Monoclonal antibodies targeting basic fibroblast growth factor inhibit the growth of B16 melanoma *in vivo* and *in vitro*

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Received March 9, 2010; Accepted April 23, 2010

DOI: 10.3892/or_00000879

Abstract. Up-regulated basic fibroblast growth factor (bFGF or FGF-2) plays an important role in the development and metastasis of melanoma; therefore, neutralizing antibodies to bFGF may suppress melanoma growth. In this study, we have developed three monoclonal antibodies against bFGF (anti-bFGF mAbs), which display remarkable anti-tumor and anti-angiogenic effects *in vitro* and *in vivo*. Anti-bFGF mAbs significantly inhibit the proliferation and induce apoptosis of B16 cells, and show inhibitory effects on the migration of B16F10 cells and the tube formation of human umbilical vein endothelial cells (HUVECs) *in vitro*. Treatment of B16 melanoma spheroids with anti-bFGF mAbs *in vivo* results in significant reduction in tumor size and prolonged survival time of animals. Moreover, TUNEL (terminal transferase dUTP nick end labeling) assay and CD31 staining confirmed the increase of apoptosis and decrease of intratumoral microvessel density in tumor sections from animals treated with anti-bFGF mAbs. Our data indicate that anti-bFGF mAbs are potential therapeutic candidates for melanoma therapy by effectively suppressing the melanoma growth through inhibition of angiogenesis and induction of apoptosis in the tumor.

Introduction

Melanoma is the most lethal form of skin cancer; it is usually resistant to both chemotherapy and radiotherapy. Therefore, biological approaches such as antibody targeted therapy and immunotherapy (cytokine and vaccine) have been recently focused on for the treatment of melanoma (1).

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Key words: melanoma, basic fibroblast growth factor, antibody, apoptosis, angiogenesis

bFGF belongs to the family of heparin-binding growth factors. Acting as a broad-spectrum mitogen and potent angiogenic agent, bFGF mediates a variety of cellular responses in embryonic development, wound healing or tumor growth (2). bFGF functions mainly by interacting with high affinity tyrosine kinase FGF receptors (FGFRs) on the surface of target cells. Upon binding to bFGF, FGFRs dimerize and are tyrosine phosphorylated, leading to the activation of downstream signaling pathways including MAPKs (mitogen-activated protein kinases)/ERKs (extracellular signal-regulated kinases) and PI3K (phosphoinositide 3-kinase)/AKT (protein kinase B) (3).

The expression of bFGF is absent in normal melanocytes, which require exogenous bFGF to maintain the growth. bFGF transduced normal melanocytes exhibit transformed phenotype resembling early-stage melanoma, indicating a critical role of bFGF in the transformation of melanocytes to melanoma (4,5). Most melanomas express high levels of bFGF and FGFRs, which form an autocrine loop and are critical for the survival and growth of melanoma cells; melanoma growth can be arrested by interfering with the production or biological activity of bFGF alone (6,7). bFGF has long been regarded as an important tumor angiogenic factor and the specific roles of bFGF in angiogenesis and spontaneous metastasis of melanoma have also been revealed (8,9). These reports suggest the potential of bFGF as a target for melanoma therapy. Antisense targeting of bFGF/FGFR-1 in primary and metastatic melanoma cells blocks the tumor growth by inducing apoptosis without activation or increased production of VEGF (7). In addition, bFGF peptide vaccines also show significant inhibition on angiogenesis and tumor growth (10). However, the efficiency and the mechanism of therapeutic antibodies targeting bFGF in melanoma therapy are still poorly known.

Antibodies targeting bFGF have been shown to inhibit the growth of several other solid tumors, including chondrosarcoma, glioma and colon carcinoma (11-13). In our previous studies we developed a panel of anti-bFGF mAbs (14). In order to further investigate the anti-tumor activity of anti-bFGF mAbs and the underlying mechanisms *in vivo*, in this study, we have examined the effects of anti-bFGF mAbs on angiogenesis, cell migration and growth of melanoma in B16 cells implanted C57 BL/6 mice.

Materials and methods

Cells and cell culture. B16 cells were obtained from ATCC and cultured in DMEM supplemented with 10% FBS, 100 μg/ml penicillin and streptomycin and 2 mM L-glutamine. B16F10 cells were obtained from ATCC and maintained in RPMI-1640 medium supplemented with 10% FBS without HEPES. HUVECs were isolated by collagenase treatment and grown in medium M199 (Sigma) with 15% FBS, 50 μg/ml Low Serum Growth Supplement (LSGS, Invitrogen), penicillin and streptomycin and 2 mM glutamine. The isolated HUVECs were used in later experiments for tube formation and FACS. All the cells were cultured in an incubator with 95% humidity and 5% CO_2 at 37˚C.

Animals. C57 BL/6 normal mice were purchased from Laboratory Animal Center of South Medical University, Guangzhou, China. Animals were taken care of according to institutional guidelines for the care and use of experimental animals.

Generation of bFGF mAbs. The generation of monoclonal antibodies against bFGF was described preciously (14). MabF7, MabF10 and MabF12 were used in this study. The antibodies were purified by a protein G column, and the isotyping of antibodies was carried out using ELISA. To determine whether the anti-bFGF mAbs recognize conformational or linear epitopes of bFGF molecule, native or heat-denured bFGF was used in ELISA.

Cell proliferation assay. B16 cells and HUVECs were seeded in 96-well plates at concentration of $1x10^4$ cells/ml. After the cells attached, the medium was changed to fresh DMEM with 0.5% FBS containing anti-bFGF mAbs (50, 100 and 200 μg/ml, respectively) or mouse normal IgG as control (200 μg/ml). After 96 h, the cell viability was determined by Cell Counting Kit-8 (CCK-8), and the inhibition of cell growth was calculated as the ratio of absorbance of IgG treated group to the absorbance of no IgG treatment group.

Apoptosis assays. B16 cells and HUVECs were plated on a 6-well plate. After 24 h, the cells were incubated for 96 h with 50, 100 and 200 μg/ml of anti-bFGF mAbs, respectively, or mouse normal IgG (200 μg/ml) as control. The fraction of apoptotic cells were marked with FITC-conjugated Annexin V and propodium iodide (PI). After 15 min of incubation at room temperature, the samples were analyzed by flow cytometry.

Tube formation assay. ECM (extracellular matrix) gel (Sigma) was thawed overnight at 4˚C and then dispensed into 96-well plate (60 μl/well). After the gel formation, HUVECs ($1x10^4$ cells/well) in M199 with LSGS were plated on top of the thin layer of gel for 2 h in the absence or presence of 100 μg/ml anti-bFGF mAbs. The formation of capillary tubes was photographed with an inverted microscope. The closed networks of vessel-like tubes were counted with exclusion of the incomplete networks. The tube formation obtained from non-IgG-stimulated cells was set as 100 as described in the respective Figure legends.

Cell migration assay. Cell migration was assayed using a Transwell chamber (8 μm pore size; Corning Costar). B16F10 cells were treated with trypsin and resuspended in serum-free medium. Cells were then added to the upper chamber ($5x10^4$ cells/well) and the medium with 10% FBS was added to the lower chamber in the presence of 100 μg/ml anti-bFGF mAbs or with mouse IgG as control. After incubation for 16 h at 37˚C, the cells in chamber were fixed with 75% ethanol and stained with Giemsa solution. The cells remained at the upper surface of the membrane were removed using a swab, and the number of cells migrated to the lower surface through the filter was counted. The number of cells obtained from control (treatment without supplement) was set as 100 as described in the respective Figure legends.

In vivo anti-tumor activity of anti-bFGF mAbs. Six- to seven-week-old male and female C57 BL/6 mice were subcutaneously inoculated in the right flank with $1x10^5$ B16 cells in 200 μl DMEM medium. One week later, when palpable tumors (≥5 mm in diameter) developed, the mice (n=8 for all groups) were subcutaneously (around tumors) injected with anti-bFGF mAbs at 5, 2.5 and 1.25 mg/ml, respectively, in 200 μl PBS. Control mice received equal amounts of normal mouse IgG or PBS in the same way as that of the anti-bFGF mAbs every 3 days for a total of five injections. Tumor size was measured every 3 days in two dimensions using a vernier caliper, and tumor volume (mm^3) was calculated as volume = $(4\pi/3)$ x (tumor width/2)^2 x (tumor length/2). Mice were sacrificed 3 weeks after tumor cell inoculation and tumor weights were measured.

Immunohistochemical analysis of CD31 in tumor tissue. The expression of CD31 in tumor tissues was analyzed to determine the intratumoral microvessel density. Tumorous and normal tissues were fixed in buffered formalin, embedded in paraffin, and cut at 5 μm. After deparaffinization, the slides were incubated with 3% H_2O_2 to quench the endogeneous peroxidase and then blocked in blocking buffer with 1.5% serum. The sections were incubated with goat anti-mouse CD31 polyclonal antibody (1:1000, Santa Cruz) overnight at 4˚C and detected by biotinylated secondary antibody and avidin-HRP. Tissue sections were then counterstained with hematoxylin and scanned at low power for hot spots of angiogenesis. Microvessels were counted in 3 areas with highest vascular density at a magnification of x200.

TUNEL assay. Apoptotic cells in tumor sections were detected using TUNEL assay kit (Beyotime, China) according to the manufacturer's manual. Briefly, tissue sections were treated with proteinase K and immersed in 3% H_2O_2. TdT was used to catalyze the addition of biotin-conjugated d-UTP to the 3'-OH ends of DNA fragments. The coloring was then done with DAB as substrate.

Effect of anti-bFGF mAbs on survival of mice implanted with B16 cells. C57 BL/6 mice were injected with B16 cells as afore-mentioned. When palpable tumors (≥5 mm in diameter) developed, the mice were randomly divided into three groups with eight mice in each group and injected with MabF7 (5 mg/ml) in 200 μl PBS, equal amount of control IgG and PBS,

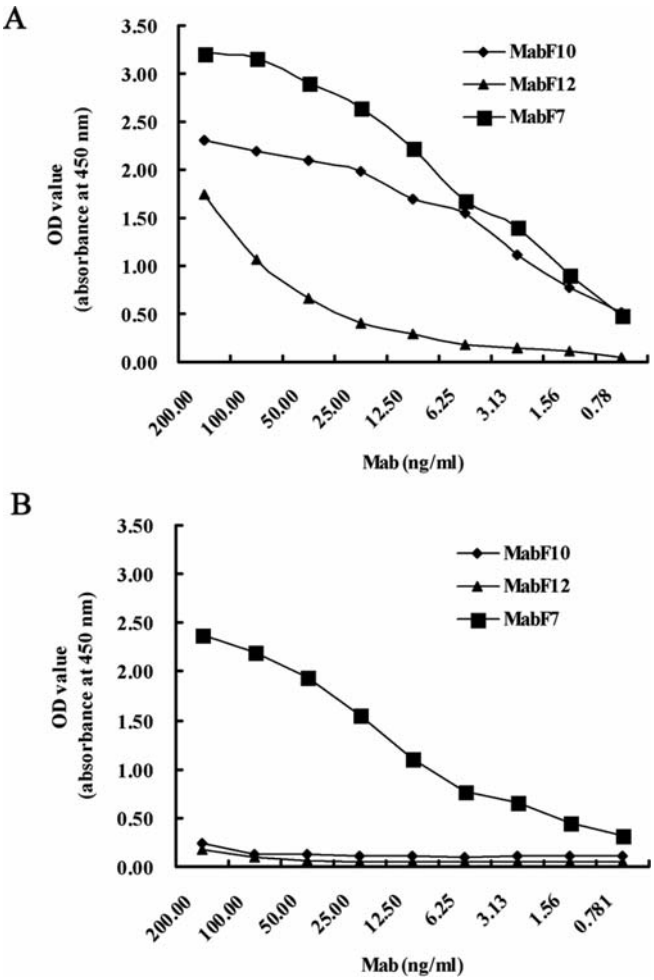

Figure 1. Determination of the continuous or discontinuous epitopes recognized by anti-bFGF mAbs. (A) Natural and (B) heat-denatured bFGF were coated on a plate at 4˚C overnight, and blocked with 5% defatted milk. The plate was then incubated with anti-bFGF mAbs and HRP-labeled goat anti-mouse polyclonal antibody for 1 h successively and colored with TMB substrate. Absorbance value was measured at 450 nm.

respectively. Mouse survival was monitored from the day of tumor inoculation until the day of animal death.

Results

Characterization of anti-bFGF mAbs. The mAbs MabF7, MabF10 and MabF12 belong to the IgG1 isotype. To determine whether anti-bFGF mAbs recognize continuous or discon-tinuous epitopes on bFGF, the binding of these mAbs to heat-inactivated and native bFGF was examined by ELISA. The result of MabF7 binding to both native and denatured bFGF implies that MabF7 recognizes a continuous epitope. MabF10 and MabF12 do not recognize the heat-denatured bFGF suggesting that they both recognize conformation-dependent epitopes on bFGF (Fig. 1).

Anti-bFGF mAbs induce apoptosis in B16 cells but not in HUVECs. B16 cells and HUVECs were treated with 50-200 μg/ml monoclonal antibodies. The proliferation of B16 cells was inhibited in a dose- and time-dependent manner (Fig. 2). Apoptosis was analyzed by flow cytometry with Annexin V/PI staining. With mAbs at a concentration of 200 μg/ml, 73.20±10% of B16 cells show apoptosis after 96 h of incubation, whereas IgG control group shows no effect compared with medium control (Fig. 3A). No significant apoptotic activity of anti-bFGF mAbs was found in HUVECs compared with control IgG (Fig. 3B).

Anti-bFGF mAbs suppress the tube formation of HUVECs. HUVECs were cultured in M199 medium containing 50 μg/ml LSGS composed of bFGF, heparin, hydrocortisone and EGF. As shown in Fig. 4, the tube formation in groups with no IgG, control IgG, MabF7, MabF10 and MabF12 are 100%, 105.93±3.15%, 56.53±3.56%, 29.23±5.27% and 12.77±2.18%, respectively. It indicates that anti-bFGF mAbs significantly inhibit the tube formation of HUVECs; however, control IgG shows no effect.

Anti-bFGF mAbs inhibit the migration of B16F10 in vitro. To examine the effect of anti-bFGF mAbs on migration of B16F10 cells, a Transwell chamber culture system was used to imitate the process of tumor cell migration. The cells in upper chamber are chemoattracted by the serum in the lower chamber, and the migration of B16F10 cells was analyzed in the presence or absence of anti-bFGF mAbs. As shown in Fig. 5, the number of migrating cells in the presence of mAbs reduces significantly compared to the control group (treatment without supplement), and the migration ratio in groups treated with no IgG, control IgG, MabF7, MabF10 and MabF12 are 100%, 109.00±9.56%, 72.14±13.57%, 34.68±14.37% and 36.56±13.60%, respectively.

Anti-bFGF mAbs inhibit the growth of B16 melanoma in vivo. As mentioned above, anti-bFGF mAbs inhibit cell proliferation and migration and induce apoptosis of melanoma cells *in vitro*. To investigate the *in vivo* effect of mAbs on melanoma growth, we have used B16 cells implanted C57 BL/6 mice. As shown in Fig. 6, the MabF7 remarkably reduces tumor burden and suppresses tumor growth by 46.40% at the concentration of 5 mg/ml. However, the inhibitions by MabF10 and MabF12 are 23.24 and 19.89%.

Anti-bFGF mAbs decrease vessel density in tumor. Since anti-bFGF mAbs inhibit the tube formation of HUVECs *in vitro*, we further analyzed the *in vivo* effect of mAbs on micro-vessel density in tumor. The microvessel density in tumor was determined by staining the tumor section with anti-CD31 antibody, and the numbers of microvessel density in tumor were reduced significantly in MabF7 group (24.75±6.70) in comparison with that in IgG control group (45.38±12.59, Fig. 7A).

Anti-bFGF mAbs increase apoptosis in tumors. As mentioned above, mAbs induce cell apoptosis in B16 cells. To examine whether the reduction in tumor size is the result of the increased cell apoptosis in the tumor, melanoma sections were analyzed with TUNEL assay. As shown in Fig. 7B, more TUNEL-positive cells with deep brown stained nuclei were detected in MabF7 treated tumors than that in the control IgG treated group.

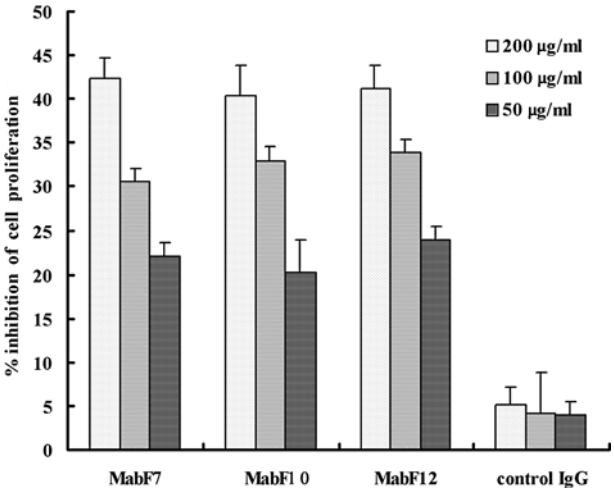

Figure 2. Inhibition of B16 cell proliferation by anti-bFGF mAbs. B16 cells were treated with 50-200 μg/ml anti-bFGF mAbs, and the cell proliferation was measured using the CCK-8 assay. The results are from three independent experiments. Column, average of a representative experiment; bar, SD.

Anti-bFGF mAbs increase survival of C57 BL/6 mice. To investigate whether the inhibition of tumor growth by MabF7 impacts the survival of animals, a survival assay was performed as shown in the Kaplan-Meier survival curve in Fig. 8. The median survival time of mice treated with MabF7 (32 days) is longer than that of mice treated with PBS (24 days) or control IgG (26 days)

Discussion

bFGF is an important survival and angiogenic factor for melanoma progression; therefore, targeting bFGF may be an effective method for melanoma therapy. In this study, we demonstrate that anti-bFGF mAbs display remarkable anti-tumor and anti-angiogenic effects in mouse model of B16 melanoma. The mAbs induce apoptosis and inhibit migration of melanoma cells, and reduce tube formation by HUVECs. Therefore, antibodies against bFGF offer a potential therapeutic approach to melanomas.

bFGF is one of the epigenetic mechanisms explaining the multidrug resistance (MDR) of tumors (15). Some previous studies have shown that bFGF prevents chemotherapy induced apoptosis, which result in chemoresistance in many cancers such as small cell lung cancer and breast cancer. This anti-apoptotic effect is mediated by MAPK/ERKs, PI3K/AKT and/or PKC that increase the expression and activation of several anti-apoptotic proteins including Bcl-2, Bcl-X_L and XIAP (16,17). Although the precise mechanisms of drug resistance in melanoma cells are largely unknown, targeted inhibition of bFGF/FGFR-1 in primary and metastatic melanoma cells blocks tumor growth by inducing apoptosis indicating a critical pro-survival role of bFGF in melanoma (7). Here we show that anti-bFGF mAbs induce significant apoptosis in B16 cells; antibody-mediated cell apoptosis is an important mechanism for killing tumor cells, and antibody-directed mechanisms include antigen crosslinking (anti-CD20), activation of death receptors (anti-TNFR) and blockade of ligand-receptor pathways for growth or survival (anti-HER2)

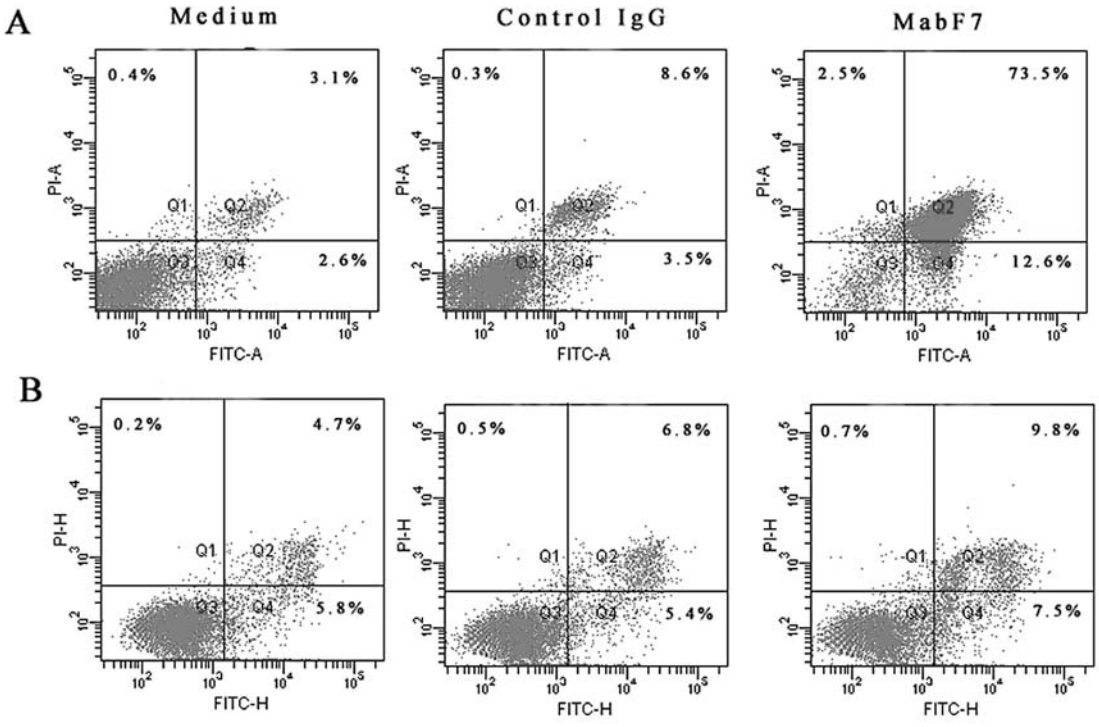

Figure 3. Apoptosis assay of B16 melanoma and HUVECs treated with anti-bFGF mAbs. (A) B16 cells and (B) HUVECs were treated with MabF7 (200 μg/ml), control IgG (200 μg/ml), and no IgG for 96 h. The apoptotic cells stained with Annexin V-FITC and PI were analyzed by flow cytometry. Cells in the lower right quadrant are Annexin-positive, early apoptotic cells. The cells in the upper right quadrant are Annexin-positive/PI-positive, late apoptotic cells. Results are representative of three independent experiments.

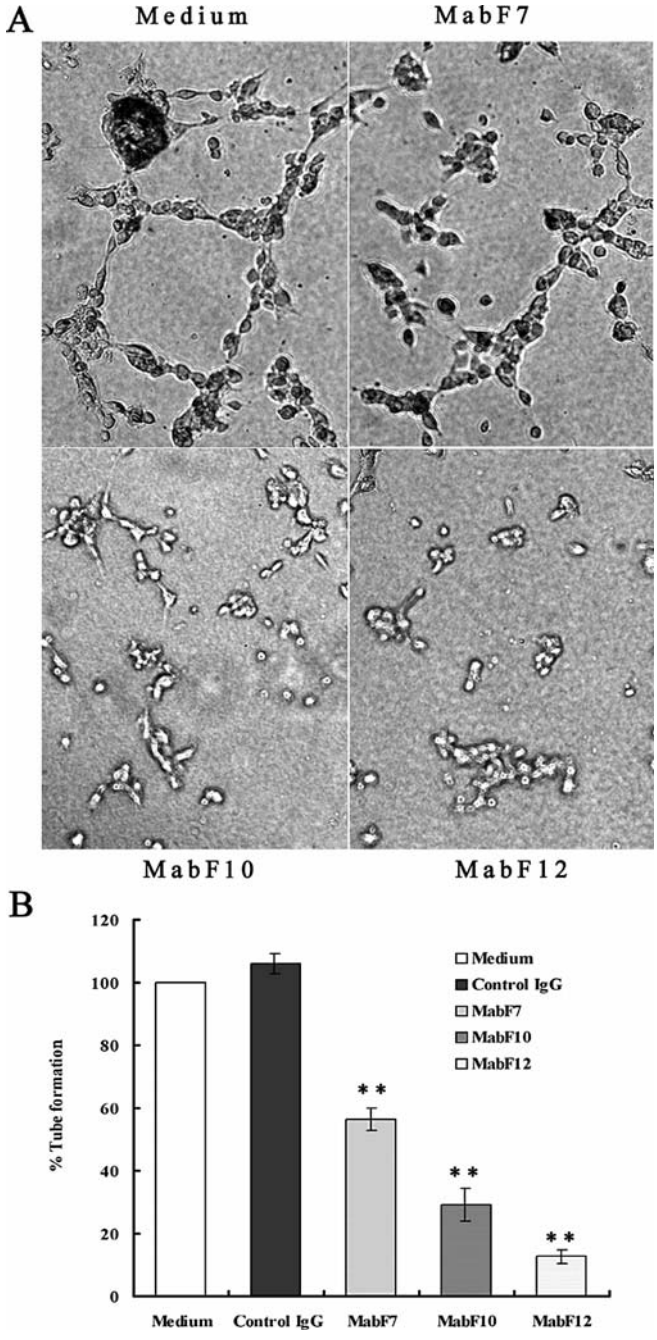

Figure 4. Inhibition of tube formation in HUVECs by anti-bFGF mAbs *in vitro*. (A) HUVECs were cultured onto the surface of ECM gel in presence or absence of MabF7. (B) The closed networks of vessel-like tubes were counted with exclusion of the incomplete networks. Tube formation is presented as relative values to that of no IgG group. The results are from three independent experiments. Column, average of representative experiment; bar, SD; $^{**}P<0.01$.

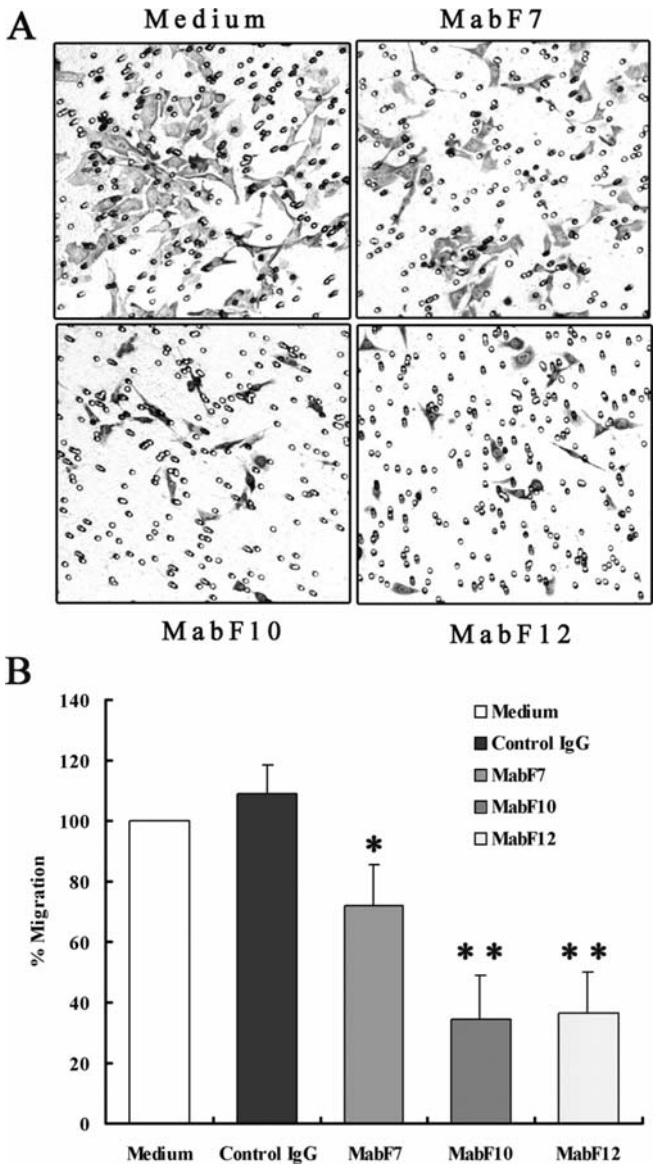

Figure 5. Inhibition of B16F10 migration by anti-bFGF mAbs *in vitro*. The inhibitory potential of anti-bFGF mAbs on the migration of B16F10 *in vitro* was evaluated in Transwell chambers. B16F10 cells in serum-free medium were treated with anti-bFGF mAbs (100 μg/ml) and seeded into the upper chamber. After incubation for 16 h at 37˚C, the number of migrated cells was stained with Giemsa and counted. The number of cells obtained from no IgG-treated cells was set as 100. Column, average of a representative experiment; bar, SD; $^{**}P<0.01$.

(18). In this study, the induction of apoptosis in melanoma cells may be caused by the blockade of bFGF mediated survival pathways.

Although the antibodies do not lead to apoptosis in HUVECs in the present study, the formation of tube-like structure of HUVECs on matrigel *in vitro* is significantly inhibited by bFGF mAbs. Because 50 μg/ml LSGS (consisting of several growth factors including bFGF) is used in the experiment, it implies that bFGF mAbs may suppress the formation of tube-like structure by blocking the exogenous bFGF but not the autocrine loop of bFGF in HUVECs. Moreover, the suppression of mAbs on migration of B16F10 *in vitro* indicates the potential inhibitory activity of anti-bFGF mAbs on tumor metastasis *in vivo*.

The data from our animal studies show that administration of MabF7 significantly inhibits tumor growth and increases survival time of mice compared to that in the control group, demonstrating the *in vivo* anti-tumor effect of anti-bFGF mAbs. The suppression of tumor growth in mice treated with anti-bFGF mAbs can be explained, at least in part, by the induction of apoptosis and decrease of microvessel density in tumor tissues. These results are consistent

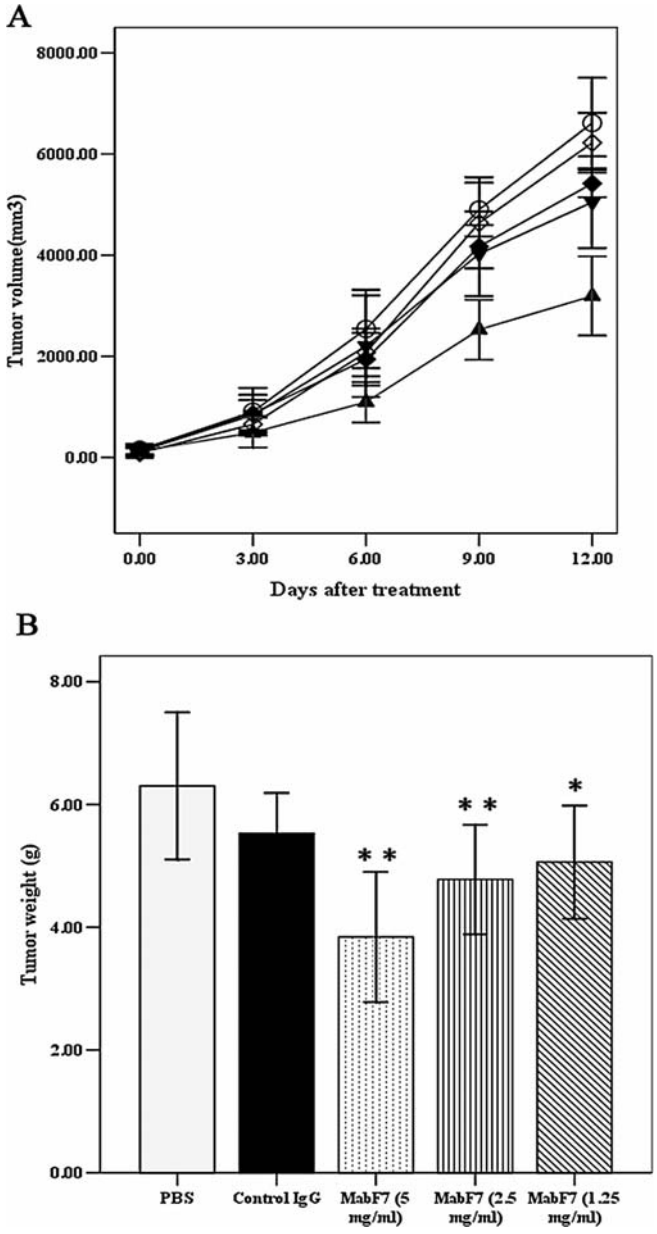

Figure 6. Inhibition of melanoma tumor growth *in vivo*. (A) treatment was started 7 days after B16 cell inoculation, and tumor volume (mm^3) was measured at different time-points after treatment. ○, s.c. injection of PBS (200 μl); ◇, s.c. injection of control antibody; ◆, s.c. injection of MabF10 (5 mg/ml); ▼, s.c. injection of MabF12 (5 mg/ml); ▲, s.c. injection of MabF7 (5 mg/ml). Results are the means ± SD (error bars) from 8 animals. (B) Anti-bFGF mAbs (MabF7) dose-dependently inhibit B16 tumor growth; tumor weight changes after treatment with PBS, control antibody or MabF7 (5, 2.5 and 1.25 mg/ml) respectively. Results are the means ± SD (error bars) from 8 animals. $^{*}P<0.05$; $^{**}P<0.01$.

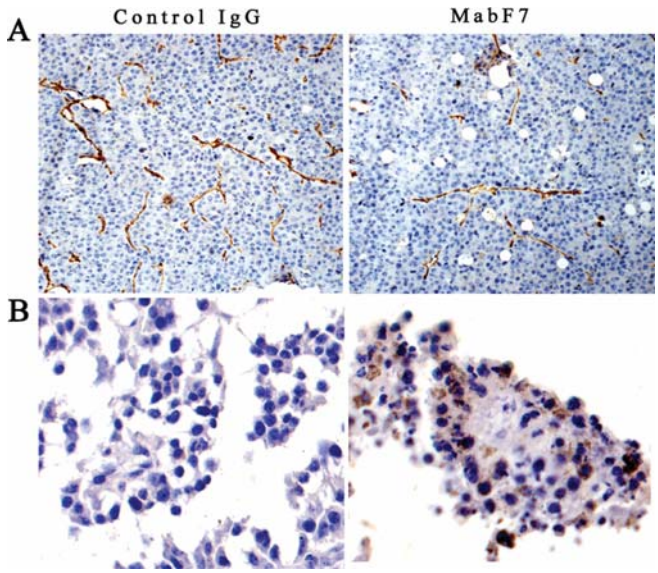

Figure 7. Blood vessel density and apoptosis in tumor tissues from treated mice. (A) Melanoma-bearing mice were treated as described in Materials and methods. The animals were sacrificed, and the tumors were removed for immunohistochemical staining for blood vessel density. The number of blood vessels at 3 high-power fields (x200) per section was counted. (B) Apoptotic cells were stained by *in situ* TUNEL and counterstained with hematoxylin. TUNEL-positive cells have brown (DAB) nuclear staining, whereas the nuclei of TUNEL negative cells are blue (DAB). Magnification, x400.

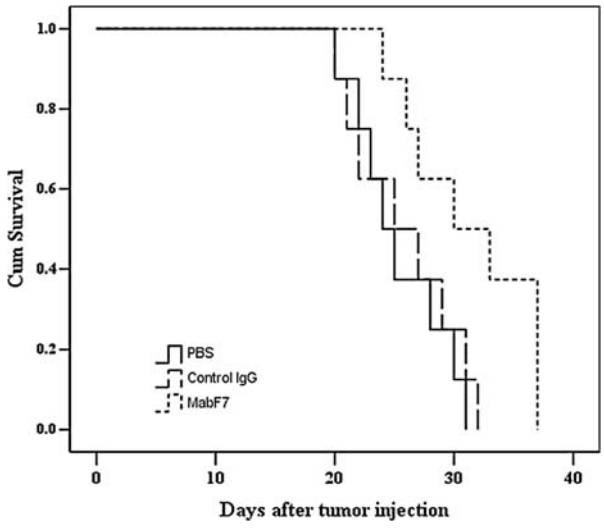

Figure 8. Survival curves of mice. Melanoma-bearing mice were treated with anti-bFGF antibodies (8 mice per group). The survival of animals was recorded starting from the injection of tumor cells. The median survival time in PBS, control IgG and MabF7 groups are 24, 26 and 32 days, respectively.

with a previous report, where goat anti-human bFGF polyclonal antibody was used to determine the role of bFGF in angiogenesis and metastasis of human melanoma xenografts, and the tumor growth, lung colonization, and spontaneous metastasis were significantly inhibited by the neutralizing antibody (19).

Nevertheless, there was no remarkable suppression of melanoma growth *in vivo* by other two antibodies MabF10 and MabF12, though they show inhibition of tumor cell growth and reduce tube formation and tumor cell migration more effectively than MabF *in vitro*. This may be influenced by the tumor type, pharmacokinetic factors and the recognizing epitope of antibodies. The anti-bFGF antibody MabF7 recognizes the linear epitope on the bFGF molecule, but MabF10 and MabF12 antibodies recognize conformation-dependent epitopes. A previous study has demonstrated that an antibody recognizing heparin epitope of bFGF cannot

exert efficient anti-tumor activity, compared with the significant anti-tumor activity mediated by the antibody recognizing receptor epitope (20). In contrast, another report shows that a heparin binding domain peptide of bFGF more remarkably inhibit angiogenesis and tumor growth compared to the receptor binding domain peptide (10). Therefore, further studies to determine the accurate epitope reaction will shed more light on our understanding of the anti-tumor mechanism of these mAbs.

In summary, we have shown that the anti-bFGF mAb MabF7 remarkably inhibits melanoma growth both *in vitro* and *in vivo*. The results from this study provide a good approach to the melanoma therapy by blocking bFGF/FGFR function. The combination of anti-bFGF mAbs with chemotherapy and radiotherapy in melanoma treatment is currently under investigation.

Acknowledgements

This research is supported by Grant of the National High Technology and Development Program of China (863 program, 2009AA02Z112) and sub-project of 863 major program (2006AA02A247). We thank Dr Guoquan Gao for his help in the culture of HUVECs and Qifang Song for purification of the antibodies.

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