Rapid Purification of a New Humanized Single-chain Fv Antibody/Human Interleukin-2 Fusion Protein Reactive against HER2 Receptor

Wei-Yun ZHANG*, Tak-Chun YIP¹, and Cheuk-Sang KWOK²

Medical School, Nanjing University, Nanjing 210093, China; ¹Department of Clinical Oncology, Queen Elizabeth Hospital, Hong Kong, China; ²Division of Radiation Oncology, City of Hope National Medical Center, Duarte, CA 91010, USA

Abstract Human embryonic kidney 293 cells were transfected with plasmid pcDNA-H520C9scFv-rhIL-2 containing a chimeric cDNA encoding the humanized 520C9 scFv/recombinant human IL-2 fusion protein (H520C9scFv-rhIL-2). The transfected cells in plateau growing phase were cultured in serum-free medium for three days. The supernatant was collected, concentrated and purified using an affinity column packed with CNBr-activated Sepharose 4B coupled with anti-rhIL-2 mouse monoclonal antibody. The purified fusion protein was analyzed by ELISA, SDS-PAGE and Western blot. The fusion protein showed only one band in both silver stained electrophoresis gel and Western blot developed by ECL chemiluminescence system. Its molecular weight was confirmed to be about 45 kD. This fusion protein possessed binding specificity against p185 positive SKOV3 and B16/neu cells, and it might stimulate IL-2-dependent CTLL-2 cell proliferation as well.

Key words single-chain Fv antibody; IL-2; fusion protein; human embryonic kidney 293 cells; affinity chromatography

Overexpression of the proto-oncogene c-erbB-2, neu or HER2 has been shown in many human tumor cells, especially in breast cancer cells [1–5], which is thought to be important in human carcinogenesis. c-erbB-2 gene encodes a 185 kD transmembrane glycoprotein p185 with tyrosine kinase activity [6]. p185 has become a promising target for antibody therapy in breast cancer [7]. Many monoclonal antibodies specific for p185 have been studied [7–9]. Because of its important role in immunity, interleukin-2 (IL-2) has been studied extensively for the immunotherapy of cancers [10,11]. Systemic administration of IL-2 has been shown to stimulate antitumor responses in vivo, but efficacy in clinic has been limited because high dose IL-2 therapy causes serious side effects, especially the induction of vascular leak syndrome (VLS) [12,13]. Targeting IL-2 to the tumor site would decrease its systemic toxicity while exerting its antitumor function better.

A chimeric cDNA encoding humanized single-chain scFv of a murine antibody 520C9 with binding specificity to p185 extracellular domain and recombinant human IL-2 (rhIL-2) has been constructed using standard recombinant DNA techniques in our previous study [14]. The resultant fusion protein (FP) possessed binding specificity against p185 and retained the immuno-stimulatory activities of IL-2. It will be of interest to demonstrate if the FP will exert potent effects against disseminated human p185 positive tumors. Some pilot animal studies have been done to test the in vivo properties of this FP, and the FP treatment significantly inhibited the tumor growth compared with the control groups [15]. However, producing large quantity of pure FP quickly remains a bottleneck in carrying out large-scale animal experiments.

In this study, a reliable and rapid purification method for producing large quantity of fusion protein of humanized single-chain scFv of a murine antibody 520C9 and recombinant human IL-2 was established. The purified
fusion protein showed high specificity against p185 positive SKOV3 and B16/neu cells.

Material and Methods

Reagents

RPMI 1640, 293 serum-free medium (SFM) II, trypsin-EDTA, geneticin (G418), GlutaMax and fetal calf serum (FCS) were obtained from Gibco, Invitrogen Corporation. Mouse anti-hIL-2 monoclonal antibody IgG1 (MAB202) was purchased from R&D Systems, Inc.. Immuno pure gentle Ag/Ab elution buffer and Turbo ELISA substrate were from Pierce. Western blotting detection kit (ECL chemiluminescence system), rainbow markers and silver staining kit were from Amersham Pharmacia Biotech. Recombinant human IL-2 was purchased from Cell Sciences. CNBr-activated Sepharose 4B was from Armershan Biosciences. Goat anti-mouse secondary antibody (Peroxidase-Conjugated) and phosphate saline (PBS, pH 7.4) were products of Sigma.

Cell line

Human embryonic kidney (HEK) 293 cells were transfected with the expression plasmid pcDNA-H520C9scFv-rhIL-2 that contained the chimeric cDNA fragment encoding the humanized 520C9 scFv and human recombinant IL-2 fusion protein (H520C9scFv-rhIL-2). Schematic structure of the chimeric cDNA was shown in Fig. 1.

![Schematic structure of the chimeric cDNA in the vector](image)

SKOV3, B16/neu and HeLa cells were cultured for antigen-binding assays. SKOV3, a human ovarian carcinoma cell line, expressed high level of p185. B16/neu, the mouse B16 melanoma cell line transfected with human HER-2/neu gene, expressed p185 as well. HeLa cells expressed little p185.

Cell culture and supernatant collection

Transfected HEK 293 cells were plated in P75 culture flasks containing RPMI 1640 medium supplemented with 10% FCS and 500 mg/ml G418. When the cells grew to approximately 70% confluence, the supernatant was discarded and the flasks were rinsed thrice with RPMI 1640 medium to remove the residual FCS. 15 ml of 293 SFM II containing 2 mM GlutaMax and 8 mg/ml G418 was added to each flask. The cells were allowed to grow to complete confluence within three days. Then the supernatant was collected. The procedures of SFM II addition and supernatant collection were repeated before the cells were discarded. The supernatants were pooled, centrifuged to remove the floating cells, and concentrated with a centrifuge-type concentrator (30 kD filter-Millipore YM-30MW). The concentrated solution was diluted with PBS (pH 7.4) and filtrated with a 0.22 mm millipore filter. The filtrate was stored at –20 °C until use.

Coupling of MAB202 to CNBr-activated Sepharose 4B

One gram of CNBr-activated Sepharose 4B was rehydrated and washed using 1 mM HCl at room temperature. The coupling buffer (0.1 M NaHCO₃, pH 8.3, containing 0.5 M NaCl) was added to the gel for prehydrolysis for 4 h in order to reduce the number of coupling groups on the matrix to preserve the structure of the binding site and to facilitate elution. 2 mg of MAB202 dissolved in 5 ml of coupling buffer was mixed with the gel. The tube was immediately capped and rocked end-to-end for 1 h at room temperature on a rotor. The supernatant was collected for detecting the unreacted MAB202, and the remnant MAB202 was washed away with 5 times gel-volume of coupling buffer.

In order to block the redundant active groups on the Sepharose 4B, the gel was further treated with 0.1 M Tris-HCl buffer, pH 8.0, for 2 h; and washed with three cycles of alternating buffers: 0.1 M acetate buffer (pH 4.0, containing 0.5 M NaCl) followed by 0.1 M Tris-HCl (pH 8.0, containing 0.5 M NaCl). Then the coupled gel was extensively washed with PBS and packed into a chromatography column.

Running the affinity chromatography

The Sepharose 4B-MAB202 column was washed extensively with PBS before protein sample was loaded onto the column. The sample was allowed to flow into the column slowly and then the flow was stopped for 30 min
to let FP adsorb fully. The column was washed extensively with 0.02 M Tris-HCl, pH 7.4, then 5 column volumes of Immuno pure gentle Ag/Ab elution buffer were added and the flow was stopped for 20 min to completely desorb the bound FP. The eluant was slowly collected under gravity at 0.5 ml per sample tube, and the optical density at 280 nm ($A_{280}$) of each collected fraction was measured with an MBA 2000 machine (Perkin Elmer). The FP contained in the appropriate fractions was concentrated with a Millipore concentrator and stored at –20 °C for further analyses.

**ELISA test**

The concentration of FP in each collected fraction was detected by indirect ELISA according to Baines [16] with some modification. Briefly, 100 µl/well serial dilutions of standard rhIL-2 and collected fractions were added into the wells of ELISA plate and incubated overnight at 4 °C. The liquid was then aspirated. The plate was washed thrice with PBS containing 0.02% Tween 20 (PBS-T), blocked with 200 µl blocking solution (3% non-fat dry milk powder in PBS-T), and incubated at 37 °C in a humidified incubator for 1 h. After repeating the same washing procedure, 100 µl/well of a primary antibody against human IL-2 (MAB202, 1:1000) was added, and the plate was incubated at 37 °C for 1 h. Following washing several times, 100 µl/well of secondary antibody (anti-mouse Ab HRP conjugated, 1:2000) was added to the plate and was incubated for 1 h at 37 °C. Washing the plate again, 100 µl/well of Turbo ELISA substrate was added. The plate was incubated at 37 °C for 15 min in a dark chamber. 100 µl/well of 1 M H$_2$SO$_4$ was added to stop the reaction. The optical density of each well was measured at 450 nm ($A_{450}$) using an ELISA plate reader.

**Electrophoresis and silver staining**

The collected eluant and culture supernatant dissolved in 2× reducing sample buffer [4% SDS, 0.25 M Tris-HCl (pH 6.8), 0.05% bromphenol blue, 20% glycerol and 10% 2-mercaptoethanol] were boiled for 5 min, cooled and centrifuged to remove any sediment, and applied to 12% SDS-PAGE. Rainbow protein markers were run simultaneously. Electrophoresis was kept at 160 V for about 1 h. The gel was stained with a silver staining kit.

**Western blot**

The eluant, culture supernatant, rhIL-2 and Rainbow markers were electrophoresed by 12% SDS-PAGE in 2× reducing sample buffer without 2-mercaptoethanol. Then, separated proteins were transferred onto a nitrocellulose membrane using a wet blotting device (Pharmacia) with a current of 0.30 A for 1 h at 4 °C. Molecular weights of protein standards were labeled according to their colors. The membrane was dried in air and blocked overnight with a blocking buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, and 5% skimmed milk powder) shaking at 4 °C. The membrane was then incubated with MAB202 solution (1:1000) in the blocking buffer for 1 h at room temperature and was washed thrice with a washing buffer TBS-T (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, and 0.05% Tween 20). Following this, the membrane was incubated in a goat anti-mouse secondary antibody solution (peroxidase-conjugated, 1:2000) for 1 h at room temperature. The membrane was then extensively washed with PBS-T thrice. The ECL chemiluminescence reagent was added onto the membrane as instructed by the manufacturer. The membrane was exposed to an X-ray film (Fuji) for 1 min before it was developed and fixed.

**Antigen-binding assays and CTLL-2 proliferation assay**

Serially diluted samples of the H520C9scFv-rhIL-2 were incubated for 1 h in a C8 Maxisorp Nunc immunomodule plate (Nunc, Roskilde, Denmark) coated overnight with 1×10$^5$ live SKOV3, B16/neu or HeLa cells into each well. After blocking with Blotto, bound FP was incubated with a rabbit anti-human IL-2 antibody (1 µg/ml) for 2 h, followed by an HRP-conjugated goat anti-rabbit IgG polyclonal antibody (0.5 µg/ml) for 2 h. Color was developed with the addition of peroxidase substrate.

Biological activity of H520C9scFv-rhIL-2 was determined by standard IL-2 dependent T-cell proliferation assay using the murine T cell line CTLL-2. CellTiter 96® AQueous Assay (Promega, Madison, WI) was adopted for measurement of cell viability according to the manufacturer’s instructions. The cells were deprived of IL-2 for 3 days before seeding into a flat-bottomed 96-well plate at 5×10$^4$ cells per well. Serially diluted samples of the FP and standard samples of rhIL-2 were incubated with the cells in each well for 2 days. 20 µl of chromogen solution (MTS/ PMS) was added to each well and incubated for 1 h before determining absorbance of the wells at 490 nm.

**Results**

**The column and affinity chromatography elution curve**

The collected supernatant of coupling matrix mixture...
was measured the $A_{280}$ and its value was 0.005. Thus the content of unreacted MAB202 was below 5 µg according to the standard solutions.

After $A_{280}$ of each eluant fraction was measured, an elution curve as shown in Fig. 2 was drawn. Fraction 1 to fraction 10 constitute peak 1, which included flow through materials. Peak 2, between fraction 20 and fraction 30, contained the FP eluted by the Immuno pure gentle Ag/Ab elution buffer.

Fig. 2 Elution curves of the affinity chromatography detected by ultraviolet and ELISA
The eluant between fraction 20 and fraction 30 contained the fusion protein.

ELISA analysis

After affinity purification, each eluant fraction was analyzed by ELISA. The test was conducted in triplicate and the mean absorbance values at 450 nm were calculated. The results are shown in Fig. 2. Fractions containing FP yielded higher absorbance than other fractions.

The flow through materials and eluant were also collected respectively after another purification. They were concentrated and washed with PBS using Millipore concentrator and then analyzed by ELISA. The FP contents in the flow through and eluant were 6.8% and 85.2% respectively when compared with the loaded sample.

SDS-PAGE analysis

The purity of the collected FP was verified by SDS-PAGE and silver staining, which showed a single band at about 45 kD (Fig. 3, lane A). The presence of FP in HEK 293 cell culture supernatant was also confirmed (Fig. 3, lane B).

Western blot analysis

To verify the presence of human IL-2 moiety in the FP, the culture supernatant and elution fractions under peak 2 of the elution curve were analyzed by Western blot. Recombinant human IL-2 was used as a control. As shown in Fig. 4, the supernatant, peak 2 elution fractions and rhIL-2 showed positive bands. The bands for the first two samples appeared at 45 kD position (Fig. 4, lane A and B), while that for the rhIL-2 at 15 kD position.

Antigen-binding specificity and IL-2 bioactivity of fusion protein

Cell-mediated ELISA using cultured SKOV3, B16/neu and HeLa cells showed that FP was able to bind specifically to p185-expressing cells in a dose-dependent manner. The binding activity to the SKOV3 cells was higher than that of B16/neu and HeLa cells (Fig. 5).

Fig. 3 SDS-PAGE analysis of the humanized scFv antibody/rhIL-2 fusion protein
A, fusion protein purified by an affinity column; B, concentrated cultured medium from transfected HEK 293 cells; M, Rainbow markers.

Fig. 4 Western blot assay of the humanized scFv antibody/rhIL-2 fusion protein expressed by HEK 293 cells transfected with H520C9scFv-rhIL-2 cDNA
A, fusion protein purified by an affinity column; B, concentrated cultured medium from transfected HEK 293 cells; C, recombinant human IL-2.
The biological activity of the IL-2 moiety in H520C9scFv-rhIL-2 was determined by the CTLL-2 cell proliferation assay and the results showed that half-maximal stimulation of the CTLL-2 cells occurred at a concentration of approximately 0.1 µg/ml rhIL-2. The H520C9scFv-rhIL-2 sample achieved the half-maximal stimulation at 0.2 µg/ml. When compared with rhIL-2 on a molar basis, H520C9scFv-rhIL-2 had an average of 50% of the bioactivity of rhIL-2.

**Discussion**

Conventional cytotoxic chemotherapy on specific cancers is known to be ineffective. One of several promising approaches to overcome this problem is immunotherapy. Lymphokine-activated killer (LAK) cells induced by IL-2 from natural killer cells can be very efficient in eliminating various tumors. However, systemic administration of IL-2 to induce LAK cells in cancer immunotherapy may cause fever, pulmonary edema and VLS [13,17,18]. IL-2 may have an indirect action through LAK cells by releasing additional cytokines that produce both the anti-tumor effect and some side effects. Thus an optimal therapy might combine IL-2 activation and tumor antigen presentation together with a tumor-specific Ab that mediates Ab-dependent cellular cytotoxicity activity.

In this study we developed a quick and efficient method to purify FP (H520C9scFv-rhIL-2) produced by HEK 293 cells transfected with a cDNA encoding the FP. The FP binds specifically to the extracellular domain of the p185 of the HER2 proto-oncogene and possesses IL-2 activities [14]. However, how to purify large quantity of the FP efficiently has been a key barrier of its in vivo testing. 293 SFM II used for culturing the transfected cells contains very low concentration of proteins and makes it easier to purify the protein of interest. Affinity chromatography is a technique for purification of a biomolecule with respect to its biological function or chemical structure. In our study, 4 h of prehydrolysis was carried to reduce the number of coupling groups on the Sepharose 4B to preserve the structure of the binding site in case steric effects reduced the binding efficiency of a large ligand. The FP was specifically and reversibly adsorbed to the ligand MAB202, immobilized by a covalent bond to the chromatographic bed material Sepharose 4B. Recovery of the FP was achieved by changing the elution buffer to favor desorption and the recovery ratio of FP is about 85%. From this study it has been confirmed that the affinity chromatography is a specific and rapid purification method to obtain the FP from the supernatant of HEK 293 cells cultured in serum-free medium. The purity of the FP from the chromatography was confirmed by SDS-PAGE. Its molecular weight was also ascertained to be 45 kD through SDS-PAGE and Western blot analysis. Our results also demonstrated that the FP was able to specifically bind to p185 positive tumor cells and retained the activity of stimulating IL-2-dependent CTLL cell proliferation.

**Acknowledgements**

The authors would like to thank Dr. Roger KC Ngan, the Consultant, Dr. Wai-Hon Lau, the former COS of Clinical Oncology, and Dr. Stephen CK Law, the present COS of Clinical Oncology, Queen Elizabeth Hospital for their supports and permission in using the Radiobiology Laboratory for most of the research work done in this study.

**References**


Edited by
Zu-Chuan ZHANG