Construction of the Single-Chain Fv from 196-14 Antibody toward Ovarian Cancer-Associated Antigen CA125

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The variable regions of heavy- and light-chains of mouse monoclonal antibody 196-14 toward ovarian cancer-associated antigen CA125 were linked with a peptide linker (GSTSGSGKSSEGKG) and a histidine tag was attached at the carboxyl terminal. This single-chain Fv (scFv) with a histidine tag was expressed in *Escherichia coli* as an inclusion body. The inclusion body was solubilized with guanidium chloride, followed by purification on nickel nitrilotriacetic acid agarose column and refolding into the active form. The scFv thus obtained bound to the Siso cells, which express CA125, and may recognize the same epitope as the parental 196-14 antibody IgG does.

Key words 196-14 antibody; ovarian cancer-associated antigen CA125; single-chain Fv (scFv)

Adenocarcinoma of the uterine cervix accounts for 5 to 20% of cervical cancers, and its incidence has recently been reported to be increasing,¹⁻³⁾ however, the carcinogenesis and biological characteristics of this adenocarcinoma remain unclear. Moreover, this type of cancer shows low sensitivity against radiation and chemotherapy in comparison with squamous cell carcinoma of the uterine cervix.^{4,5)} Therefore, it is important to determine its oncogenesis, biological characteristics and sensitivity against anti-cancer reagents. Ovarian cancer-associated antigen CA125, originally defined by OC125 antibody, is detected in the sera of ovarian cancer patients in high frequency and is one of the tumor markers.^{6,7)} The following contradiction is pointed out in the characteristics of CA125: the secretion of CA125 is remarkably suppressed by anti-cancer reagents though tumor growth is not affected by various of these reagents.⁸⁾

Monoclonal antibodies against various cancer-associated antigens have been produced and used widely for evaluation of histology, serodiagnosis and detection of tumors *in vivo*.⁹⁻¹¹⁾ An endometrial adenocarcinoma cell line HOUA-1 was established from a 55-year-old endometrial cancer patient and two monoclonal antibodies, designated 196-14 and 196-28, were generated by immunization of a BALB/c mouse with HOUA-1 cells.^{6,7)} Monoclonal antibodies, 196-14 and 196-28, are reactive to the antigen CA125, and 196-14 antibody in particular has been reported suitable for diagnosis and therapy of CA125-producing endometrial or ovarian cancers.¹²⁾ Also, a mouse-human chimeric antibody of 196-14 was constructed, which was reported to retain the similar binding affinity as the parental mouse IgG antibody.¹³⁾ It is necessary to reduce the size of the antibodies in consideration of retardation of immunogenicity and permeability to the affected part *in vivo*.

The architecture of single-chain Fv (scFv) has the variable regions of heavy chain and light chain (VH and VL, respectively) of the antibody which are connected by a peptide linker.^{14,15)} In Fv molecule, VH and VL associate by the non-covalent bond, and these two molecules easily dissociate under physiological condition. This disadvantage, however, is overcome by constructing scFv.

A structure-based rational drug design has been proposed: a pharmaceutical molecule designed on the basis of tertiary structure of the complex of target protein and ligand.^{16,17)} In proteins with high molecular weight (e.g., IgG) it is difficult to analyze the tertiary structure by X-ray crystallography or NMR; it is important for structure-based drug design to miniaturize the target protein without diminishing the physiological activity. As described above, the carcinogenesis and biological characteristics of this adenocarcinoma still remain unclear. It might be meaningful to analyze expression and molecular characterization of a tumor antigen on the basis of the tertiary structure. Here we report on the construction of anti-CA125 antibody 196-14 scFv for application in diagnosis and to describe its biological characteristics.

MATERIALS AND METHODS

Materials Restriction enzyme, T4 polynucleotide kinase, T4 DNA polymerase and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan) or Toyobo Co., Ltd. (Osaka, Japan). A dye primer cycle sequencing kit for nucleotide sequencing was purchased from Perkin-Elmer Applied Biosystems (CA, U.S.A.). *N*-Hydroxysuccinimido (NHS)-biotin for biotinylation of scFv and centricon 10 were from Pierce (IL, U.S.A.) and Amicon, Inc. (MA, U.S.A.), respectively. Fluorescein 5-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and R-Phycoerythrin (PE)-streptavidin were from Sigma Co., Ltd. (St. Louis, U.S.A.). The other chemicals used were of analytical or biochemical grade.

Strains and Bacteriophages *E. coli* JM110¹⁸⁾ and BW313¹⁹⁾ were used for M13 cloning and site-directed mutagenesis. *E. coli* RR1²⁰⁾ was used for plasmid cloning, and *E. coli* KP3998 was used as a host strain for the *E. coli* overexpression system with a vector pKP1500.²¹⁾ Bacteriophage M13 mp18¹⁸⁾ was used for the construction and DNA sequencing of scFv.

Manipulation of DNA Procedures for purification of DNA, restriction enzyme digestion, ligation, agarose-gel electrophoresis were as described previously.²²⁾ DNA se-

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Fig. 1. Construction of 196-14 ScFv

H6, histidine hexamer region; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; H, *Hind*III; K, DNA polymerase I Klenow fragment; Sm, *Sma*I; Xb, *Xba*I; start, start codon; stop, stop codon; *Ptac*, *tac* promoter; Ap^r, ampicillin resistant gene (= β -lactamase); VH, the variable region of heavy chain of 196-14 antibody; VL, the variable region of the light chain of 196-14 antibody.

Table 1. Synthetic Oligonucleotides for the Construction of the ScFv-Expression Vector

AB-1 and 2:	5'-GTCACCGTCTCTCCTCATAATAGGATCCG	-3'
	3'-GCAGAGGAGTATTATCCTAGGCTCGA-5'	
	(<i>Bst</i> EII) stop <i>Bam</i> HI (<i>Hind</i> III)	
AB-3	5'-AGGTCCAGACTCCTGAAGCTTGACCTGACTC-3' (anti-sense)	
	<i>Hind</i> III	
AB-4 and 5:	5'-GATCAAAGGCTCTACTTCCGGAAGCGCAAATCCTCTGAAGGCAAAGGTCAGGTCA	-3'
	3'-TTTCCGAGATGAAGGCCTTCGCCGTTTAGGAGACTCCGTTTCCAGTCCAGTTCGA-5'	
	(<i>Bgl</i> II) G S T S G S G K S S Q G K G Q V K (<i>Hind</i> III)	
AB-6	5'-CAGCTGAATGTCATAGAATTCGTAATC-3' (anti-sense)	
	start <i>Eco</i> RI	
AB-7 and 8:	5'-GTCACCGTCTCTCCTCACATCACCATCACCATCACTAATAGCTCGAG	-3'
	3'-GCAGAGGAGTGTAGTGGTAGTGGTAGTGATTATCGAGCTCCTAG-5'	
	(<i>Bst</i> EII) H H H H H H stop <i>Xho</i> I (<i>Bam</i> HI)	

quencing was carried out with an Applied Biosystems DNA sequencer 373A and Dye Primer Cycle Sequencing Kit (Applied Biosystems). Site-directed mutagenesis on M13 phage vector was performed by the method of Kunkel *et al.*¹⁹⁾

Construction of 196-14 ScFv Expression Plasmid The outline and eight oligonucleotides for the scFv-construction are shown in Fig. 1 and Table 1, respectively. Variable region genes of heavy and light chain (VH and VL, respectively) were linked with each other on M13 phage vector, and a start codon (ATG) and double stop codon (TAATAG) were introduced on 5' and 3' terminals, respectively. VH and VL genes obtained from hybridoma producing anti-CA125 196-14 antibody by reverse transcription (RT)-PCR were inserted into a *Hinc*II site of M13 mp18 and pHS399²³⁾ to yield HA418 and HA416, respectively. To link VH gene to VL gene on the same M13 vector, *Hind*III (blunt-ended)-*Xba*I fragment of HA416 was inserted between *Xba*I and *Sma*I sites of HA418, yielding phage TN2-4. Double stop codons TAATAG and *Bam*HI site were introduced between *Hind*III and *Bst*EII sites downstream from the VH gene with two oligonucleotides AB-1 and AB-2, yielding phage TN12. After introduction of *Hind*III site into the VH gene by site-directed mutagenesis with an oligonucleotide AB-3 (TN14), 212-linker DNA cod-

ing amino acid sequence GSTSGSGKSSEGKG²⁴⁾ with two oligonucleotides AB-4 and AB-5 was inserted between *Bgl*II and *Hind*III sites of phage TN-14, yielding phage TN24. Start codon ATG and an *Eco*RI site were introduced upstream from the VL gene, followed by introduction of DNA coding histidine-hexamer peptide downstream from the VH gene using oligonucleotides AB-7 and AB-8. The scFv gene of the resultant recombinant phage TN112-8 was inserted between the *Eco*RI and *Bam*HI sites of *E. coli*-expression vector pKP1500, designated plasmid TN116. The nucleotide sequence of the scFv gene of TN112-8 was confirmed by PE-ABI DNA sequencing.¹³⁾ For protein expression, *E. coli* KP3998 was transformed with the expression plasmid TN116. Because of the temperature-sensitive copy control and regulatable expression of the *tac* promoter, pKP1500 has been used to express foreign genes in *E. coli*, especially in cases where the gene products are toxic or overproduction is deleterious to the host bacteria.²¹⁾

Expression of 196-14 ScFv 196-14 scFv expression-strain was cultivated according to the method of Miki *et al.*²¹⁾

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) SDS-PAGE was performed as previously described²⁵⁾ using 15 % acrylamide in slab gels

and stained with Coomassie brilliant blue R-250.

Purification of ScFv Cells were harvested by centrifugation. Cell pellets were resuspended in sonication buffer (50 mM 3-(N-morpholine)-2-hydroxypropane sulfonic acid (MOPSO), 0.2 mM Pefabloc SC, pH 7.0) and lysed by sonication on ice. Soluble and insoluble cell lysate, namely inclusion body, were collected by centrifugation at $24000\times g$ for 10 min. Solubilization of the inclusion body was achieved in buffer A (6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 8.0). To remove insoluble material, the solution was centrifuged at $24000\times g$ for 20 min. The supernatant was adsorbed on nickel-nitrilotriacetic acid (Ni-NTA) agarose column, which was equilibrated with buffer B (6 M guanidine hydrochloride, 20 mM Tris-HCl, pH 8.0), and eluted with buffer C (6 M guanidine hydrochloride, 250 mM imidazole, 50 mM Tris-HCl, pH 8.0).²⁶⁾

Renaturation of ScFv Renaturation was carried out by the method described by Buchner *et al.*²⁷⁾ with a slight modification. Three milliliter of denatured protein solution (1.4 mg/ml , $\epsilon=59180\text{ M}^{-1}\text{cm}^{-1}$) was prepared, diluted in 100 ml of buffer D (0.1 M Tris-HCl, 0.5 M L-arginine, pH 8.0) and incubated at 4°C for 24 h. To remove arginine and guanidine, dialysis was performed against buffer E (20 mM Tris-HCl, 0.1 M urea, pH 8.0). The scFv protein in supernatant after centrifugation was recovered by salting-out with 65% saturated ammonium sulfate.

Biotinylation of ScFv The scFv recovered by salting-out was dissolved in buffer (50 mM NaHCO_3 , pH 8.2), followed by incubation of scFv and NHS-biotin at 4°C for 14 h. Non-reacted NHS-biotin was removed by dialysis against buffer (20 mM Tris-HCl, pH 8.0) or by filtration with Centricon 10.

Binding Assay toward the Siso Cells by Flow Cytometric Analysis Flow cytometric analysis was carried out as previously described.⁸⁾ The Siso cells (1×10^5 cells), which express the tumor-associated antigen CA125, were harvested upon mild trypsinization and suspended in washing buffer (0.3% bovine serum albumin, 0.05% NaN_3 , 1.9 mM NaH_2PO_4 , 8.1 mM Na_2HPO_4 , 0.15 M NaCl, pH 7.4) containing 40% goat serum for 20 min. Then, the cells were incubated with biotinylated scFv on ice for 60 min. After washing 3-times with washing buffer, cells were incubated with 40% goat serum and PE-streptavidine on ice for 60 min, then washed 3-times with washing buffer. Flow cytometric analysis was performed using the Coulter Epics flow cytometry Epics Elite (Coulter, Hialeah, Florida). As negative control cells, K562²⁸⁾ and MM28,²⁹⁾ which does not express the antigen CA125, were used instead of Siso cells.

Competitive Assay toward Siso Cells by Flow Cytometric Analysis These techniques were carried out as previously described.⁸⁾ After the Siso cells (1×10^5 cells) were suspended in washing buffer containing goat serum, anti-CA125 IgG (10 pmol) and scFv were added and the mixture was incubated on ice for 60 min. The cells were washed 3-times with washing buffer, then incubated with FITC-conjugated goat anti-mouse antibody on ice for 60 min, then washed 3-times with washing buffer, and flow cytometric analysis was performed. As a negative control scFv, anti-hen lysozyme scFv, derived from LKS103,³⁰⁾ was used.

Fig. 2. SDS-PAGE of Proteins from 196-14 ScFv Expression Strain TN116/KP3998

Lane 1, total cell; lane 2, supernatant after cell-disruption; lane 3, precipitate after cell-disruption (=inclusion body). The arrow shows scFv protein.

Fig. 3. Chromatography of Inclusion Body Dissolved in Guanidine Hydrochloride on Ni-NTA Agarose Column

The column was equilibrated with 20 mM Tris-HCl (pH 8) containing 6 M guanidine hydrochloride. The arrow shows the elution point with the same buffer containing 6 M guanidine hydrochloride and 250 mM imidazole.

RESULTS

Expression of 196-14 ScFv Isopropyl-1-thio- β -D-galactoside (IPTG) was added to a final concentration of $1\times 10^{-4}\text{ M}$ at $\text{OD}_{660}=1.0$ of cell growth, followed by additional cultivation for 3 h at 40°C . The cell paste (5 g per liter) was obtained by centrifugation, and the expression of scFv was confirmed by SDS-PAGE (Fig. 2).

Purification and Renaturation of ScFv As histidine hexamer was added at the carboxyl terminal of the scFv by gene engineering, the scFv could be purified on Ni-NTA agarose column.³¹⁾ The inclusion body was solubilized with the guanidine hydrochloride solution, and applied to Ni-NTA agarose column (Fig. 3). The adsorbed fraction was mainly scFv protein on SDS-PAGE under the reduced condition because the theoretical molecular mass of scFv protein is 28 kDa (Fig. 4). The scFv adsorbed on the column was 50 mg per liter. Multimers other than monomer of scFv were also observed under the non-reduced condition (Fig. 4). This fractionated scFv was refolded in the presence of arginine by rapid dilution method, followed by salting-out.²⁷⁾ The scFv thus obtained was almost monomer (Fig. 4). The yield of re-

Fig. 4. SDS-PAGE of Proteins at Progressive Stages of Purification

Lane 1, non-adsorbed fraction on Ni-NTA column (reduced); lane 2, adsorbed fraction on Ni-NTA column (reduced); lane 3, adsorbed fraction on Ni-NTA column (non-reduced); lane 4, supernatant after salting-out followed by dialysis (non-reduced).

Fig. 5. Binding Affinity of 196-14 ScFv against Siso Cell

(A) Flow cytometric analysis. Siso cell was incubated with various amounts of biotinylated 196-14 scFv, followed by staining with PE-streptavidin. Stained cells were analyzed by Epics Elite. (a) to (f) represent the flow cytometric profiles on 1.2, 3.7, 11.1, 33.3, 100 and 300 pmol of biotinylated scFv, respectively. (B) Plotting of positive peak area shown in (A) relative to total area. Affinities against Siso, K562 and MM28 cells are represented by open circles, filled circles and open triangles, respectively.

natured scFv was 3 mg per liter of culture medium. Purified scFv was confirmed by amino acid composition analysis (data not shown).

Binding to the Siso Cell Expressing Antigen CA125

The scFv was biotinylated using NHS-biotin. Binding between the biotinylated scFv and the Siso cell was detected with PE-streptavidin, which carries the fluorescent probe, using flow cytometry (Fig. 5). The scFv was confirmed to specifically bind to the Siso cell expressing the antigen

Fig. 6. Competition Assay of 196-14 ScFv against Siso Cell

(A) Flow cytometric analysis. Siso cells were incubated with the 196-14 IgG (10 pmol) and various amounts of 196-14 scFv, followed by staining with FITC-conjugated goat anti-mouse IgG. Stained cells were analyzed by Epics Elite. (a) to (h) represent the flow cytometric profiles on 0, 12.5, 25.0, 50.0, 100, 200, 400, and 800 pmol of scFv, respectively. (B) Plotting of positive peak area shown in (A) relative to total area. 196-14 scFv and anti-lysozyme scFv (negative control) are represented as open and filled circles, respectively.

CA125. K562²⁸⁾ and MM28²⁹⁾ cells, in contrast, did not bind at all to the Siso cell. The association constant (K_a) was $6.5 \times 10^7 \text{ M}^{-1}$ from Fig. 5. K_a of the parental antibody 196-14 IgG to Siso cell was $1.0 \times 10^9 \text{ M}^{-1}$ (data not shown). Kanda *et al.* reported that K_a of the parental antibody 196-14 IgG to A549 cell was $1.2 \times 10^9 \text{ M}^{-1}$.¹³⁾ K_a of the scFv against Siso cell decreased to 1/15 that of the parental IgG against the same cell.

Competitive Assay of ScFv and the Parental IgG
Competition of the scFv and the parental IgG were determined against the Siso cells at various concentration of the scFv (Fig. 6). As the concentration of the scFv was increased, the binding of the parental IgG to the cell was inhibited. The anti-lysozyme antibody LKS103 scFv,^{30,32)} which is not concerned with the 196-14 scFv at all, did not compete with the binding of the 196-14 IgG, showing that the scFv may recognize the same site as the parental IgG does.

DISCUSSION

In this report, we constructed the scFv of the 196-14 IgG,¹³⁾ which specifically recognizes the ovarian cancer-associated antigen CA125. ScFv, where the VH and the VL regions were connected with a peptide linker, retained a similar antigen-binding capability as the parental IgG antibody.

The 196-14 scFv was expressed as an inclusion body inside the cell. The histidine tag at the carboxyl terminal of the scFv facilitated the purification efficiently in the denaturation condition.³¹⁾ Various methods have been developed to refold the denatured scFv into the active form.^{27,30,33,34)} Maeda *et al.* reported a method to refold a denatured protein efficiently and in a large quantity using dialysis.³⁰⁾ We applied this method to refold 196-14 scFv, but the molecules were multi-

merized and it ended in failure. Buchner *et al.* developed the rapid dilution in the presence of arginine for protein-refolding.²⁷⁾ This method was applied to refold 196-14 scFv. The concentration of monomer could be raised to 1 mg/ml and the final yield of the renatured scFv was 3 mg per liter of culture medium (Fig. 4).

Binding activity of the 196-14 scFv to the CA125 positive Siso cell was evaluated using flow cytometry. Because the scFv could not be detected directly, it was biotinylated in advance for staining with PE-streptavidin. Biotinylated scFv was found to bind the Siso cell specifically. However, K_a of the 196-14 scFv to the Siso cell decreased to 1/15 that of the parental IgG to the same cell (6.5×10^7 , $1.0 \times 10^9 \text{ M}^{-1}$, respectively).¹³⁾ An order of 10^7 to 10^8 is seen in the K_a of some scFv's reports.^{15,34)} Binding affinity generally shows a tendency to decline by miniaturization; this 196-14 scFv showed a similar tendency. The scFv may recognize the same epitope as the parental 196-14 antibody IgG does (Fig. 6). An inverse experiment was also carried out to examine whether the affinity changes. Cells were stained with the biotinylated scFv, and the inhibition of the scFv-cell binding by the cold IgG was assayed (data not shown). Half inhibition of the maximum (*ca.* 30%) of binding efficiency was 40 pmol (IgG) for 100 pmol (biotinylated scFv), while half inhibition from Fig. 6 was 60 pmol (scFv) for 10 pmol (IgG). That is to say, the mole ratio of the scFv for the IgG was approximately 2.5–6 through both experiments. This result shows that affinity between the scFv and Siso cells is similar to that between the parental IgG and Siso cells.

ScFv has a tendency to form the multimer when the concentration of scFv is high.^{35,36)} This may be the reason why the refolding method by Maeda *et al.*³⁰⁾ was not successful. Various trials to suppress multimer-formation have been reported.^{37,38)} In addition, scFv forms multimers with time. As well as the 196-14 scFv, a multimer was formed when the scFv was stored for a long time. Thus, in this study, all experiments were performed as soon as the scFv was purified. If we can elicit a more stable and soluble 196-14 scFv monomer, it will be a more useful tool to evaluate histology and serodiagnosis, immunoscintigraphy of cancers, and so on.

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