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Anti-HER2/neu Peptide Producing Vector System for Biologic Therapy - Is It Possible to Mass-produce the Peptide?

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A humanized monoclonal antibody against HER2 has been using in a clinical setting and has been shown to possess therapeutic properties. A mimetic peptide against HER2 was also reported to bind to the HER2 receptor with some therapeutic potential.

Based on a previous report and the sequence of Herceptin, we designed oligonucleotides of anti-HER2 mimetic peptides, named V2 and V3 peptides, in order to develop a peptide-producing vector system for biologic therapy against HER2-overexpressing cancers. We also adopted the sequence of a previously reported mimetic peptide, V1 (Park BW et al. Nat. Biotechnol, 2000, 18:194-198), as a reference peptide. We examined the effects of the V2 and V3 peptides against the HER2-overexpressing cell lines, SK-BR-3 and T6-17. Transient transfection of the construct expressing V1 and V2 inhibited cell proliferation in HER2-overexpressing cell lines by 20 - 30%, but had no effect on the HER2-negative NIH3T3 cells. The proliferation inhibition shown by V2 was slightly better than that shown by V1. Recombinant peptides V2 and V3 were produced on a large scale in an *E. coli* system, and the V2 peptide showed anti-HER2-specific tumor cell proliferation inhibition of 10% to 30%.

Current results suggest that anti-HER2 mimetic peptides, overexpressed by a constitutive promoter or produced in an *E. coli* system, could specifically inhibit the proliferation of HER2-expressing cancer cells. Further efforts to augment the biologic specificity and efficacy and to develop new technologies for the purification of the peptide from the E coli system are needed.

Key Words: HER2/neu; mimetic peptide; biologic therapy

INTRODUCTION

HER2/neu (HER2, also called c-erbB-2) belongs to a receptor tyrosine kinase family group that is characterized by ectodomains with two cysteine-rich sequences.[1,2] Unlike other members of this family, the ligand for HER2 remains unknown and its activity is exerted mainly by heterodimerization with other members of the family.[3,4] Because continual expression of neu (rat homologue of HER2) is necessary for the maintenance of the neoplastic phenotype of neu-transformed cells[5] and anti-neu monoclonal antibody (mAb) 7.16.4 causes phenotypic reversal of neu-overexpressing tumor cells *in vitro* and *in vivo*,[6,7] it has been an attractive target for tumor therapy.[8]

With extensive studies being undertaken on the potential use of anti-HER2/neu monoclonal antibodies in the diagnosis and treatment of cancer, a lot of mouse monoclonal anti-HER2 monoclonal antibodies have been developed,[9-11] and finally one of these antibodies has been humanized and was approved for clinical use in 1998.[12]

There are some limitations in the clinical use of full-length mAb, due to the expense or difficulty of commercial-scale production, the limited ability for mAb to penetrate cells and tissues,[13] and the possibility of severe side effects in terms of its

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immune responses.[14] Smaller peptides, reduced in size, have been highlighted as potential therapeutic molecules[15-20] for clinical application.

With a structure-based approach, an exocyclic anti-HER2 mimetic peptide was developed and which demonstrated similar anti-tumor activity but lower binding affinity compared to the humanized monoclonal antibody.[14,21] We designed peptides based on this previous report[14] and tested their bioactivities. In this study, we examined the possibility of performing mass-production of a small peptide displaying bioactivity in an *in vitro* system.

MATERIALS AND METHODS

Cell lines

T6-17 cells (a gift from Dr. Mark I. Greene at the University of Pennsylvania, Philadelphia, PA, U.S.A.) were derived from NIH3T3 cells transfected with HER2. SK-BR-3 cells, T6 - 17 cells, NIH 3T3 cells were maintained in DMEM containing 10% (vol/vol) FBS, 1% L-glutamine, and 1% penicillin/streptomycin at 37℃, 95% humidity, and 5% CO_2.

Design of olignucleotides and vector

We made three oligonucleotides for the V1, V2, and V3 peptides. Peptide V1 is an anti-HER2 mimetic peptide previously described in the literature.[14] For the formation of its intramolecular cyclic structure, peptide V3 contains an additional 17 amino acids sequence at both the N- and C-termini of V1, giving rise to a coiled-coil structure, which plays a role in the tetramer formation of the lac repressor. Peptide V2 has the same structure as peptide V3, except that it has the epitope binding sequence of Herceptin, instead of the anti-HER2 mimetic peptide (Table 1). Each oligonucleotide was inserted in between the HindIII and ApaI sites of the pSecTag2/zeocin vector (Invitrogen, Carlsbad, CA, USA). The oligonucleotides for V2 and V3 were also inserted in between the BamH I and Sal I sites of the pQE30 vector (Invitrogen, Carlsbad, CA, USA) for the production of recombinant protein.

Cell proliferation assay

We analyzed the cell proliferation using the MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} assay for mitochondria viability in an adherent cell assay. Proliferation was measured by standard MTT assay.[22] Cells were plated in 96-well plates (3,000 cells/well) in DMEM-10% FBS with indicated amount of peptide, and were incubated for 24 hours. Then a total of 25 uL of MTT solution (5 mg/ml in PBS) was added to each well, and after 4 hours of incubation at 37℃, 100 uL of lysis buffer (20% w/v of SDS, 50% N,N-dimethyl formamide, pH 4.7) were added. After overnight incubation at 37℃, the optical density was measured at 570 nm to assess cell proliferation using an ELISA reader. The number of cells used in these assays was determined to be within the linear range for these cell types. Each cell line was examined in triplicate in three separate experiments.

Generation of transient transfectants and cell proliferation assay

Transient tansfectants were generated by transfecting the pSec2Tag2 (vector only), -V1, or -V2 plasmid into the indicated cells by using Lipofectamine-plus (Gibco BRL, Gaithersburg, MD, USA), followed which cell proliferation was measured by means of the MTT assay.[22] For the transient transfection, cells were plated at a density of 3,000 cells/well. On the next day, transfection was performed with 0.1 ug of pSec2Tag2

Table 1. Sequences of Peptides

Peptide	Amino acid sequence
V1	**FCDGFYACYMDV**
V2	RALADSLMQLARQVSRL**WGGDGFYAMDVSP**RALADSLMQLARQVSRL
V3	RALADSLMQLARQVSRL**WFCDGFYACYMDVSP**RALADSLMQLARQVSRL

(vector only), -V1, or V2 plasmid construct. Transfection was performed using Lipofectamine-plus transfection reagent (Gibco BRL, Gaithersburg, MD, USA) for 3 hours according to the manufacturer's instructions. After 2 days, cell proliferation was assessed by means of the MTT assay.

Preparation of recombinant V2 peptide

Recombinant V2-peptide was expressed in *E. coli* SG13009. Expresssion plasmid of pQE30-V2 was prepared by inserting V2-oligonucleotide into the BamHI/SalI sites of pQE30 (Invitrogen, Carlsbad, CA, USA). The bacteria, freshly transformed with expression vector, were grown to mid-log phase, and the peptide was induced for 6 hours with 0.5 mM or 1mM of isopropyl-1-thio-B-D-galactopyranoside (IPTG). The bacteria were harvested by centrifugation and disrupted by sonication. The recombinant peptide, containing the histidine-tag, was purified to homogeneity by Ni-NTA-agarose (Qiagen, Hilden, Germany) chromatography. The purity of the recombinant peptide was verified by means of SDS-polyacrylamide gel electrophoresis, followed by Coomassie Brilliant Blue staining, and the concentration was measured by Bradford protein assay.

RESULTS

Design of peptide

For the formation of the intramolecular cyclic structure, an additional 17 amino acids sequence (C-terminal α-helix of lac repressor, residues 341-357) were added to both the N- and C-termini of the epitope-binding core sequences, which produces the peptides V2 and V3 (Table 1). The C-terminal α-helix of the lac repressor residue has a coiled-coil structure, which plays a role in the tetramer formation of the lac repressor, and which was expected to increase the cyclization of the peptide.

Cell proliferation inhibition with transient transfection

We analyzed the cell proliferation of the peptide-treated cells using the standard MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} assay.[22] A transient transfection of the constructs expressing V1 and V2 resulted in 5.5-27.3% and 17.6-28% inhibition of cell proliferation in SK-BR-3 and T6-17, respectively, compared with that in NIH3T3 cells (Fig. 1). The cell proliferation inhibition effect of V1 was not significant on the SK-BR-3 and NIH3T3 cells, but was significant on the T6-17 cells. V2 showed

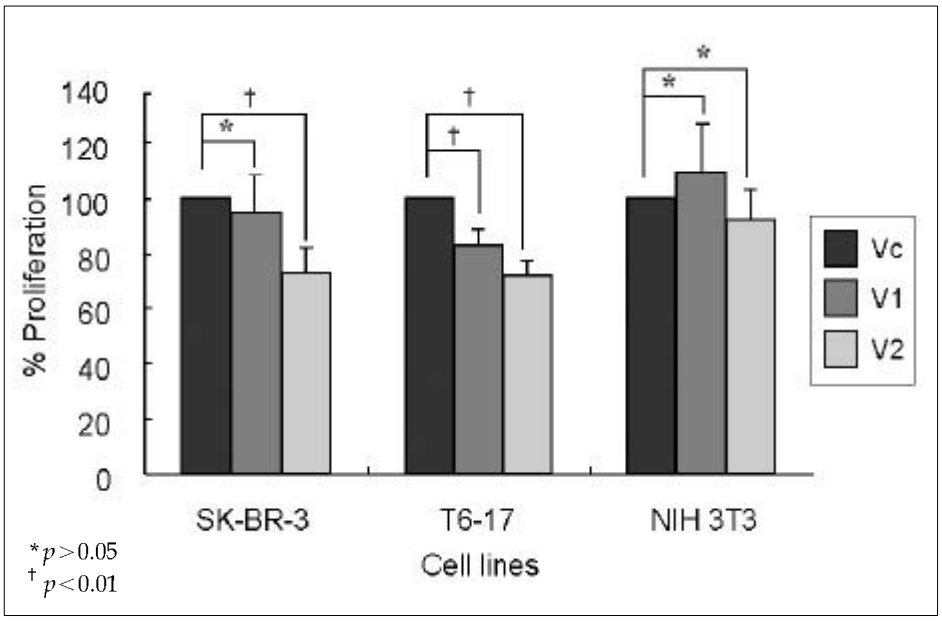

Fig. 1. Inhibition of cell proliferation by transient transfection of the constructs expressing V1 and V2. Effect of V1 and V2 transfection on proliferation of cells expressing HER2-SK-BR-3 and T6-17 cells, and HER-negative NIH3T3 cells, as compared with empty vector Vc. Cell proliferation was measured by the standard MTT assay. Each cell line was examined in triplicate in three separate experiments.

significant cell proliferation inhibition on both the SK-BR-3 and T6-17 cells, but showed no significant inhibition on the NIH3T3 cells (Fig. 1). Both V1 and V2 were more potent on the T6-17 cells than on the SK-BR-3 cells and V2 showed a slightly better effect than V1.

Production of recombinant V2 peptide

To investigate the possibility of mass-producing the mimetic peptide, we transformed *E. coli* SG13009 using pQE30-V2 plasmid. The 9kD-sized recombinant peptide of V2 was induced with 0.5 or 1mM of IPTG and its presence was confirmed in the supernatant as well as in the cell lysate. (Fig. 2A) The induction was much more efficient with 1 mM IPTG. The induced peptide was purified using Ni-NTA-agarose (Qiagen, Hilden, Germany) chromatography and the 9kD-sized peptide was isolated, with the disappearance of most of the non-specific peptide bands (Fig. 2B). The peptide was eluted in phosphate buffered saline (PBS) buffer with 4 M of UREA and 250 mM imidazole, and was diluted in the same volume of PBS. The final concentration of the peptide was 3 ug/ul of PBS with 2M urea and 125 mM immidazole. This result suggests that a rationally designed mimetic peptide can be produced in the *E. coli* system. Peptide V3 was also induced by the same procedure but the efficiency was poor and the peptide showed a solubility problem, as reported by others.[21]

Cell proliferation inhibition by recombinant peptide

We analyzed the growth characteristics of the recombinant peptide treated cells using the MTT assay for mitochondrial viability.[22] Each cell line was examined in triplicate in three separate experiments. In the two cell lines overexpressing HER2 receptors, treatment with recombinant peptide V2 at various concentrations resulted in 17 - 23% inhibition of cell proliferation in a dose-dependent manner, but the inhibitory effect on the NIH3T3 cells, was not significant (Fig. 3). These data suggest that the recombinant peptide V2 specifically inhibits the proliferation of HER2-overexpressing cells.

Poor solubility of peptidomimetics is often reported as a limitation of their clinical use[21] and a precipitation problem was observed in the treatment of a high-dose, over 3 ug/well, of the recombinant peptide V2. We also made a recombinant

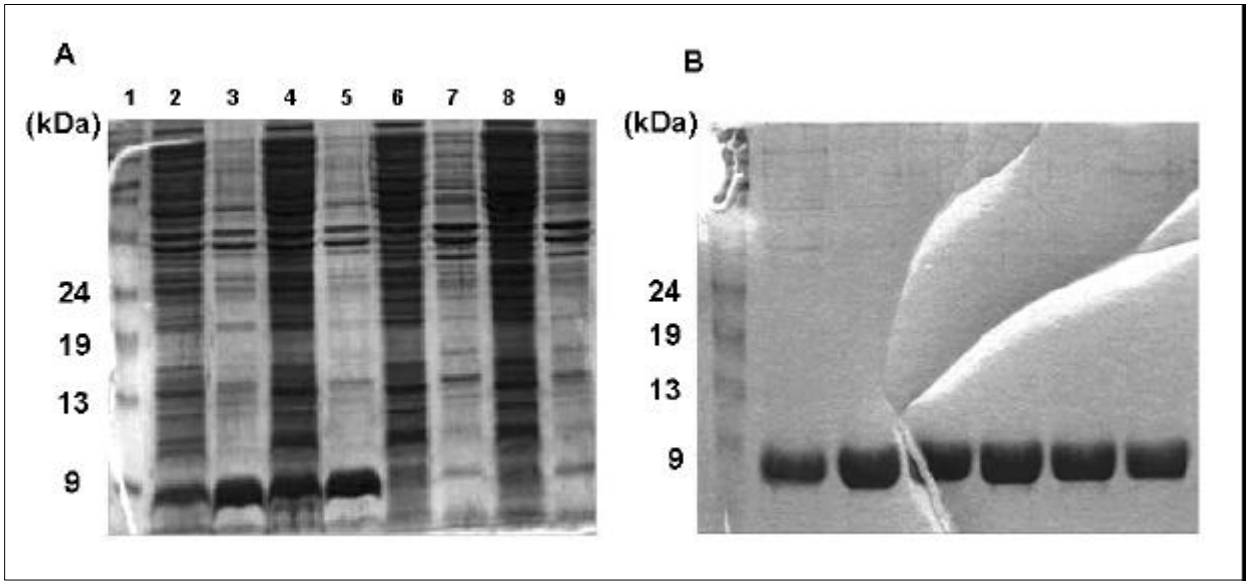

Fig. 2. Induction and purification of recombinant peptide V2. (A) Induction of recombinant peptide V2 by IPTG. Lane 1 showed size ladders, lanes 2-5 were induced with 1mM of IPTG and lanes 6-9 by 0.5mM IPTG. Lanes 2, 4, 6, and 8 showed bands from supernatant and lanes 3, 5, 7, and 9 from cell lysates. (B) Purification of recombinant peptide V2 with Ni-NTA-agarose. All the lanes showed a 9kD-sized peptide band and most non-specific bands disappeared.

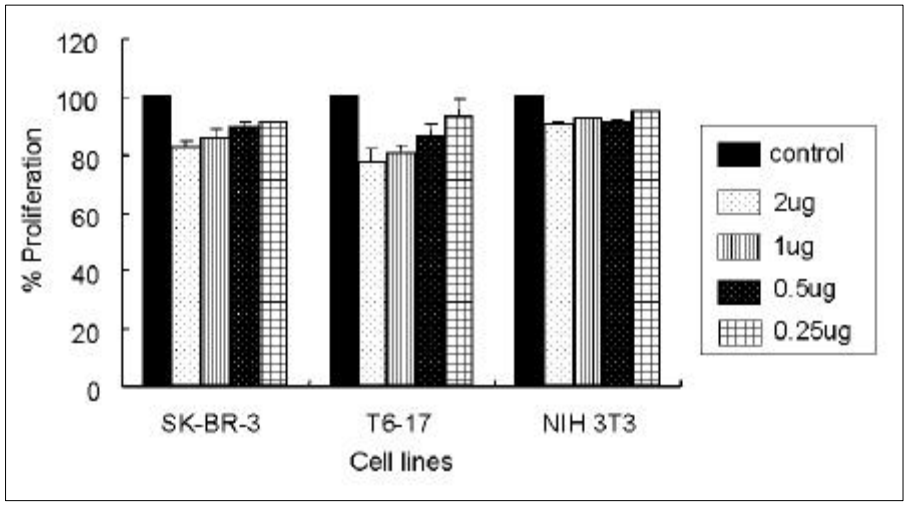

Fig. 3. Inhibition of cell proliferation by peptide V2. Effect of peptide V2 on proliferation of cells expressing HER2-SK-BR-3 and T6-17 cells, and HER-negative NIH3T3 cells. Cell proliferation was measured by the standard MTT assay. Each cell line was examined in triplicate in three separate experiments.

peptide V3, but this peptide gave rise to much more serious precipitation problems.

DISCUSSION

Extensive studies on the clinical use of anti-HER2/neu monoclonal antibodies finally led to a humanized monoclonal antibody, Herceptin, being produced, and in 1998, it was approved for clinical use. Since then several investigators demonstated improved survival rates of the metastatic breast cancer patients by using Herceptin in combination with other chemotherapeutic agents.[23,24]

Because of some limitations of full-length mAbs in clinical use,[13,14,25] it has been suggested that smaller peptides would act as more appropriate therapeutic molecules for clinical application.[15-20] In general, epitopes can be sequential or conformational. Similar conformations might be be possible, even if they contained sequentially unrelated protein segments,[26] and an exocyclic mimetic peptide with similar conformation to mAb was developed.[14]

Since small peptides give rise to a variety of conformations in solution, the cyclization of peptides has been adopted as a method of constraining the conformation, which, in some cases, also leads to the peptide improved binding affinity,[27] although in other cases it can give rise to a conformation which is unable to recognize the receptor.[26]

Although the tridimensional structure of HER2 is complex, an exocyclic anti-HER2 mimetic peptide, showing anti-tumor activity similar to that of mAb, has been reported.[14,21] However, it is also very expensive and is not easy to mass-produce. In this study, we attempted to develop an *in vitro* system of peptide-production, which could be used on a large scale, and which would therefore have the possibility of being used for the mass-production of the mimetic peptide.

The transient transfection of pSecTag-V1 and pSec-Tag-V2 induced 5.5 - 27.3% proliferation inhibition for SK-BR-3 cells and 17.6 - 28% for T6-17 cells, respectively. However, no significant difference in the proliferation inhibition of HER2-negative NIH3T3 cells was observed between pSecTag, pSecTag-V1 and pSecTag-V2. As shown in Fig. 1, V1 and V2 induced proliferation inhibition in the HER2-expressing cells but not in the HER2-negative cells, which indicated receptor specificity. There was a slight difference in length between V1 and V2. V2 has two more amino acids in addition to the core sequence, which may give slightly more stability to the peptide and may the cause of the small difference in the bioactivity of the two peptides.

Although cyclization might lead to a conformation unable to be recognized by the antibody, causing it to lose its inhibitory function,[26] we adopted a strategy of cyclization based on that described in a previous report.[14] Since similar conformations could be formed by sequentially unrelated protein segments, depending on the amino acid composition,[26] 17 amino acids of the

C-terminal α-helix of the lac repressor were added to the core epitope-binding sequence, in order to improve the cyclization. Finally, a 9Kd-sized peptide was induced with IPTG and was purified on a large scale in the *E. coli* system. In this study, we induced a small peptide using a recombinant technique in an *E. coli* system, which showed 7-23% of cell proliferation inhibition in a dose-dependent manner for HER2- overexpressing cell lines, but showed no effect on the HER2-negative NIH3T3 cells.

Preliminary results suggest that a receptor-specific peptide could be designed and that a bioactive peptide might be produced on a large-scale in *E. coli* system.

Further studies are required to assess the binding affinity of the peptide to the HER2 receptor and to verify the tri-dimensional structure of the peptide. New peptide designs providing better solubility and bioactivity should be considered and new techniques for isolating the peptide from the *E. coli* system need to be developed.

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