Cytokine 96 (2017) 161–165

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Short communication

# BFGF neutralization stimulates VEGF secretion in melanoma B16 cells

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ARTICLE INFO

*Keywords:*
Basic fibroblast growth factor (bFGF)
Autocrine
Vascular endothelial growth factor (VEGF)
Melanoma

## 1. Introduction

Autocrine bFGF plays a key role in tumor angiogenesis [1]. The bFGF-targeted therapies, including both tyrosine kinase inhibitors and blocking antibodies, can suppress tumor growth in certain pre-clinical models, including melanoma [2]. Thus, it seems to indicate that the inhibition of bFGF signaling represents a promising strategy for the treatment of malignant melanomas. Compelling evidence indicates that resistance of tumors to antiangiogenic therapies can occur via multifarious mechanisms, including upregulation of other proangiogenic factors [3]. Although bFGF can stimulate VEGF secretion and act as an escape mechanism for VEGF inhibition [4], the influence of bFGF blockade on VEGF expression remain uncertain.

We have previously reported that blocking of bFGF could inhibit tumor growth and induce cancer cells apoptosis in B16 cells by using a neutralizing monoclonal antibody MabF7 [5]. The strategy is worthy of further investigation to improve antitumor effects of bFGF blockade in melanoma cells. It has been reported that the inhibition of bFGF increased VEGF expression in lung cancer cells [6], suggesting a possibility that upregulation of VEGF might be involved in other tumors. Here, we investigated the effect of bFGF inhibition on VEGF secretion in B16 cells, and the results showed that anti-bFGF therapy enhanced VEGF level and synergized with VEGF blockade for melanoma cell killing.

## 2. Materials and methods

### 2.1. Cells lines and reagents

Mouse melanoma B16 cells (ATCC) were cultured in DMEM/10% FBS (Gibco). A neutralizing human/mouse bFGF monoclonal antibody (mouse) and a neutralizing human/ mouse VEGF monoclonal antibody (mouse) were obtained as previously described [5]. A Trypan Blue exclusion assay kit was from Beyotime Biotech Co. Ltd (Shanghai, China). An annexin V-fluorescein isothiocyanate apoptosis kit was from 4A Biotech Co.Ltd (Beijing, China). A VEGF enzymatic assay kit was from Neobioscience Biotech Co. Ltd (Shenzhen, China). All phosphor-specific antibodies were from CST (Beverly, USA). An anti-VEGFA antibody was from EnoGene Biotech Co. Ltd (Nanjing, China). An anti-actin antibody was purchased from Santa Cruz Biotechnology (Dallas, USA).

### 2.2. Western blot

Cells were collected and lysed in RIPA lysis buffer (Keygene, China) supplemented with proteinase and phosphatase inhibitor cocktails, and protein content was measured by BCA protein assay. The Western Blot was performed with little modification as previously reported study [7]. Briefly, the samples were separated through SDS-PAGE, transferred to PVDF membranes, and probed with indicated primary antibodies. After the HRP-labed secondary antibodies incubation, the immunoreactive bands were visualized by ECL reagent (Wanlei, Shenyang, China) and exposed to X-ray film.

### 2.3. ELISA assay

Culture supernatants of different experimental groups were collected and VEGF level was checked using ELISA kit (NeoBioscience, China) according to the manufacturer's manual. Briefly, different concentrations of standards (100 μL/well) and samples (100 μL/well) were added to a 96-well coated plate and incubated for 90 min at 37 °C. After rinsing 5 times, added 100 μL polyclonal anti-mouse VEGF

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http://dx.doi.org/10.1016/j.cyto.2017.04.014
Received 5 February 2017; Received in revised form 8 April 2017; Accepted 11 April 2017

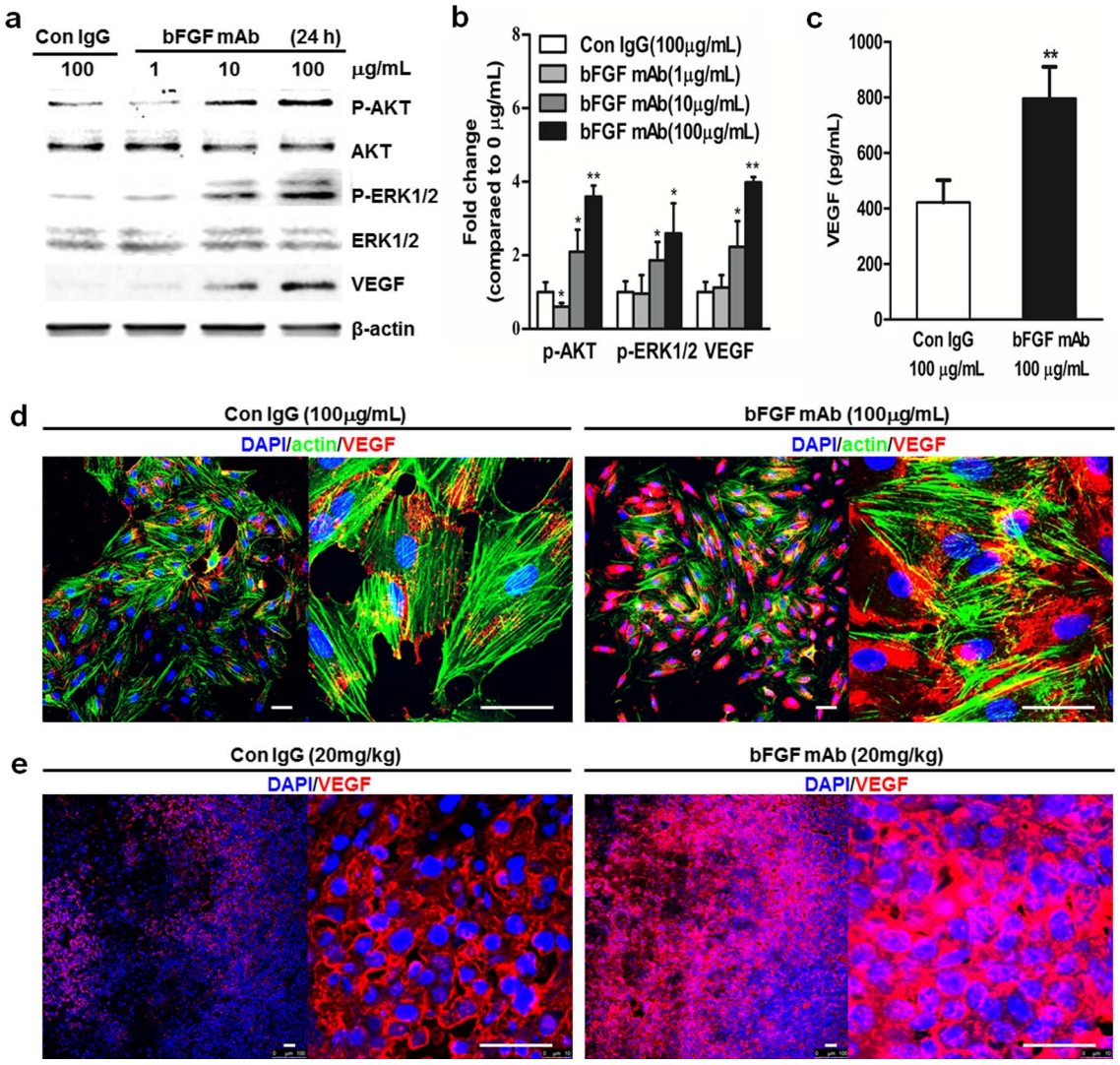


**Fig. 1. Enhanced VEGF expression following bFGF blockade.** (a and b) Effects of different concentration of anti-bFGF mAb on expressions of phosphorylated AKT (Ser473), AKT, phosphorylated ERK1/2, ERK1/2 and VEGFA in melanoma B16 cells. (c) VEGF level in melanoma B16 cells treated with anti-bFGF mAb or mouse IgG at the concentration of 100 μg/mL. The expression of the no treatment cells was regarded as 100%. $*P < 0.05$, $**P < 0.01$. (d) Expression of VEGF was examined under a confocal microscope in the cells after treating with 100 μg/mL, anti-bFGF mAb or 100 μg/mL mouse IgG, bar, 25 μm. (e) Expression of VEGF in the tumor tissues treated with 20 mg/kg anti-bFGF mAb or 20 mg/kg mouse IgG, bar, 30 μm.

antibody conjugated horseradish peroxidase to each reaction well. After an additional incubation for 60 min 37 °C, the plate was again rinsed 5 times, and 100 μL of chromogenic agent (TMB) was added. The reaction was stopped by adding sulfuric acid (100 μL) to each well, and the absorbance was measured at 450 nm.

### 2.4. Immunofluorescence assay

After treatment with anti-bFGF mAb or control IgG for 2 d, the cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked with normal goat serum (10% in PBS). Incubation with polyclonal rabbit anti-VEGF antibody (dilution, 1:200) for 1 h was followed by incubation with Alexa Fluor 555-labeled antimouse IgG secondary antibody (dilution, 1:400). The slides were examined using a confocal microscopy (Leica TCS SP5).

### 2.5. Cell viability assay

The cells were seeded in 96-well plates ($2 \times 10^3$ cell/ well) and incubated at 37 °C overnight. After treating with control IgG, anti-bFGF mAb, anti-VEGF mAb, or the combination of anti-bFGF mAb and anti-VEGF mAb, the Trypan Blue exclusion assay was performed and the survival fractions of the tumor cells were calculated.

### 2.6. Apoptosis detection assay

Whether apoptosis occurred in the cells after treatment was determined using an annexin V-FITC apoptosis kit according to the previously reported study with little modification [8,9]. The cells were analyzed using a FacsCalibuo flow cytometer (BD, USA), and the data analysis was performed using the Cell Quest software.

### 2.7. In vivo assay

C57BL mice (6–8 weeks old) were from the Animal Experimental Center of the Guangdong Medical University, and the animal experiments were approved by its Animal Care and Use Committee. B16 cells ($1 \times 10^6$) in 100 μL no serum medium was injected into each mouse's right flank. The treatments started on day 7 after B16 cell inoculation and continued twice a week for 4 weeks, mice were sacrificed after anesthesia and the tumors were weighted respectively. For the immunohistochemistry of 4% paraformaldehyde (PFA) perfusion-fixed