Production of a Single-chain Variable Fragment Antibody Recognizing Type III Mutant Epidermal Growth Factor Receptor

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The type III deletion mutant of the epidermal growth factor receptor (EGFR) is a potential target in diagnostic and therapeutic approaches for those glioblastomas characterized by its expression. We previously raised a mouse monoclonal antibody, 3C10 (IgG2b) specifically recognizing this mutant EGFR. In this study, a single-chain variable fragment (scFv) antibody was produced. Partial determination of its N-terminal amino acid sequence and preparation of adequate primers for variable heavy chain (VH) and variable light chain (VL) genes were performed to allow cloning by means of reverse transcriptase-polymerase chain reaction. The genes cloned were assembled with a linker, (Gly4Ser)3, and ligated into a bacterial expression vector to express the scFv as cytoplasmic inclusion bodies. After appropriate refolding, the antibody activity of the VH-VL scFv was examined in an enzyme-linked immunosorbent assay. 3C10 scFv showed a selective reactivity with the mutant peptide, similarly to the parental 3C10 antibody. A mouse transfectant expressing the type III mutant EGFR and a glioblastoma with type III deletion-mutant EGFR were positively stained by immunofluorescence. By Biacore analysis, the affinity (Kd) of the parental 3C10 for the mutant peptide was 9.7×10⁻⁹ M⁻¹, while that of 3C10 scFv was 2.45–2.48×10⁻⁹ M⁻¹, being approximately 4-fold weaker. The results together suggested that the scFv antibody retained the appropriate structure to recognize a conformational epitope of the mutant receptor, similarly to the parental antibody.

Key words: scFv — Epidermal growth factor receptor — Type III deletion mutant — Glioblastoma

The epidermal growth factor receptor (EGFR) gene is amplified and overexpressed in about 40% of glioblastoma cases. This amplification is frequently related to structural rearrangement, resulting in in-frame deletion mutations in the extracellular domain. Such deletions in EGFR have been classified into three types, based on size and location.1-7 Type III has been identified in about 17% of glioblastomas, and is characterized by an 801 bp in-frame deletion, which creates a unique sequence with a glycine residue at the fusion junction between amino acid residues 5 and 274. Since the sequence around the fusion junction is expressed only in glioblastoma cells, it is a potential target for diagnostic and therapeutic approaches. Accordingly, several laboratories including our own8-11 have attempted to produce mouse monoclonal antibodies with specificity for the type III mutant. Wikstrand et al.8) and Hills et al.9) obtained antibodies by immunization with a synthetic peptide, named Pep3, covering the fusion junction of the type III deletion mutant which had been used by Humphrey et al.10) for production of polyclonal rabbit anti-serum. Using the same peptide, we also succeeded in producing an antibody, 3C10, showing specificity for type III mutant EGFR.11)

With the recent advances in technology involving cloning of immunoglobulin (Ig) genes, generation of recombinant/chimeric Ig genes, and their expression in a variety of systems, clinical application of a variety of antibody molecules appears feasible. One advance has been the development of a recombinant single-chain variable fragment (scFv) antibody composed of a variable heavy chain (VH) amino acid sequence tethered to a variable light chain (VL) sequence by a designed peptide which links the carboxyl terminus of the VH to the amino terminus of the VL or vice versa. Preclinical studies with scFv antibodies have demonstrated improved tissue penetration efficiency, faster blood clearance, and lower immunogenicity than the parental mouse monoclonal antibodies.12, 13)

In 1996, Lorimer et al. described a scFv against the type III EGFR mutant (named MR1), isolated from a phage display library prepared from spleen cells of a mouse.
immunized with Pep3 and purified truncated EGFR. In this study, we attempted to produce a scFv from our 3C10 mouse monoclonal antibody and established a relatively efficient refolding protocol to yield a scFv with potent antibody activity from cytoplasmic inclusion bodies expressed in *Escherichia coli* (*E. coli*).

**MATERIALS AND METHODS**

**Cell lines, monoclonal antibody and synthetic peptides** The ERMs cell line is derived from mouse NIH/3T3 fibroblasts, which do not express wild-type EGFR, transfected with a cDNA from the human glioma xenograft GL-5 featuring an 801 bp in-frame type III EGFR deletion. A431 is a human squamous carcinoma cell line overexpressing intact EGFR.

The 3C10 hybridoma producing a mouse monoclonal antibody (IgG2b, κ) was established by immunization with a synthesized 14-amino-acid peptide (named Pep3 according to the report by Humphrey et al.). LEEKKQNYVVTDHC corresponding to the fusion junction of the truncated EGFR, coupled to keyhole limpet hemocyanin (KLH). A 17-amino-acid peptide corresponding to the fusion junction without the glycine (LEEKKVACPRNYVVTDHC) was used as a negative control peptide. These peptides were chemically synthesized with a peptide synthesizer (ABI 431A, Applied Biosystems, Foster City, CA) and the amino acid sequences were confirmed using a protein sequencer (ABI 477A, Applied Biosystems).

**Purification and amino acid sequence analysis of the monoclonal antibody** The anti-Pep3 monoclonal antibody, 3C10, was purified from ascites of athymic nude mice (KSN Scl, Shizudokyo, Mishima, Shizuoka) bearing hybridoma cells using a 2-fluoro-1-methylpyridinium toluene-4-sulfonate activated Cellulofine gel (Seikagaku Co., Ltd., Tokyo) conjugated with the Pep3 peptide according to the report by Humphrey et al.

An enzyme-linked immunosorbent assay (ELISA), a mixed hemadsorption assay (MHA), fluorescence-activated cell sorter (FACS) analysis and immunohistological staining were employed to detect antigens with 3C10 scFv and parental whole antibodies using the methods described previously. In the immunostaining analysis, Ab-1 antibody which reacts with intact EGFR...
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Table I. Oligonucleotides Used for Cloning of Murine Ig V Genes

(a) Primers used for the primary amplification of $V_H$

| Primer   | Sequence                        |
|----------|---------------------------------|
| VH1      | GAGATCCAGCTGCGACAGTCTGG         |
| VH2      | CTGGACAGGGATCCAGAGTTCCA         |

(b) Primers used for the primary amplification of $V_L$

| Primer   | Sequence                        |
|----------|---------------------------------|
| VL1      | CAGTCTCCACTCACTTATCGGTCGC       |
| VL2      | ATACAGTTGCGACAGCATCACGC         |

(c) Re-amplification primers (to introduce the linker fragment and restriction sites in the assembled cassette)

| Primer   | Sequence                        |
|----------|---------------------------------|
| VH3      | GCCATGGCTGGGATCCGGGATACACATATGAGATCCAGTCCAGCTGGGGCAGAATTGTGAAG |
| VH4      | GTCCATGGCCAAACCTCTTAAATTCGGGACCACCCACCACCGGACCACCCCTCTCCAGGAGACTGTAGGTG |
| VL3      | GCCATGCTCCCGGAGGTGGTGGTCACATATGAGATGATTGATGACCCAGTCTCCACTCCTCA |
| VL4      | TTCATGGCCGCAAAGCTTATATGATGCGCAGCGACCTCGACCCTGACGGTTTATCTCCAGCTGGTCCCTCACC |

Dotted lines: linker sequences.

(Oncogene Science Diagnostic, Cambridge, MA) was used as the positive control. In the case of scFv, a mouse anti-His-tag antibody (RGS-His Antibody, QIAGEN, San Diego, CA) was used as the second antibody after incubation of scFv with target peptides or cells, followed by further reaction with peroxidase- or fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig or MHA indicator cells. Concentrations of reagents for these assays are described in the figure legends.

For estimation of antibody specificity, competition MHA was performed. In preliminary experiments, antibody activities of 3C10 whole and scFv antibodies were determined using ERM5 cells expressing type III mutant EGFR as target cells, and the minimal amount of antibody concentration to show nearly 100% positivity was selected and incubated with 4-fold serial dilutions of Pep3 and negative control peptides. Thereafter, the remaining antibody activity was tested by MHA assay as described previously.11) Then the amount of each peptide showing approximately 50% inhibition in MHA assay was estimated, and the ratio of the two peptides was calculated.

RESULTS

Cloning of 3C10 scFv genes Genes encoding $V_H$ and $V_L$ of 3C10 hybridoma cells producing a monoclonal antibody against the type III EGFR deletion mutant were amplified by RT-PCR and assembled to form a scFv construct with a 15-residue (Gly4Ser)3 linker (Fig. 1). In order to design the set of oligonucleotide primers, the N-terminal amino acid sequences of the $\kappa$ light and G2b heavy chains of the 3C10 antibody were partially determined. Based on the results shown in Fig. 2, 5′ primers (VH1 and VL1) were prepared (Table I). 3′ primers (VH2 and VL2) were based on Kabat’s database of the constant regions of heavy (CH1) and light chain (CL) genes.

After first-strand synthesis, the cDNA was subjected to PCR. One reaction mixture contained VH1 and VH2 primers for amplifying $V_H$, while another contained VL1 and VL2 primers for $V_L$. After amplification, both reaction tubes showed a major band of approximately 350 bp (data
not shown). Subsequently, to introduce restriction enzyme sites and linker fragments, reamplification of \(V_h\) cDNA and \(V_l\) cDNA was conducted with VH3 and VH4, and VL3 and VL4 primers, respectively. \(V_h\) and \(V_l\) cDNAs thus obtained were assembled into the pRSET B plasmid vector using three enzyme sites, \(Nde\)I, \(Acc\)III and \(Vsp\)I, with 3C10 scFv expressed as a His-tag protein. The DNA sequence of the cloned \(V_h-L-V_l\) scFv gene construct was determined (Fig. 2). The authenticity of the cDNAs was proven by comparing the partial N-terminal protein sequence of the 3C10 heavy and light chains with the amino acid sequences deduced from the reading frames of the cloned genes. The DNA sequence also showed that the 3C10 heavy chain is a member of the mouse class II C, while the light chain belongs to the \(\kappa\) class II subgroup (Table II).

**Protein expression and refolding** Expression of the \(V_h\)-\(L-V_l\) 3C10 scFv gene in *E. coli* was achieved as described in “Materials and Methods.” About 10% of the total protein of the IPTG-induced culture was the recombinant product, deposited in cytoplasmic inclusion bodies. The purified inclusion bodies contained the protein with the expected molecular mass of 26 kD (Fig. 3).

**Fig. 1.** Strategy for the cloning of \(V_h\) and \(V_l\) genes for the 3C10 monoclonal antibody and construction of plasmids for expression of 3C10 scFv. The plasmid pRSET B contains \(Nde\)I and \(Vsp\)I ligation sites and expresses a His-tag protein. The sequences of the PCR primers (VH1–VH4 and VL1–VL4) are shown in Table I. L indicates the region encoding the (Gly4 Ser)3 linker. PT7 indicates the T7 promoter and His the Histidine (6) tag.

After several attempts with different procedures, that described in “Materials and Methods” was found to be the most effective for refolding of recombinant 3C10 scFv. Briefly, inclusion bodies after sonication were solubilized with 8 M urea and then the recombinant product was affinity-purified with His-tag agarose. Thereafter, it was slowly refolded with redox solution containing 2 mM reduced glutathione and 0.5 mM oxidized glutathione.

**Serological activity of recombinant scFv** After refolding, the antibody reactivity of recombinant 3C10 scFv was...
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tested against the type III mutant EGFR peptide, Pep3, by ELISA. For detection of 3C10 scFv, an anti-His-tag antibody was used as the second antibody. 3C10 scFv showed a clear reactivity to Pep3, but also reacted to a control peptide, albeit much more weakly (Fig. 4), similarly to the parental 3C10 whole antibody.11) In order to obtain the same degree of reactivity with Pep3, the 3C10 scFv required about 10 times the concentration of whole 3C10 antibody. However, this does not necessarily mean that 3C10 scFv has one-tenth of the binding activity, because the binding of the second antibody must be taken into account.

Next, FACS analysis was conducted to examine the reactivity of 3C10 scFv to the native epitope of the mutant EGFR using ERM5 mouse transfectant expressing type III mutant EGFR as target cells. 3C10 scFv clearly stained ERM5 cells, although more weakly than the parental 3C10 (Fig. 5). 3C10 scFv was also tested against 5 human glioblastoma specimens. As shown in Fig. 6, only the specimen from patient 5, known to have the type III EGFR deletion mutation,11) was positively stained. Finally, the reactivity was tested by using an MHA assay, known to be very sensitive for detecting surface antigens. It showed a positive reaction to ERM5 cells, whereas the A431 human squamous cancer cell line over-expressing wild-type EGFR was almost negative (Fig. 7), despite the positive ELISA reaction with the control peptide described above (Fig. 4).

The specificity of the 3C10 whole and scFv antibodies against native mutant EGFR was further studied with a competition MHA assay. An appropriate amount of each antibody was preincubated with various amounts of Pep3 and negative control peptides, and then the remaining activity against ERM5 cells was tested to estimate the amount of each peptide that would show approximately 50% inhibition in MHA assay. The ratio of the two peptides was then calculated (Table III). The value for 3C10 whole antibody was 50, while two different lots of 3C10 scFv antibody gave values of 11 and 6, severalfold lower than that of the parental 3C10.

**Biacore analysis** We first showed by gel filtration that 3C10 scFv antibody was a monomer (data not shown). Then, the binding of 3C10 scFv with immobilized Pep3 peptide was studied by surface plasmon resonance (Table IV). Two different lots of scFv showed similar values of binding constant ($K_A$, $2.45 \times 10^7 M^{-1}$ and $2.48 \times 10^7 M^{-1}$), while $K_A$ of 3C10 whole antibody was $9.7 \times 10^7 M^{-1}$, approximately 4-fold larger.

**DISCUSSION**

We document here the production and characterization of an scFv derived from the 3C10 hybridoma producing a monoclonal antibody against the truncated EGFR resulting from the type III EGFR deletion mutation. Lorimer et al. earlier reported production of MR1 scFv antibody specific for the type III mutant EGFR by using a phage display library prepared from an immunized mouse to bypass the hybridoma step and fusion with *Pseudomonas* exotoxin A.14) As shown by the comparison in Table IV, the comple-
mentarity-determining regions (CDRs) of the \( V_H \) and the \( V_L \) of 3C10 and MR1 scFv are quite different: 3C10 has IIC \( V_H \) and II \( V_L \) subgroups, while MR1 employs IIID \( V_H \) and VL, although both were originally derived from immunization with the same Pep3 peptide. Combined application of anti-mutant EGFR scFv with different \( V_H \) and \( V_L \) usage may show synergistic effects on immuno-imaging and immuno-targeting therapy. In this regard, Schmidt et al. reported interesting findings that 14E1 scFv antibody against intact EGFR fused with Pseudomonas exotoxin A displayed killing activity against cells expressing type III mutant EGFR up to 100-fold higher than that against cells expressing intact EGFR, pointing to the benefit of using reagents simultaneously targeting different forms of EGFR.17, 18)

In the system reported by Lorimer et al., MR1 scFv protein is expressed in periplasmic inclusion bodies.15) The cytoplasmic inclusion body approach utilized here is generally believed to have advantages for large-scale preparation, but the cytoplasm maintains a reducing milieu wherein cysteinyl residues cannot form the disulfide bonds that are essential for the integrity of native variable domains. Accordingly, in vitro protein refolding is indispensable to obtain functional scFv, but this remains a formidable problem.19) In this study, several refolding methods were attempted without success, the results suggesting that refolding under reducing conditions is not suitable for 3C10 recombinant scFv. Finally, relatively efficient production of immunoreactive 3C10 scFv was achieved with a procedure based primarily on disulfide restricted refolding. In this context, the report by McCartney et al. that MOPC 315 scFv could be produced by a disulfide restricted refolding procedure, but not by dilution or redox refolding methods is of interest.20) According to the Chou-Fasman secondary structure prediction model,21) the \( V_H \) of 3C10 would be expected to have an extended shape, resembling the \( V_L \) of the MOPC 315 antibody, which might require disulfide restricted refolding. Further studies are apparently necessary to ascertain whether the secondary structure of the variable region can predict what kind of procedures are appropriate for refolding recombinant Ig molecules.

We previously reported that 3C10 whole antibody shows a weak but significant reaction to a control peptide
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Fig. 5. FACS analysis of 3C10 whole and scFv antibodies tested against ERM5 cells. Cells were harvested after EDTA treatment of monolayer cultures, reacted with mouse myeloma protein MOPC-21 (A) as a negative control, 3C10 scFv (B) or 3C10 whole antibody (20 µg/ml) (C), stained by indirect immunofluorescence and analyzed. For 3C10 scFv, an anti-His-tag antibody was used as the second antibody (1 µg/ml) and a fluorescein isothiocyanate-conjugated anti-mouse Ig (10 µg/ml, BIOSOURCE Inter.) as the third antibody. ERM5 cells were stained with 3C10 scFv, but to a lesser extent than with 3C10 whole antibody.

Fig. 6. Immunohistological staining of human glioblastoma specimens with 3C10 scFv antibody. Frozen sections from glioblastoma samples that had been analyzed for abnormality of type III deletion-mutant EGFR were reacted with Ab-1 antibody reactive with normal EGFR (5 µg/ml) (A, D), 3C10 whole antibody (5 µg/ml) (B, E) or 3C10 scFv (20 µg/ml) (C, F). For 3C10 scFv, an anti-His-tag antibody was used as the second antibody (1 µg/ml). The section from patient 5 with the type III deletion-mutant EGFR (A, B and C) bound all of the antibodies, whereas that from patient 4 with amplification of wild-type EGFR (D, E and F) was only immunoreactive with Ab-1 antibody.

Fig. 7. MHA reactivity of 3C10 whole and scFv antibodies. 3C10 whole and scFv antibodies were tested against the mouse ERM5 transfectant expressing type III deletion-mutant EGFR (□) and human A431 cell line over-expressing wild-type EGFR (○). For detection of 3C10 scFv, an anti-His-tag antibody was used as the second antibody (1 µg/ml). Both 3C10 whole and scFv showed a positive reaction with ERM5 cells, but not with A431 cells.
Table III. Antibody Specificity of 3C10 Whole and scFv Antibodies Studied by Competitive MHA

| Antibody (concentration) | Peptide concentration to show 50% inhibition | Relative ratio |
|--------------------------|---------------------------------------------|----------------|
| whole (100 ng/ml)        | 0.4 ng/ml                                   | 100/20 = 50    |
| scFv (lot 1) (1 µg/ml)   | 20 ng/ml                                    | 11             |
| scFv (lot 2) (1 µg/ml)   | 60 ng/ml                                    | 6              |

Table IV. Kinetic Analysis of 3C10 Whole and scFv Antibodies Binding to Pep3 Peptide

| 3C10 antibody | $k_{on}$ (M$^{-1}$ s$^{-1}$) | $k_{off}$ (s$^{-1}$) | $K_d$ (M$^{-1}$) |
|---------------|-----------------------------|----------------------|-----------------|
| whole         | 1.30±0.03×10$^8$            | 9.8±1.7×10$^{-2}$     | 9.7±2.9×10$^3$  |
| scFv (lot 1)  | 2.95±0.21×10$^7$            | 1.21±0.02×10$^{-2}$   | 2.45±0.01×10$^7$|
| scFv (lot 2)  | 3.00±0.05×10$^7$            | 1.22±0.01×10$^{-2}$   | 2.48±0.01×10$^7$|

The $k_{on}$, $k_{off}$, and $K_d$ values were determined from seven sensorgrams using antibody at different concentrations between 1.37–1000 nM.

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