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Expression of $VEGF_{121}$ in gastric carcinoma MGC803 cell line

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INTRODUCTION

Vascular endothelial growth factor (VEGF) which is also known as vascular permeability factor (VPF) is a heparin-binding, dimeric polypeptide growth factor and a potent mitogen for endothelial cells. VEGF can stimulate the endothelial cell growth and enhance the motility through its two known receptors flt-1 and KDR[1]. Acting through these receptors, VEGF may stimulate angiogenesis and promote tumor progression. $VEGF_{121}$, as one of the four VEGF protein isofo rms containing the least number of amino acids, has all the biological function of VEGF and is the ideal isoforms for further studying VEGF at molecular levels[2]. In this study, we cloned $VEGF_{121}$ cDNA from gastric carcinoma cell MGC803 and had it expressed in *E.coli* bacterial. Further identification of its expression products was carried out with anti-VEGF monoclonal antibodies. This gives new evidence to clarify the source of VEGF in tumor tissues.

MATERIALS AND METHODS

Materials

Balb/c mice, female, 6-8 weeks old, was purchased from the Animal Center of the Chinese Academy of Medical Sciences. HUVEC was obtained from the Academy of Pre ventive Medical Sciences and cultured in RPMI1640 with 15% fetal cow serum (full growth medium) at 37℃ with 5% CO_2. MGC803 cell line was established from a primary poorly differentiated mucoid adenocarcinoma of human stomach. Recombinant human $VEGF_{121}$ was obtained from the Department of Immunology, Beijing Medical University. ^{3}H-thymidine was purchased from Shanghai Institute of Atomic Energy.

Preparation of $VEGF_{121}$ monoclonal antibodies

Balb/c mice were immunized subcutaneously with the purified fusion protein GST-$VEGF_{121}$ and hybridoma cell clones were obtained by traditional hybridoma technology. ELISA was used to screen hybridoma clones with recombinant fusion protein GST-VEGF and GST-P21 as antigen. The clones which reacted with GST-VEGF, but not with GST-P21, were subcloned. After three rounds of subcloning by limited dilution, $VEGF_{121}$, which had proliferation activity on HUVEC, was used as antigen to select the positive clones. The antibodies were purified through protein A-Sephasrose CL-4B chromatography and their subclasses were measured.

Inhibition of the monoclonal antibody on HUVEC proliferation induced by $VEGF_{121}$

Assay of ^{3}H-thymidine incorporation was used on HUVEC for neutralizing the activity of anti-$VEGF_{121}$ monoclonal antibody. HUVEC was seeded at a density of 2×104 per well of 24 well plates and incubated with full growth medium for 48 hrs at 37℃. The cells were then incubated with serum free medium for 24 hrs, and the testing groups were added with $VEGF_{121}$(10 μg/L) and anti-$VEGF_{121}$ monoclonal antibody at various concentrations. After 30 hrs culture, ^{3}H-thymidine (37 KBq/mL) was added, and after 6 hrs, the cells were collected and measured in a liquid scintillation counter.

RT-PCR of $VEGF_{121}$ from MGC803 cells and HUVECs

Total RNA of both cell lines was extracted respectively by TRISOLV™ isolation of RNA kit (GIBCO BRL). First-strand cDNA was synthesized using the Superscript™-II Preamplification System for First Strand cDNA Synthesis Kit (GIBCO BRL) with 5 μg total RNA in a 20 μL reaction volume. Two μL cDNA was used as template in a 100 μL-PCR reaction volume. The primer for VEGF reverse transcription was oligodT. The cDNA

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encoding VEGF was amplified using forward primer (5'-GGG GGA TCC GCC TCC GAA ACC ATG AAC TT-3' containing Bam-HI restriction endonuclease site) and reverse primer (5'-CCC GAA TTC TCC TGG TGA GAG ATC TGG TT-3', containing Eco-RI restriction endonuclease site), and PCR was carried out in a DNA Engine™- Peltier Thermal Cycler Model PTC-200 (MT Research Inc, USA) at 95 ℃ for 5 min first, then at 94 ℃ for 45sec, 55 ℃ for 40sec, and 72 ℃ for 1min for 36 cycles, followed by 5 min at 72 ℃.

Recombinant of VEGF on plasmid PGEX-2T vector

The PCR products were purified with DNA purified kit (QIAEGN), digested with Bam-HI/Eco-RI and ligated with fusion protein prokaryotic expression plasmid PGEX-2T. E. coli-XL-1 blue was transformed and the positive clones were selected by restriction endonuclease mapping.

Expression and identification of GST-VEGF$_{121}$ protein

Positive clones were selected and cultured with LB until the OD_{600} reached to 1.0, then the 5 mmol/L IPTG was added for 4 h culture to induce the protein expression. The expression products were analysed by SDS-PAGE, and Western blot was carried out for further identification of the products.

RESULTS

Preparation and identification of anti-VEGF monoclonal antibodies

After three rounds of subcloning and selecting by ELISA with recombinant fusion protein GST-VEGF$_{121}$ and GST-P21, six clones stably secreting anti-VEGF$_{121}$ antibodies were obtained. Screened with purified VEGF$_{121}$, clone 5C$_5$, which showed more specific binding activity to VEGF, was selected for pre paring ascites. The ascites were purified through chromatography with ProteinA- Sepharose CL-4B column. Its subclass is IgG2b.

Neutralization activity of anti-VEGF$_{121}$ monoclonal antibody

We measured the effects of anti-VEGF antibody 5C$_5$ at various concentrations on HUVEC proliferation induced with VEGF at a concentration of 2 μg/L. The results from analysis of ^{3}H-thymidine incorporation showed that the VEGF antibody 5C$_5$ neutralized the activity of VEGF in a dose-dependent manner and blocked the VEGF-induced cell growth completely at a concentration of 10 mg/L (Figure 1).

Amplification of VEGF$_{121}$ cDNA

The VEGF$_{121}$- cDNA was amplified with its specific primers from MGC803 cells. Following 0.8% agarose gel analysis, a clear band about 550bp, which m arched the predicated size, was generated (Figure 2, lane 2). No specific band was obtained from HUVEC (Figure 2, lane 3).

Recombinant of VEGF$_{121}$ and its expression

The recombinant PGEX2T-VEGF$_{121}$ digested with Eco-RI/Bam-HI released a fragment about 550 bp (Figure 2, lane 4). After induced by IPTG, the positive transformed E. coli XL-1 blue can stably express fusion protein GST-VEGF at the molecular weight about 40 KD. The proportion of expressed VEGF$_{121}$ to total bacterial protein was about 25% and it existed in the inclusi on body (Figure 3).

Western blot analysis

Induced by IPTG, further identification of the expressed product was carried out by Western blot analysis. The results showed that 5C$_5$ can be specifically reacted with denatured GST-VEGF$_{121}$ (Figure 4).

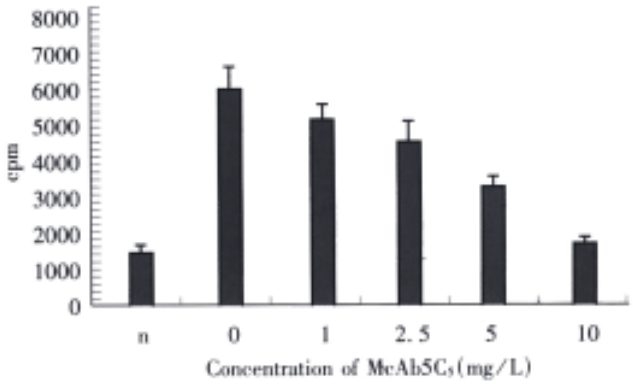

Figure 1 Neutralization of 5C$_5$ to the VEGF-induced HUVEC growth.
n: no VEGF or antibody added, 0.10: VEGF 2mg/L and antibody 5C$_5$ in various concentration were added.

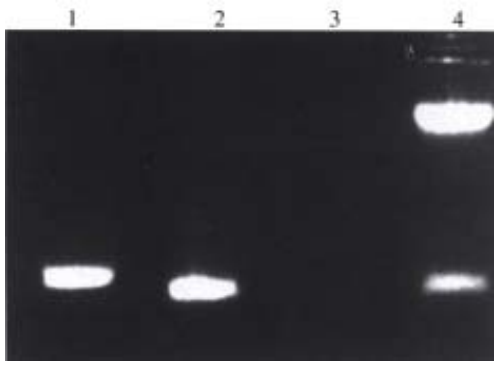

Figure 2 RT-PCR of VEGF$_{121}$ cDNA and identific ation of recombinant PGEX2T-VEGF.
1. 585/985bp DNA marker, 2. PCR product of VEGF from MGC803 cells, 3. PCR product of VEGF from HUVEC, 4. PGEX2T-VEGF digested with *Bam*-HI/*Eco*-RI.

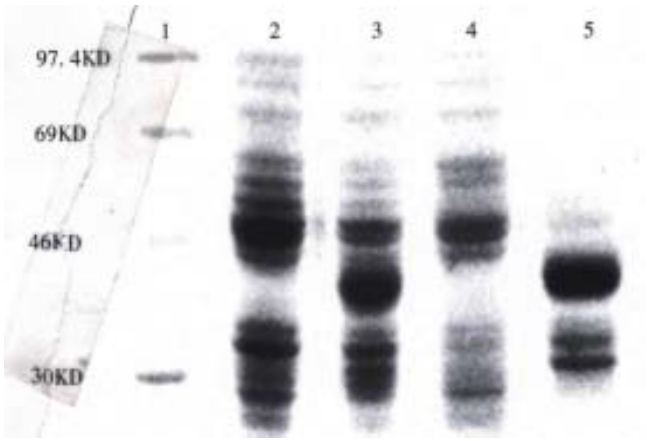

Figure 3 SDS-PAGE analysis of GST-VEGF expressed in E. coli XL-1 blue-1

Standards of protein molecular weight, 2. Total proteins from bacterial transformed with PGEX2T-$VEGF_{121}$ without induced IPTG, 3. Proteins from bacterial induced by IPTG, 4. Protein pellet of bacterial lysis without induced IPTG, 5. Protein pellet of bacterial lysis induced by IPTG.

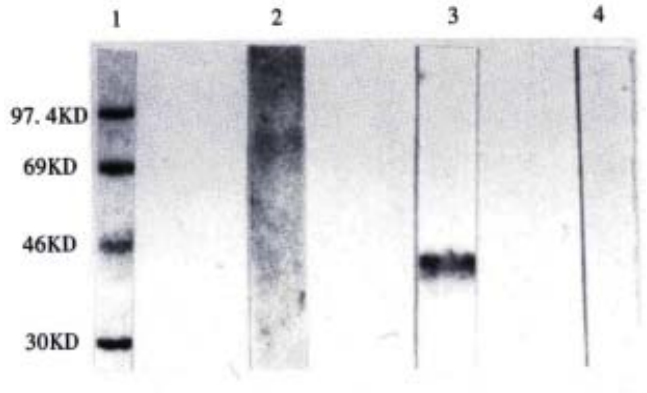

Figure 4 Western blot analysis of the bacterial expr essed GST-$VEGF_{121}$ by $5C_5$.

1. Standards of protein molecular weight, 2. Uninduced bacterial protein treated with $5C_5$, 3. Induced bacterial protein treated with $5C_5$, 4. Induced bacterial protein treated with normal mouse IgG.

DISCUSSION

Inhibition of tumor blood vessel growth is an important research area for tumor biotherapy in recent years. The process of angiogenesis involves stimulation of endothelial cell growth, motility and the release of proteases and the degradation of extracellular matrix. Blocking the overexpression of VEGF in tumor tissues and neutralizing its activities by monoclonal antibodies cast much light on VEGF related tumor therapy[3]. In this study, by using anti-VEGF monoclonal antibodies, we successfully neutralized the VEGF-induced HUVEC growth. These also clearly made the specificity of the prepared monoclonal antibodies.

There are different opinions on which kind of cells in tumor tissues can express VEGF. Wizigmann *et al*[4] proved that VEGF is mainly expressed by tumor cells. It can bind to its receptors on HUVEC and stimulate cell growth by paracrine ways. However, Plate Hetal[5] discovered that VEGF can be expressed by HUVEC in tumor tissues. Brown[6] said that the HUVECs both in the tumor tissue and the normal tissue can express VEGF. In our study, we demonstrated, by RT-PCR, the expression of VEGF in gastric carcinoma MGC803 cells. We also found, by ^{3}H-thymidine incorporation that the supernate of MGC803 cell s can promote the proliferation of HUVEC (data not shown). These suggested that MGC803 can express VEGF, but we failed to amplify VEGF cDNA from HUVEC.

To make clear about which kind of cells in tumor tissue can express VEGF is important for the blocking of its expression at genetic levels. We are preparing human anti-VEGF monoclonal antibodies by the phage display method to obtain the useful anti-VEGF antibodies for further research and its clinical application.

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