Down-regulation of vascular endothelial growth factor expression by anti-her2/neu single chain antibodies

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Abstract HER-2/neu is overexpressed in 25–30% of
breast tumors. Signaling through HER-2/neu leads to an
increase in the production of vascular endothelial growth
factor (VEGF) and enhances angiogenesis. We evaluated
the effects of three specific anti-HER2/neu single chain-Fv
(scFv) antibodies on the expression level of VEGF in
HER2/neu-expressing breast cancer cell lines. A nonim-
munized human scFv library was panned against three
epitopes of HER2/neu. BT-474 human breast cancer cell
line was treated with three specific anti-HER2/neu scFv
antibodies and the amount of VEGF gene transcript was
determined by quantitative real-time PCR. The expression
of VEGF protein was analyzed by western blot. All three
scFv antibodies along with their combination inhibited
VEGF expression at both the gene and protein levels. Our
results show that anti-HER2/neu recombinant antibodies
can be considered as anti-angiogenic agents in HER2/neu-
positive breast cancers.

Keywords Single-chain antibody · HER2/neu · VEGF ·
Angiogenesis · Breast cancer

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Introduction

HER-2/neu (Human epidermal growth factor receptor 2,
also known as ErbB2) is a 185-kDa transmembrane protein
and a member of the human epidermal growth factor
receptor (EGFR) family of tyrosine kinase receptors [1].
Overexpression of this protein has been observed in
25–30% of breast cancers due to its gene amplification [2].
The ErbB family plays vital roles in cell growth and dif-
ferentiation processes in embryonic and adult tissues.
These receptor proteins are normally coexpressed at dif-
ferent levels in diverse tissues, excluding the hematopoietic
system. HER2/neu is expressed in numerous normal and
malignant tissues of epithelial origin, including breast,
ovary, lung, gastrointestinal, endometrium, and central
nervous system [3]. HER2/neu has no known ligand and
can form dimers when overexpressed. In contrast, other
receptors of the ErbB family have several known ligands
that promote dimer formation on binding. The ErbB
receptors can form both homo- and heterodimers with
HER2/neu acting as the preferred dimer partner. The
activation of these receptors either by overexpression or by
ligand binding leads to the activation of intracellular
growth-promoting pathways and thus promotes tumor
growth [4, 5]. Overexpression of HER2/neu dysregulates
several critical pathways in the cell, including cell growth,
proliferation, migration, and cell adhesion [6, 7]. More-
over, HER2/neu signaling leads to an increase in vascular
endothelial growth factor (VEGF) and, therefore, is thought
to enhance angiogenesis [8].

Preclinical data have identified a critical role for VEGF
in endothelial cell proliferation, migration, and invasion
[9]. In addition, VEGF has anti-apoptotic effects for
endothelial cells, stimulating various inhibitors of pro-
grammed cell death [10–13]. Finally, VEGF is a potent

regulator of vascular permeability. Research has shown that VEGF-overexpressing cancer cells are capable of inducing disruption of the endothelial cell basement membrane, possibly contributing to the ability of breast tumors to undergo successful metastasis [14].

Monoclonal antibodies are the most widely used form of cancer immunotherapy at this time. Herceptin, a humanized monoclonal antibody, is one of the antibodies that has been approved by the United States Food and Drug Administration (FDA) for the treatment of patients with HER2/neu-overexpressing breast cancers [15, 16]. However, only 30% of HER2/neu-overexpressing breast cancers respond to Herceptin as a single agent, and when it is used in combined chemotherapy, patients show secondary resistance [17, 18]. In addition to Herceptin, lapatinib is the only therapy approved by the FDA for use in patients with HER2/neu-positive metastatic breast cancer. Lapatinib, a small molecule, is a dual tyrosine-kinase inhibitor of HER1 and HER2/neu [19]. However, treatment with these drugs causes multiple life-threatening side effects that limit their use [20–22]. Thus, because of deficiencies in current therapies, the search for a new therapeutic agent is still ongoing.

Advances in recombinant DNA technology facilitated the production of smaller recombinant antibody fragments. Recombinant antibodies are available in a wide spectrum of formats. The most popular format appears to be the single-chain variable fragment (scFv). ScFv molecules are the smallest antibody fragments (26–27 kDa) [23]. Human scFv antibodies have improved pharmacokinetic properties because of their lower retention times in nontarget tissues, better target tissue penetration and clearance, and non-human anti-mouse antibody response [24, 25]. Because of these properties, human scFvs perform significantly better than antibody molecules in therapeutic applications.

In this study, we assessed the effects of three specific anti-HER2/neu scFv antibodies on the expression of VEGF in the HER2/neu-expressing breast cancer cell line.

Materials and methods

Selection of anti-HER2/neu scFv

A phage antibody display library and scFv antibodies were produced essentially as described previously [26]. The specific anti-HER2/neu antibodies were selected by panning the phage library against three HER2/neu epitopes. Peptides [10 μg/ml in phosphate buffered saline (PBS)] were used to coat an immunotube (Nunc, Roskilde, Denmark) overnight at 4°C. The tubes were washed with PBS and blocked 2 h at 37°C with 10% fetal calf serum (FCS) and 2% skimmed milk in PBS. Then, the tubes were

washed four times with PBS-Tween 20 (PBST) and four times with PBS. Phage supernatant (10^9 plaque-forming units (pfu) per ml) was added and incubated for 1 h at room temperature. The bound phagemids were eluted by adding log phase TG1 *E. coli* and incubating at 37°C for 1 h. The tubes were centrifuged and the pellets were plated onto LB agar plates. Helper phage M13KO7 was used to rescue the phage-transformed *E. coli*. Four rounds of panning were performed to select specific and high-affinity scFv antibodies against three HER2/neu epitopes (epitope I: TGRCEKCSKPCARV-GYGL, epitope II: KIFGSLAFLPESFD, and epitope III: PPFCVARCPSG). MvaI DNA fingerprinting was performed on 20 colonies of each panned library to confirm enrichment of scFv clones. After PCR amplification of the scFv inserts, in order to select specific scFv against each peptide using the common fingerprinting pattern, each product was digested with MvaI (Roche Diagnostic GmbH, Mannheim, Germany) at 37°C for 2 h and run on 2% agarose gel.

Purification of scFv antibodies

The selected scFv antibody against each peptide was precipitated and purified by PEGylation. Phage rescue supernatant (30 ml) was mixed with 7.5 ml 20% PEG-8000/2.5 M NaCl and incubated on ice for 30 min. The mixture was centrifuged at $11,000g$ for 20 min then the supernatant was discarded and the pellet, containing scFv antibodies, was resuspended in 500 μl STE buffer and transferred to a fresh eppendorf tube. The tube was centrifuged at $14,000g$ for 10 min to remove the PEG and the supernatant was collected.

Measuring scFvs concentration

The scFv concentration in each phage rescue supernatant was measured by adding 10 μl of phage antibody supernatant to 1 ml TG1 *E. coli* in a logarithmic growth phase and incubating with shaking at 37°C for 1 h, The cultures were diluted serially and plated onto 2TY/ampicillin plates. The number of colonies per dilution was counted and scFv concentration titer per ml was determined.

Cell culture and antibody treatment

BT-474 HER2/neu-positive human breast cancer cell line (NCBI, Pasteur Institute, Tehran, Iran) was grown in RPMI-1640 medium (BioSera, Ringmer, UK) and supplemented with 15% fetal bovine serum (BioSera), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were grown at 37°C in an incubator with 5% CO_2. The cells were treated with anti-HER2/neu scFv antibodies ($IC_{50} = 500$ scFv/cell) and Herceptin ($IC_{50} = 150$ ng/ml) as the positive

control [27]. After 48 h, real-time RT-PCR and western blot assays were performed.

Quantitative real-time RT-PCR

Total RNA was extracted from 10^6 cultured cells using TRIzol solution (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were estimated by spectrometry. Total RNA (5 μg) was used for cDNA synthesis with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Quantitative real-time RT-PCR was performed in triplicate (Chromo4 Real-time PCR Detector, Bio-Rad, CA, USA) using SYBR Green I. The amount of 18s RNA housekeeping gene transcripts was used as a reference for the level of VEGF gene expression. Amplification was carried out in a total volume of 20 μl containing 0.5 μg cDNA prepared as described above, 3.0 pmol each of VEGF (sense: 5$'$-CCCACTGAGGAGTC CAACAT-3$'$ and antisense: 5$'$-TTTCTTGCGCTTTCGT TTTT-3$'$) and 18s RNA (sense: 5$'$-CGAACGTCTGCCCT ATCAACTT-3$'$, and antisense: 5$'$-ACCCGTGGTCACCA TGGTA-3$'$) primers, 1× reaction mixture consisting of FastStart DNA polymerase, reaction buffer, dNTPs, DNA Master SYBR Green I (Applied Biosystems, CA, USA). Primers were designed by Primer3 open source software (SourceForge, USA).

Thermal cycling for both genes was initiated with a denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 30 s and elongation at 60°C for 40 s. Melting curve analysis of the amplification products was performed at the end of each PCR by cooling the samples to 60°C and then increasing the temperature to 95°C at 0.2°C/s.

The experiment was repeated thrice for statistical analysis.

Western blot analysis

A RIPA lysis buffer [containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0] was used to lyse 10^6 cells. Protease inhibitor cocktail (1×) (Sigma-Aldrich, St. Louis, MO, USA) was added to the RIPA buffer immediately before use. The lysates were centrifuged at 12,000 rpm for 20 min to pellet the cell debris, and the supernatants were collected. The protein concentration was determined by the Bradford assay. Protein (20 μg) was loaded onto a 12% gel for SDS-PAGE. After electrophoresis, the gels were blotted onto PVDF membranes. The blots were blocked with 5% skim milk in Tris-buffered saline Tween-20 buffer (10 mM Tris, pH 8.0,

150 mM NaCl, and 0.05% Tween-20) for 2 h at room temperature. The membranes were then incubated with the primary anti-VEGF antibody (ab1316, Abcam, Cambridge, MA, USA) and anti-β-actin antibody (ab6276, Abcam) in fresh 5% skim milk-PBST buffer at 4°C overnight. The membranes were washed and incubated with secondary HRP-conjugated goat anti-mouse IgG antibody (ab6728, Abcam). The bands were visualized in a Pierce ECL chemiluminescence system and quantified by densitometry with GelPro software.

Statistical analysis

The level of VEGF transcript expression was determined from the ΔCt and $2^{(-\Delta Ct)}$ formulas. Data were analyzed with the Mann–Whitney U test to compare gene expression level between antibody-treated and untreated cells. All data are presented as the mean $\pm$ standard error. A P value <0.05 was considered statistically significant.

Results

Anti-HER2/neu scFv antibodies

Figure 1 shows DNA fingerprinting of 20 panned clones against peptide I (A), peptide II (B), and peptide III (C). Common patterns were obtained for all colonies panned against each peptide, and one colony from each pattern was used for further investigation.

Inhibition of VEGF gene expression by anti-HER2/neu scFvs

As shown in Fig. 2, a significant down-regulation of VEGF gene expression was obtained after 48 h of treatment with 500 scFv/cell anti-HER2/neu scFv-I, scFv-II, scFv-III, and their combination ($P < 0.05$). The levels of VEGF gene expression were 2.2×10^{-4}, 2×10^{-4}, 2.6×10^{-4}, and 2.1×10^{-4}, respectively, and expression in untreated cells was 5.2×10^{-4}.

Reduction of VEGF protein expression by anti-HER2/neu scFv

The expression of VEGF protein in the HER2/neu-positive breast cancer cell line, BT-474, was measured by western blot analysis. As shown in Fig. 3, after 48 h of treatment with 500 scFv/cell anti-HER2/neu scFv-I, scFv-II, and scFv-III separately and in combination, VEGF protein expression decreased.

Fig. 1 DNA fingerprinting pattern of 20 panned clones (*Lanes* 1-20) of scFv-I **a**, scFv-II **b**, and scFv-III **c**. M: Marker
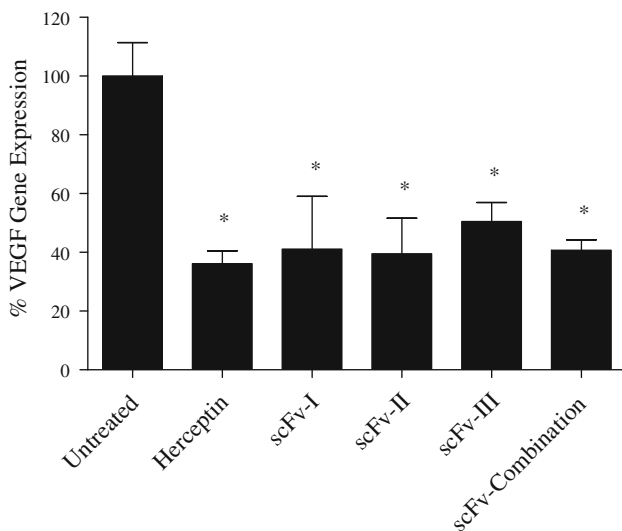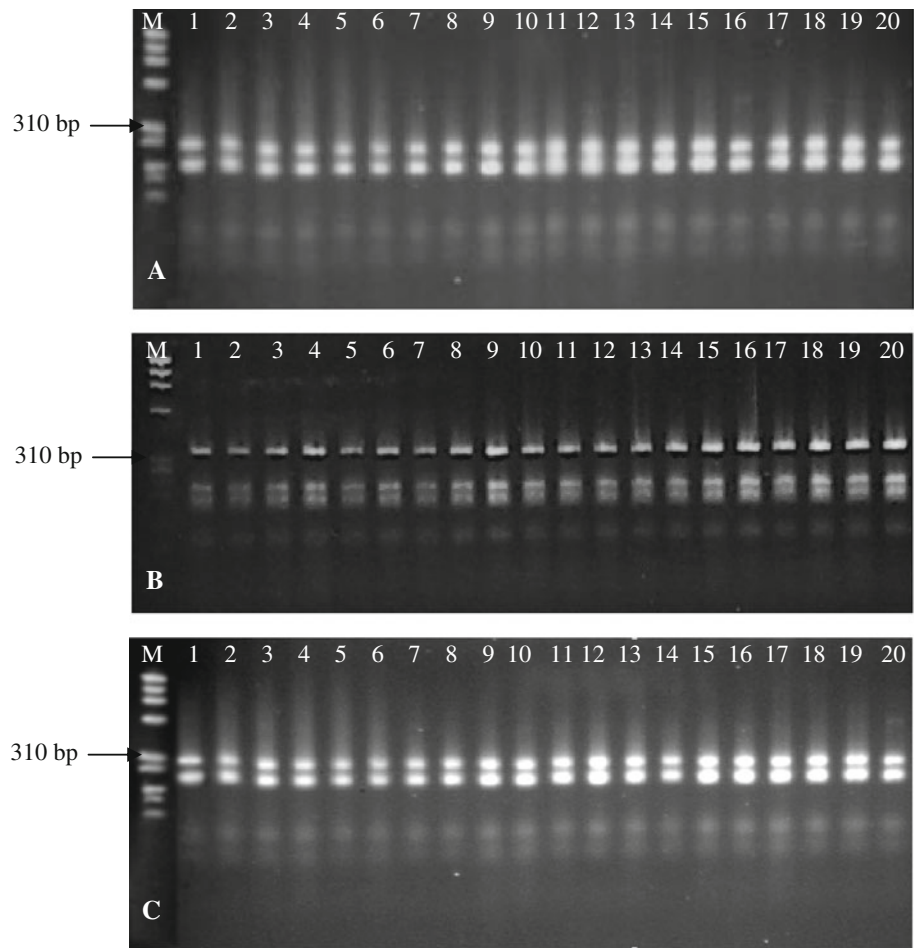

Fig. 2 Down-regulation of VEGF gene expression by anti-HER2/neu scFv antibodies in BT-474 human breast cancer cell line. Cells were treated with single-chain Fv-I, II and III separately and in combination and also Herceptin (*positive control*). The data are presented as the mean ± standard error. *$P < 0.05$

Discussion

HER-2/neu overexpression is associated with markedly aggressive forms of cancer. Although the precise signaling pathways by which HER2/neu regulates more aggressive clinical phenotype are not fully understood, angiogenesis is well established as a vital step for tumorigenesis and metastasis [28, 29]. VEGF is one of the most important inducers of tumor angiogenesis [30, 31]. Clinical studies have shown that VEGF overexpression in the early-stage breast cancer is related to augmented metastatic potential [32, 33]. The majority of HER2/neu-positive breast cancers overexpress VEGF, and elevated serum VEGF has been reported in invasive breast cancers [34–38], suggesting that VEGF-induced angiogenesis may be vital for this type of breast tumor. Moreover, two important transcriptional factors, hypoxia-inducible factor 1 and Sp1, which both play a critical role in the PI3K-AKT pathway, are responsible for the up-regulation of VEGF by HER2/neu signaling [39]. Therefore, blocking HER2/neu signaling with anti-HER2/neu antibodies is a practical approach to
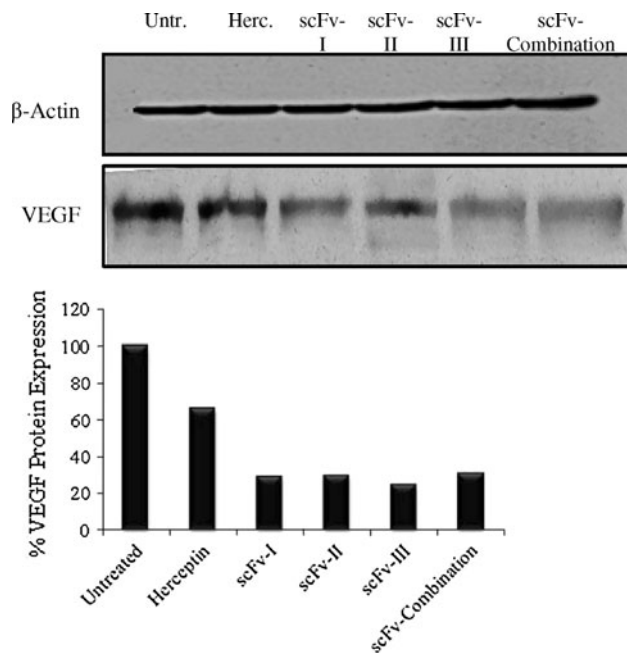
Fig. 3 Reduction of VEGF protein expression induced by anti-HER2/neu scFv antibodies in BT-474 human breast cancer cell line. After 48 h of treatment, cell lysates were immunoblotted with antibodies specific for VEGF and β-actin (internal control). Anti-HER2/neu scFv antibodies decreased the VEGF protein expression

antiangiogenic tumor therapy. Wen et al. [40] showed that activation of HER2/neu up-regulates the expression of two proangiogenic factors, VEGF and IL-8, and one antiangiogenic factor, TSP-1, in vitro and in vivo. Their results also indicated that inhibition of HER2/neu signaling by the anti-HER2/neu monoclonal antibody, trastuzumab (Herceptin), down-regulates VEGF production by inhibiting the PI3-AKT pathway. Although bevacizumab (Avastin), a humanized recombinant monoclonal antibody, is a VEGF-neutralizing antibody that specifically inhibits the binding of VEGF to its high-affinity receptors [41], it has been demonstrated that blocking of HER2/neu decreases the expression of VEGF in HER2/neu-overexpressing breast cancers [38, 42]. According to a hypothesis proposed by Pegram et al. [38], the up-regulation of VEGF in HER2/neu-overexpressing breast cancers contributes to the aggressive phenotype observed in HER2/neu-positive cases, and the angiogenic switch associated with HER2/neu can be attenuated by Herceptin. Therefore, a potentially useful approach is to target HER2/neu and VEGF simultaneously using anti-HER2/neu antibodies.

This study examined the effects of three specific scFv antibodies against HER2/neu on the expression of VEGF at both the gene and protein levels in comparison to the effects of Herceptin. All three scFv antibodies individually and in combination were able to significantly inhibit VEGF gene expression in the BT-474 cell line. Yen et al. [39]

have shown that in the HER family, HER1/HER2 and HER2/HER3 heterodimer formations are the most potent inducers of VEGF gene expression. It has been also shown that monoclonal antibodies may suppress HER2/neu signaling in vitro by inhibiting dimerization [43]. Therefore, it seems that selected scFv antibodies block HER2/neu signaling through the inhibition of HER2/neu dimerization.

The results of real-time PCR showed that the gene expression levels of VEGF in cells treated with scFv-I, -II,-III, and their combination decreased by 41, 39, 50, and 40%, respectively, compared to untreated cells ($P < 0.05$). Levels of VEGF gene expression in scFv antibody-treated cells did not differ significantly from Herceptin-treated cells (positive control). This finding suggests that the inhibitory effect of scFv antibodies on VEGF gene expression is the same as the effect of Herceptin. The reduction in VEGF protein expression after treatment with scFv-I, scFv-II, scFv-III, and their combination was documented by western blot assay. These data were consistent with the real-time PCR results.

Our results document the inhibition of VEGF expression at both the mRNA and protein levels by anti-HER2/neu scFv antibodies. These results support a possible role for these recombinant antibodies as anti-angiogenic agents in HER2/neu-positive breast cancer immunotherapy.

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