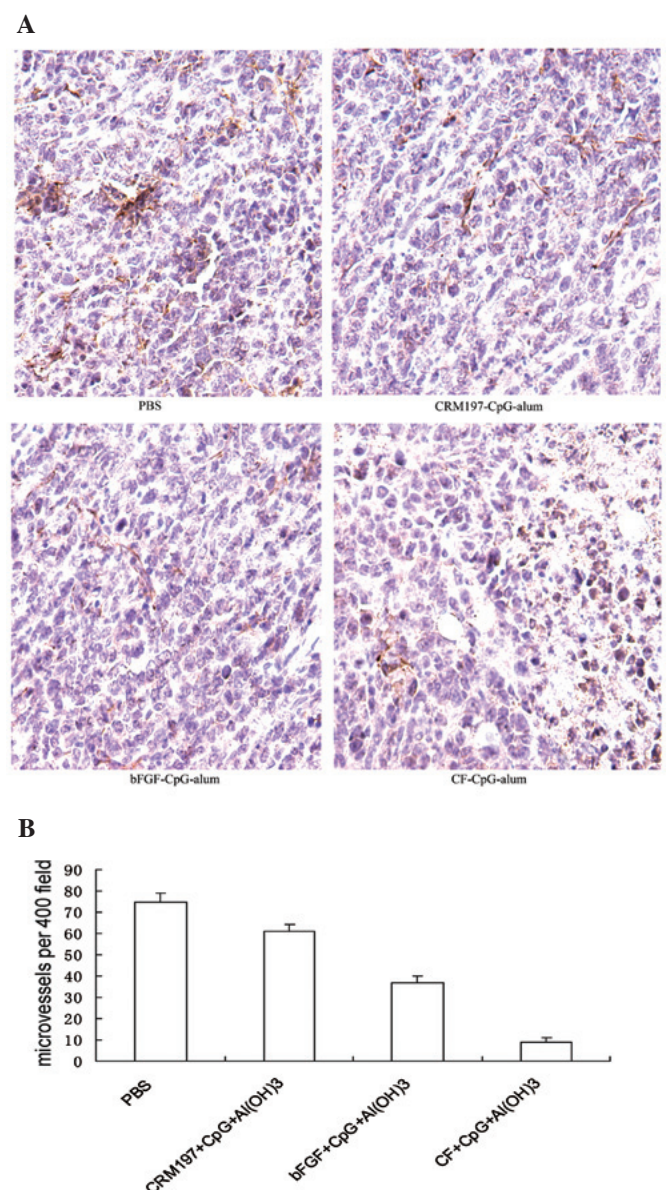
Figure 6. Inhibition of angiogenesis within tumor tissue stained by CD31. On day 62, tumor tissues were sectioned and stained with CD31. Vascular density was estimated by counting the number of microvessels per high power field (magnification, x400). (A) CD31 staining of microvessels in each group. (B) Vascular density in each group.

Discussion

It has become increasingly clear that a number of diseases including cancer depend on angiogenesis. More than a century ago, it was observed that tumor growth was associated with increased vascularity (33). Based on this, a large number of studies concerning tumor angiogenesis were carried out. In 1945, it was proposed by Algire *et al* that 'the rapid growth on tumor transplants is dependent upon the development of a rich vascular supply' (34). Subsequently, Folkman found that tumor tissue could not exceed a size limit of 2-3 mm^3 without involving angiogenesis, and that the size was limited by the diffusion of nutrients and gases including CO_2 and O_2. He therefore proposed that development of vasculature was critical for tumor growth and that anti-angiogenesis may be an effective strategy for cancer therapy (3,35).

Tumor angiogenesis is a complex process mediated by an 'angiogenic switch'. Angiogenic switch is the transition from the latent to the invasive phase of malignancy. In tumor development, this switch can be mediated by the upregulation of proangiogenitors or the downregulation of antiangiogenitors. The proangiogenitors include the platelet-derived growth factor (PDGF), VEGF, interleukin-8 and bFGF, whereas the antiangiogenitors include angiostatin and endostatin proteins (36-39). This indicates that tumor growth can be repressed or terminated by inhibiting or neutralizing the angiogenic stimulators (17). In the past decade, the approach has been confirmed by Hori *et al* and Coppola *et al* with monoclonal Ab and receptor antagonists (40,41).

In these processes, investigators have noted that bFGF plays an important role in angiogenesis and tumor progression (17,19,20). The angiogenic activity of bFGF has been demonstrated in the gelatin sponge, the corneal eye pocket, mouse cornea, and subcutaneous Matrigel injection (42-44). Similarly, a low-metastatic renal carcinoma cell line that was transfected with the bFGF gene was capable of expressing bFGF, resulting in an increased angiogenic response in an experimetal animal model (45). Furthermore, elevated serum bFGF levels have been observed in non-small cell lung cancer, head and neck cancer, lymphoma, prostate carcinoma, and soft tissue sarcoma (15,46). Therapy based on bFGF may therefore represent an effective treatment modality for solid tumors.

It has been established that there are approximately five levels on which to inhibit or neutralize the activity of bFGF (47). In the past decade, researchers have utilized various methods to demonstrate this therapy in several tumor models. Hori *et al* reported that an anti-bFGF monoclonal antibody was capable of inhibiting angiogenesis and tumor growth (41). Schilling-Schon *et al* revealed that neutralizing anti-FGFR antibodies could block bFGF-mediated angiogenesis *in vivo* (48). Wang and Becker demonstrated that antisense cDNAs for bFGF and FGFR-1 resulted in inhibition of angiogenesis and tumor growth in nude mice implanted with human melanomas (49). Moreover, Plum *et al* indicated that the heparin binding domain peptide of bFGF, presenting in a liposome/adjuvant formulation, could effectively inhibit angiogenesis and tumor growth in murine B16BL6 melanoma and Lewis lung carcinoma (17). However, due to its short half-life *in vivo*, the biological activity of free-form bFGF is limited. It appears to be difficult to sustain therapeutic concentrations *in vivo* (22).

In addition to this, we have observed through our previous studies that bFGF alone cannot stimulate the body to produce a strong immune response due to its low immunogenicity. In the present study, CF did not exhibit any proliferation of NIH-3T3 cells *in vitro*. *In vivo*, we observed that CF was capable of enhancing immunogenicity with lower concentrations of bFGF and regulating the subclasses and quantities of IgG. Moreover, we observed that CF together with CpG and alum as an adjuvant could inhibit tumor growth more effectally than other groups. TUNEL and CD31 staining of were performed for each group to define the anti-tumor mechanism. The results revealed that once tumor angiogenesis was suppressed and the diffusion of nutrients and gases to the tumor was halted, tumor cell apoptosis would occur, and tumor growth would consequently be inhibited.

In summary, until recently, studies of CRM197 as an intramolecular adjuvant to treat tumors have been rarely reported. Our data demonstrate that CF together with CpG and alum as an adjuvant enhances the immunogenicity of bFGF and exerts an inhibitory effect on tumor angiogenesis and growth. They have also clarified the immunological mechanism and contribution of this combined vaccine in tumor therapy. Our findings collectively indicate that CRM197 may be an effectual intramolecular adjuvant and that CF may be considered as a new treatment approach for solid tumors.

Acknowledgements

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