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Inhibiting angiogenesis with human single-chain variable fragment antibody targeting VEGF

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a highly specific angiogenesis factor which has crucial roles in the angiogenesis of tumors. Anti-angiogenesis agents can inhibit growth and metastasis of tumor cells. Single-chain variable fragments (scFv) have the same affinity as whole antibodies and smaller size, thus result in more tissue permeability and higher production yield.

In this research we aim to isolate a human scFv antibody against VEGF that inhibits angiogenesis. For that, we have used human scFv phage library to isolate a specific scFv antibody against binding site of VEGF. The human scFv phage library was amplified according to the manufacture protocol and panned against recombinant VEGF. ScFv antibody was isolated after five rounds of panning. Phage ELISA was used for detection of the highest affinity binder (HR6). Soluble HR6 scFv was expressed in non-suppressor strain of *Escherichia coli* HB2151 and purified using Ni-NTA chromatography. *In vivo* and *in vitro* function of the HR6 scFv was analyzed by chorioallantoic membrane assay and endothelial cell proliferation assay on VEGF stimulated HUVECs. Result of the cross reactivity showed that HR6 scFv specifically bounds to VEGF. The affinity was calculated to be 1.8×10^{-7} M. HR6 could stop HUVEC proliferation in a dose dependent manner and anti-angiogenesis activity was observed using 10 μg of HR6 in chorioallantoic membrane assay.

In this work, we demonstrate that a HR6 scFv selected from human library phage display specifically blocks VEGF signaling, furthermore, this scFv has an anti-angiogenesis effect and because of its small size has more tissue diffusion. The HR6 antibody was isolated form a human library thus, it is not immunogenic for humans and could serve as a potential therapeutic agent in cancer.

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Introduction

One of the most important mechanisms in tumor growth, especially in solid tumors is the angiogenesis process (Folkman, 2006). Angiogenesis is defined as an expansion of the blood vessels from previously existing blood vessels, and plays a significant role in the growth and metastasis of cancerous tumors (Folkman, 1971; Asano et al., 1999). In order to grow beyond 1–2 mm, solid tumors require specific blood source which is generated by angiogenesis process from the surrounding veins. Thus, inhibition of this process limits the supply of nutrient and oxygen to the tumor cells and is considered to be one of the effective strategies to battle cancer (Asano et al., 1999).

There are several factors contributing to the angiogenesis process, among them, vascular endothelial growth factor (VEGF) plays the most important role in both cancerous and normal physiologic conditions (Tammela et al., 2005). Because of its significance in the angiogenesis process, inhibition of VEGF is the most appropriate method to limit angiogenesis in various diseases (Pinedo and Slamon, 2000).

There are significant research data presented in the inhibition of VEGF expression or signaling pathway (Neufeld et al., 1999; Hicklin and Ellis, 2005). Several monoclonal antibodies have also been produced against VEGF which resulted in prevention or significant reduction of angiogenesis (Muller et al., 1998). However, there are limitations and difficulties in mass production and utilization of these antibodies. Production of mouse or humanized antibodies could be problematic because of their; dependence on the eukaryotic expression systems, long production time, difficulty of genetic manipulation, production of anti-mouse antibodies in human patients, and limited diffusion into solid tumors which reduces their application in treatment of cancer (Köhler and Milstein, 1975; Schroff et al., 1985; Hudson and Kortt, 1999; Ahmad et al., 2012).

Because of these limitations production of antibody fragments is becoming a more popular choice (Table 1). Smaller size and ease of genetic manipulation, expression in prokaryotic and eukaryotic systems and

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Table 1
Comparison between production criteria of whole monoclonal antibody and scFv antibody fragments (Hoogenboom et al., 1998; Greenwell and Rughooputh, 2001; de Marco, 2011; Schirrmann et al., 2011).

Items	Whole monoclonal antibody	scFv antibody fragment
Host cell	Hybridoma	Bacterial
Production time	Several months	Several weeks
Operation	Complicated	Relatively simple
Immune injection	Necessary	Avoidable
Humanized antibody	−	+
Cost	High	Low
Production capacity	Limited	Unlimited

compatibility with selection techniques such as phage display method, make production of antibody fragments more appropriate (de Marco, 2011; Ahmad et al., 2012; Ebrahimizadeh et al., 2013).

ScFv fragments are single chain variable domains of antibodies that can be easily expressed in a vast range of hosts including bacteria and can recognize their target with high affinity. Smaller size which increases tissue penetration and diffusion, ease of production and lower immunogenicity are among many of scFv antibodies advantages (Nelson and Reichert, 2009; Weisser and Hall, 2009; Tohidkia et al., 2013). Because of these advantages over whole antibodies, in this study we aimed to isolate a human scFv antibody that recognizes VEGF and prevents the angiogenesis process using phage display method.

Materials and methods

Preparation of scFv phagemid library

ScFv phagemid library was obtained from Tomlinson I + J (Life Sciences, Cambridge, UK) and transferred into *Escherichia coli* TG1. Transformed bacteria were cultured in a 30 ml SB medium (Super broth: Yeast extract 2%, Tryptone 3%, MOPS 1%) containing 100 μg/ml ampicillin. For release of phage particles expressing scFv antibodies, when the OD_{600} reached 0.5, 10^{12} pfu M13K07 helper phage (Amersham Pharmacia Biotech, USA) were added and culture was incubated in 37 °C for 30 min and then another 30 min at 37 °C/250 rpm. Kanamycin was added at final concentration of 50 μg/ml and the culture was incubated for 18 h at 37 °C/250 rpm. In order to isolate the recombinant phage particles, the bacterial cells were centrifuged at 5000 rpm for 20 min and the supernatant was collected. The supernatant was mixed with 20% (*v*/*v*) PEG solution (Polyethylene glycol 6000 (20%) in 2.5 M NaCl) and incubated for 2 h on ice. The recombinant phage particles were collected by centrifugation at 12,000 rpm for 20 min at 4 °C and then resuspended in 1% of the original volume in TBS (50 mM Tris–Cl, 150 mM NaCl, pH 7.5) + 1% BSA. Cell debris were removed by centrifugation at 12,000 rpm for 5 min. The solution was passed through a 0.45 μm filter and stored at 4 °C until further use.

Bio-panning

Recombinant VEGF was coated overnight at 4 °C in ELISA microplate wells using TBS buffer. For the first round of panning, 10 μg of VEGF was used. Wells were washed three times with 200 μl of TBS-T (TBS + 0.05% Tween-20) and blocked using 3% BSA in TBS. After one hour incubation at 37 °C and washing the wells using TBS-T, 100 μl of recombinant phage library was added to each well and the plate was incubated for 1 h at 37 °C. Then, wells were washed several times with TBS-T. Attached phages were eluted with 100 μl of Glycine-HCl (pH 2.2). Eluted phages were neutralized with 40 μl of Tris buffer (pH 9) and then added to 30 ml culture of *E. coli* TG1 bacteria at OD_{600} of 0.5. Phages were propagated as described under "Preparation of scFv phagemid library". For isolation of high affinity antibodies, after each round of panning, the intensity of washing buffer was increased by addition of 0.01% Tween-20.

Polyclonal phage-ELISA

Bio-panning was monitored using polyclonal phage-ELISA. VEGF and BSA (as control) were coated as previously described under "Bio-panning" in separate wells. Wells were washed and blocked as before. Phages obtained from each round of panning were added to the corresponding wells. After two hour incubation at 37 °C, the wells were washed three times, 100 μl anti-M13 antibody conjugated to HRP (Amersham) was added to each well. ELISA microplate was incubated for 1 h at 37 °C and washed three times using TBS-T. TMB substrate (3,3′,5,5′-Tetramethylbenzidin) (Sigma, USA) was added to each well. After 10–15 min incubation at 37 °C, the reaction was stopped with 100 μl of 2.5 N H_2SO_4. The absorbance was read at 450 nm.

scFv selection

The bio-panning round that showed the highest absorbance in the polyclonal phage-ELISA was used for selection of monoclonal scFv antibody. *E. coli* TG1 bacteria transformed with phagemids were plated on LB agar (Luria Bertani Agar) containing 100 μg/ml of ampicillin. Single colonies were picked and cultured in LB medium containing 100 μg/ml ampicillin. Phages were propagated as described under "Preparation of scFv phagemid library". Monoclonal phage-ELISA was carried out using phages obtained from each colony as described under "Polyclonal phage-ELISA". The clone with the highest affinity (HR6) was selected for production of soluble scFv antibody.

Expression and purification of HR6 clone

HR6 Phagemid was extracted using plasmid purification kit (Bioneer, South Korea) and transferred into non-suppressor HB2151 bacteria. The transformed bacteria were cultured in SB medium at 37 °C/250 rpm. Expression was induced with 1 mM IPTG when OD_{600} reached 0.5. Cells were collected after 4 h and expression was analyzed on 12% SDS-PAGE. ScFv was purified using Ni-NTA chromatography (Qiagen).

Specificity of HR6

To evaluate the specificity of the HR6 scFv antibody, 10 μg of various antigens including PSMA (Prostate specific membrane antigen), NMP-22 (Nuclear matrix protein 22), HER-2 (Human epidermal growth factor receptor 2), SK (Streptokinase) and BSA was coated in ELISA

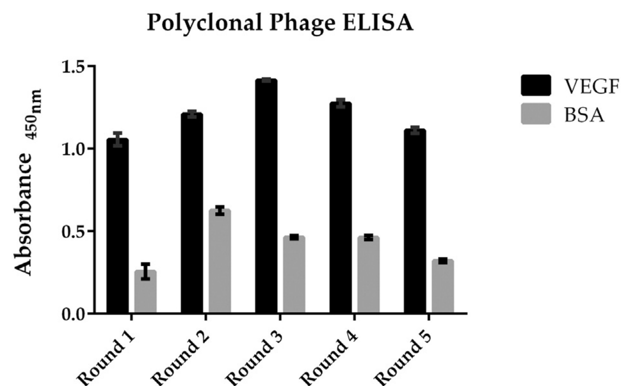

Fig. 1. Results of polyclonal-phage ELISA. Phages obtained after each round of panning were tested against rVEGF by ELISA. Round three showed the highest absorbance and was used for selection of monoclonal scFv antibodies.

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