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

Norihisa Nakayashiki,[1,2] Kazuhiro Yoshikawa,[1,5] Kazuyasu Nakamura,[3] Nobuo Hanai,[3] Kenta Okamoto,[2] Sho Okamoto,[2] Masaaki Mizuno,[2] Toshihiko Wakabayashi,[2] Sinsuke Saga,[1] Jun Yoshida[2] and Toshitada Takahashi[4]

[1]Second Department of Pathology, Aichi Medical University School of Medicine, Yazako, Nagakute, Aichi 480-1195, [2]Department of Neurosurgery, Nagoya University School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, [3]Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahi, Machida, Tokyo 194-8533 and [4]Laboratory of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681

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 (V_H) and variable light chain (V_L) genes were performed to allow cloning by means of reverse transcriptase-polymerase chain reaction. The genes cloned were assembled with a linker, $(Gly_4Ser)_3$, and ligated into a bacterial expression vector to express the scFv as cytoplasmic inclusion bodies. After appropriate refolding, the antibody activity of the V_H-V_L 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 (K_A) of the parental 3C10 for the mutant peptide was 9.7×10^7 M^{-1}, while that of 3C10 scFv was $2.45–2.48\times10^7$ 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 own[8–11] 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 (V_H) amino acid sequence tethered to a variable light chain (V_L) sequence by a designed peptide which links the carboxyl terminus of the V_H to the amino terminus of the V_L 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

[5] To whom correspondence should be addressed.
E-mail: yoshikaw@amugw.aichi-med-u.ac.jp

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB030249.

immunized with Pep3 and purified truncated EGFR.[14] 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 ERM5 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.[4] A431 is a human squamous cell carcinoma cell line overexpressing intact EGFR.[15]

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*.[2, 10]; LEEKKGNYVVTDHC) corresponding to the fusion junction of the truncated EGFR, coupled to keyhole limpet hemocyanin (KLH).[11] A 17-amino-acid peptide corresponding to the fusion junction without the glycine (LEEKKVCPRNYVVTDHC) 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 Slc, 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 manufacturer's instructions. Antibodies were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through 12.5% PAGE, transferred electrophoretically onto polyvinylidine difluoride membranes (Millipore Co., Ltd., Bedford, MA), and stained with Coomassie brilliant blue R250. Bands to be sequenced were cut out. Searches for an Ig variable region subgroup with similar amino acid sequences were performed using Kabat's database (Johnson *et al*.,[16] http://immuno.bme.nwu.edu/).

cDNA of 3C10 Total cellular RNA was extracted from 1×10^9 3C10 hybridoma cells by means of the cesium chloride density gradient method as described previously.[11] Poly(A)+ RNA was purified with an oligo(dT) column (Pharmacia Biotech. Co., Ltd., Uppsala, Sweden) converted to cDNA using random primers (Pharmacia Biotech. Co., Ltd.) and then subjected to the reverse transcriptase-polymerase chain reaction (RT-PCR) with V_H or V_L cloning primers and a thermal cycler (TP3000, TaKaRa, Ohtsu) (Table I). All primers were purchased from Biologica Co., Ltd. (Nagoya, Aichi). The sequences of cloned genes were confirmed by DNA sequencing (ABI 310 Genetic Analyzer, Applied Biosystems).

Cloning vectors and scFv expression To express 3C10 scFv, the pRSET B plasmid driven by the T7 promoter derived from the M13/T7 phage (Express system, Invitrogen Co., Ltd., San Diego, CA) was used (Fig. 1). Vectors containing the V_H-*L*-V_L *scFv* gene (L, linker) and a stretch of six histidine residues were transfected into the host strain, *E. coli* JM109. The bacteria were grown at 37°C in 1 liter of SOB broth, induced in the logarithmic phase at A_{600}=0.3 with 1 m*M* isopropyl β-D-thiogalactoside (IPTG), and cultured at 30°C for 1 h. After infection with the M13/T7 phage at an MOI (multiplicity of infection) of 5 pfu/cell, they were cultured at 30°C for 5 h and harvested. The recombinant protein was obtained as cytoplasmic inclusion bodies.

Refolding method Cytoplasmic inclusion bodies in 40 ml of sonication buffer (50 m*M* Tris-HCl pH 5.0, 50 m*M* NaCl, 1 m*M* EDTA) were sonicated 5 times for 150 s at 80 W in an ice-cold bath with a sonicator (Sonifier B-12, BRANSON Co., Ltd., Danbury, CT), and then centrifuged at 9000 rpm at 4°C for 9 min. Approximately one-tenth of the white pellet obtained from 1 liter of *E. coli* culture was spread on the wall of a 50-ml centrifuge tube, dissolved in 45 ml of pH 8.0, 8 *M* urea solution (8 *M* urea, 50 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA) and then centrifuged at 9000 rpm 4°C for 2 min. The supernatant was transferred into a new 15-ml tube, mixed with 2.5 ml of histidine-tagged (His-tag) affinity agarose (Ni-NTA-agarose, QIAGEN GmbH, Max-Volmer-Straße, Hilden, Germany) and rotated for 30–45 min at room temperature in a rotator (RT-50, TAITEC Co., Ltd., Koshigaya, Saitama). Thereafter, His-tag binding proteins were eluted with 3 ml of pH 3.5, 8 *M* urea solution (8 *M* urea, 50 m*M* Tris-HCl pH 3.5, l m*M* EDTA), and diluted to 0.3 mg/ml protein concentration with pH 9.0, 8 *M* urea solution. A 5 ml aliquot of this solution was transferred to a new 15-ml tube containing 10 ml of redox solution (50 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA, 2 m*M* reduced form glutathione, 0.5 m*M* oxidized form glutathione) and then rotated at 10 rpm 4°C for 5 days. Thereafter, this mixed refolded solution was dialyzed slowly against dialysis buffer (50 m*M* Tris-HCl pH 7.6, 1 m*M* EDTA) for 2 days to obtain functional 3C10 scFv.

Serological assays 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.[11] In the immunostaining analysis, Ab-1 antibody which reacts with intact EGFR

Table I. Oligonucleotides Used for Cloning of Murine Ig *V* Genes

(a) Primers used for the primary amplification of V_H

VH1: GAGATCCAGCTGCAGCAGTCTGG

VH2: CTGGACAGGGATCCAGAGTTCCA

(b) Primers used for the primary amplification of V_L

VL1: CAGTCTCCACTCACTCTATCGGTCGC

VL2: ATACAGTTGGTGCAGCATCAGC

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

VH3: GCCATGGCTGGATCCGGTGGCGGAGGATCACATATGGAGATCCAGCTGCAGCAGTCTGGGGCAGAACTTGTGAAG
*Nde*I

VH4: GTCCATGGCGCAAAGCTTATTAATTCCGGAACCACCACCACCGGAACCACCACCTCCTGAGGAGACTGTGAGAGTGGT
*Acc*III

VL3: GCCATGGCTTCCGGAGGTGGTGGTTCACATATGGATGTTGTGATGACCCAGTCTCCACTCACTCTA
*Acc*III

VL4: TTCCATGGCGCAAAGCTTATTAATGGATCCGCCGCCACCTGATCCGCCGCCTCCTGACCGTTTTATCTCCAGCTTGGTCCCTCCACC
*Vsp*I

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.

Biacore analysis of 3C10 scFv The binding kinetics of 3C10 whole and scFv antibodies to Pep3 peptide was measured using a Biacore biosensor (Biacore AB, Rapsgatan, Uppsala, Sweden). The peptides were immobilized onto a sensor chip with 0.05 *M* N-hydroxysuccinimide and 0.2 *M* N-ethyl-N′-(dimethylaminopropyl)carbodiimide hydrochloride, and 3C10 antibody and two different lots of 3C10 scFv antibody were injected at 7 different concentrations between 1.37 to 1000 n*M*. Sensorgrams were obtained at each concentration and evaluated using the BIA evaluation 3.0 program to determine the association and dissociation rate constants [k_{assos} (M^{-1}, s^{-1}) and k_{diss} (s^{-1})]. K_A (M^{-1}) was calculated from the ratio of k_{diss}/k_{assos}.

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 $(Gly_4Ser)_3$ linker (Fig. 1). In order to design the set of oligonucleotide primers, the N-terminal amino acid sequences of the κ 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 κ 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 product with the expected molecular mass of 26 kD (Fig. 3).

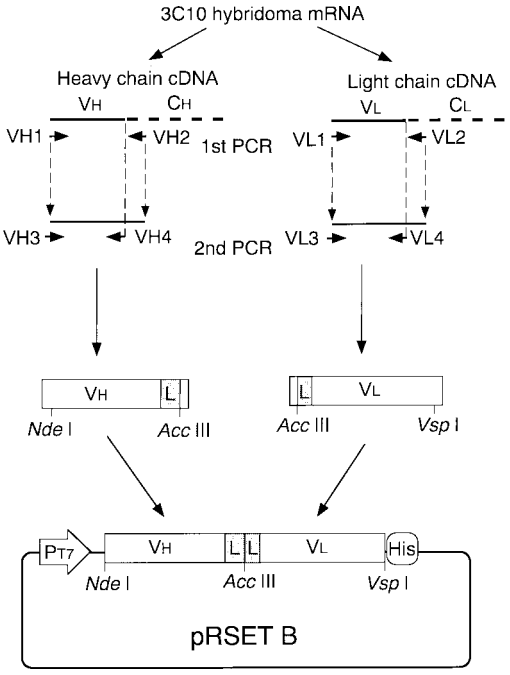

Fig. 1. Strategy for the cloning of V_H and V_L genes for the 3C10 monoclonal antibody and construction of plasmids for expression of 3C10 scFv. The plasmid pRSET B contains *Nde*I and *Vsp*I ligation sites and expresses a His-tag protein. The sequences of the PCR primers (VH1–VH4 and VL1–VL4) are shown in Table I. L indicates the region encoding the $(Gly_4Ser)_3$ linker. PT7 indicates the T7 promotor 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 m*M* reduced glutathione and 0.5 m*M* oxidized glutathione.

Serological activity of recombinant scFv After refolding, the antibody reactivity of recombinant 3C10 scFv was

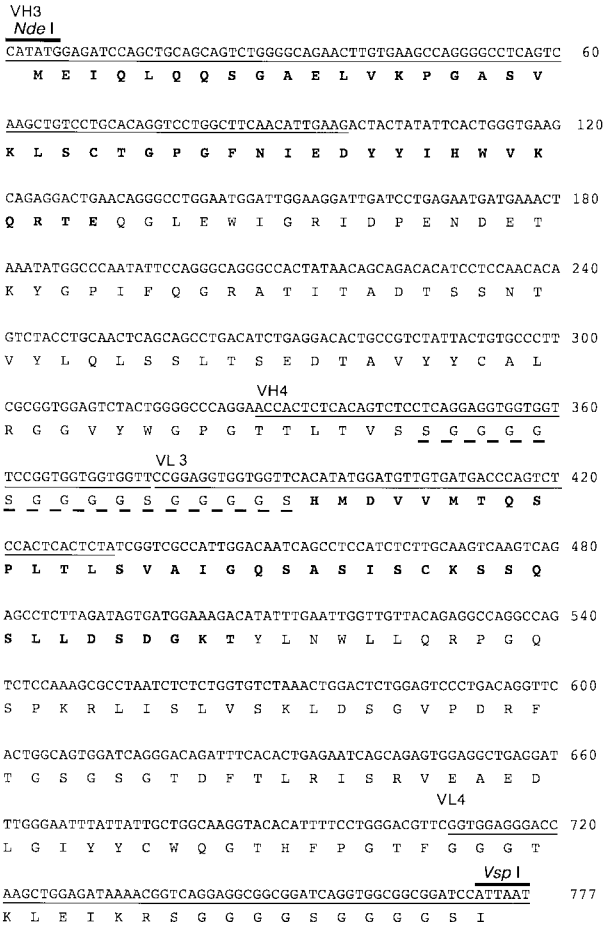

Fig. 2. Nucleotide and amino acid sequences of the V_H and V_L gene regions of the 3C10 antibody. The V_H coding region extends from position 1 to 345, V_L from 394–778, and the linker from 346–393. Restriction enzymes for recombinations are shown above, and the restriction sites are thick-underlined. PCR re-amplification primers used for cloning and assembling V_H (*VH3* and *VH4*) and V_L (*VL3* and *VL4*) genes are underlined (see Table I). The deduced amino acid sequence (one letter symbols) is shown in Roman letters; thick Roman letters are the partial protein sequence determined by Edman sequencing of the G2b heavy chain and the κ-light chain of the 3C10 antibody.

Table II. Comparison of Deduced Amino Acid Sequences of Two scFv Antibodies against Type III Mutant EGFR

(a) Heavy chain V region

	FR1	CDR1	FR2	CDR2
3C10 (IIC)	HMEIQLQQSGAELVKPGASVKLSCTGPGFNIE	DYYIH	WVKQRTEQGLEWIG	RIDPENDET
MR1(IIID)	--QVK.....GG.......L....VTS..TFR	KFGMS	..R.TSDKR...VA	SISTGGYN.

	FR3	CDR3	FR4
3C10	KYGPIFQG RATITADTSSNTVYLQLSSLTSEDTAVYYCAL	------RGGVY	WGPGTTLTVSS
MR1	Y.SDNVK. .F..SRENAK..L...M...K.....L...TR	GYSSTSYAMD.	..Q...V....

(b) Light chain V region

	FR1	CDR1	FR2	CDR2
3C10(II)	HMDVVMTQSPLTLSVAIGQSASISC	KSSQSLLDSDGKTYLN	WLLQRPGQSPKRLIS	LVSK
MR1(V)	--.IEL....AS....T.EKVT.R.	--MTS--TDI-DDDMN	.YQ.K..EPP.F...	EGNT

	FR3	CDR3	FR4
3C10	LDS GVPDRFTGSGSGTDFTLRISRVEAEDLGIYYC	WQGTHFPGT	FGGGTKLEIKR
MR1	.RP ...S..SS..T....VFT.ENTLSEDV.D...	L.SFNV.L.	..D.......-

( ): subgroup number in Kabat's data base.

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, 2.45×10^7 M^{-1} and 2.48×10^7 M^{-1}), while K_A of 3C10 whole antibody was 9.7×10^7 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-

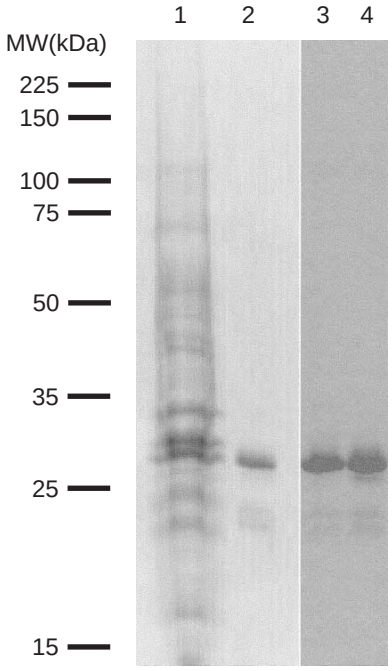

Fig. 3. SDS-PAGE and western blot analyses of 3C10 scFv of V_H-L-V_L. Cytoplasmic inclusion bodies produced by *E. coli* infected with the plasmid containing the 3C10 *scFv* gene construct were sonicated, solubilized with 8 *M* urea, affinity-purified with an His-tag agarose column and then refolded under non-reducing conditions (see "Materials and Methods"). Lanes 1, 3, total cell pellet; lanes 2, 4, affinity-purified protein with His-tag agarose column: lanes 1, 2, Coomassie blue staining; lanes 3, 4, western blotting with anti-His-tag antibody.

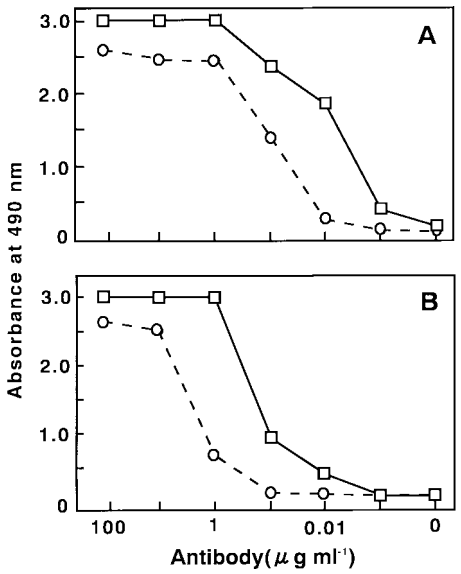

Fig. 4. ELISA reactivity of 3C10 whole and scFv antibodies tested against the immunizing peptide, Pep3, and a control peptide. Serially diluted 3C10 whole (A) and scFv (B) antibodies were assayed using immunoplates precoated with 5 μg/ml of Pep3 (□) or the control peptide (○). In the scFv case, a mouse anti-His-tag antibody (1 μg/ml) was used as a second antibody after incubation of scFv with peptide antigens, for final demonstration of binding with peroxidase conjugated anti-mouse Ig (10 μg/ml, BIOSOURCE Inter., Camarillo, CA). Absorbance values represent the means of duplicate determinations.

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 V_L, 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.[14] 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

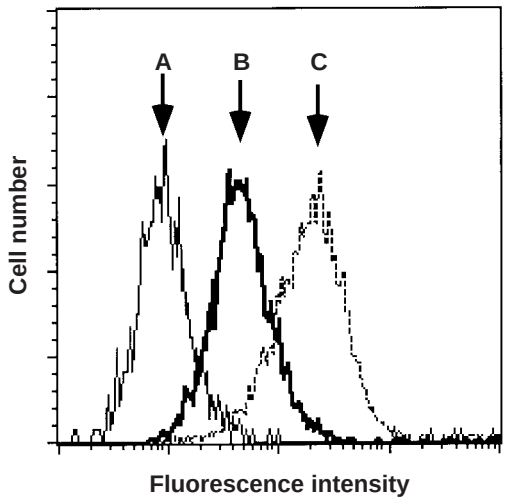

Fig. 5. FACS analysis of 3C10 whole and scFv antibodies tested against ERM5 cells. Cells were harvested after EDTA treatment of monolayer cultures, reacted with mouse myeloma protein MOPC-21 (A) as a negative control, 3C10 scFv (B) or 3C10 whole antibody (20 μg/ml) (C), stained by indirect immunofluorescence 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.

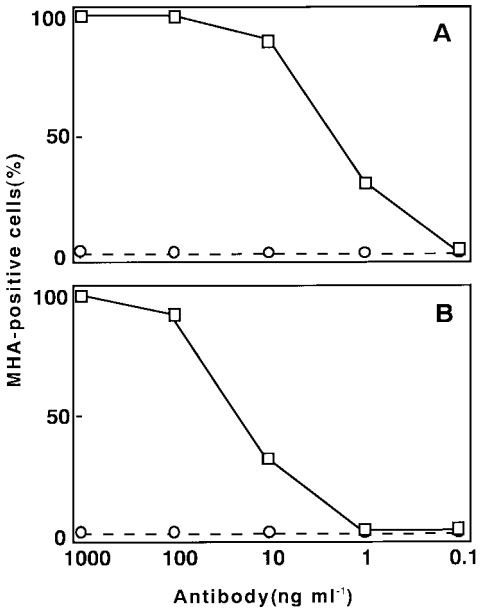

Fig. 7. MHA reactivity of 3C10 whole and scFv antibodies. 3C10 whole and scFv antibodies were tested against the mouse ERM5 transfectant expressing type III deletion-mutant EGFR (□) and human A431 cell line over-expressing wild-type EGFR (○). For detection of 3C10 scFv, an anti-His-tag antibody was used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

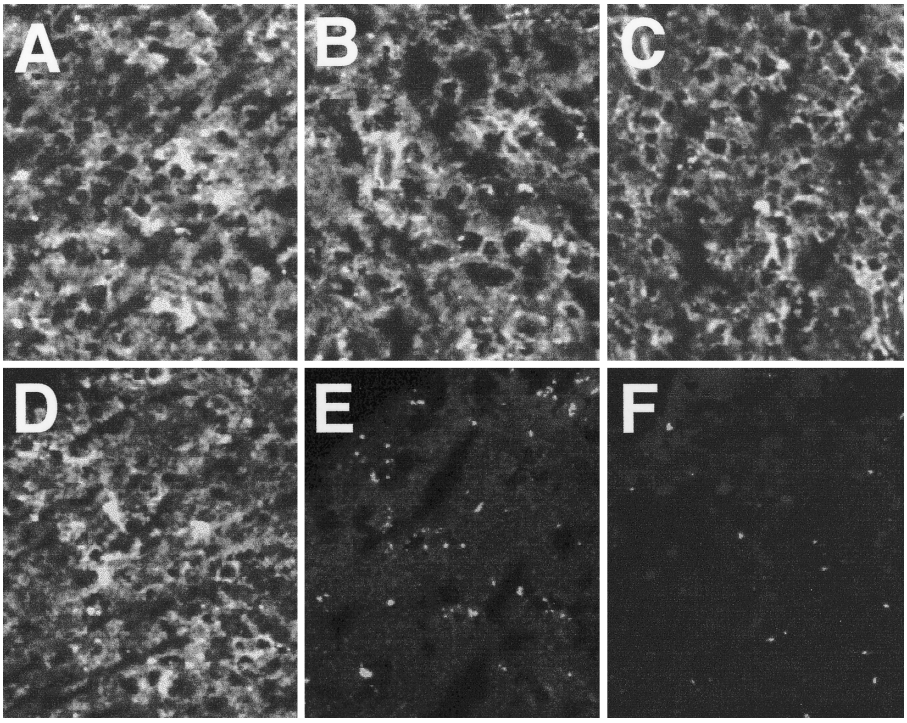

Fig. 6. Immunohistological staining of human glioblastoma specimens with 3C10 scFv antibody. Frozen sections from glioblastoma samples that had been analyzed for abnormality of type III deletion-mutant EGFR[11] were reacted with Ab-1 antibody reactive with normal EGFR (5 μg/ml) (A, D), 3C10 whole antibody (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.

Table III. Antibody Specificity of 3C10 Whole and scFv Antibodies Studied by Competitive MHA

Antibody (concentration)	Peptide concentration to show 50% inhibition[a)]		Relative ratio
	Pep3	Control peptide	Control/Pep3
whole (100 ng/ml)	0.4 ng/ml	20 ng/ml	50
scFv (lot 1) (1 μg/ml)	20 ng/ml	220 ng/ml	11
scFv (lot 2) (1 μg/ml)	60 ng/ml	360 ng/ml	6

a) The minimal antibody concentration to show nearly 100% positivity was selected and incubated with 4-fold serial dilutions of Pep3 and control peptides. Thereafter, the remaining antibody activity was tested by MHA assay, and the peptide concentration giving approximately 50% inhibition was estimated.

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

3C10 antibody	k_{assos} (M^{-1}, s^{-1})	k_{diss} (s^{-1})	K_A (M^{-1})
whole	$1.90\pm0.03\times10^{5}$	$9.8\pm1.7\times10^{-2}$	$9.7\pm0.9\times10^{7}$
scFv (lot 1)	$2.95\pm0.21\times10^{5}$	$1.21\pm0.02\times10^{-2}$	$2.45\pm0.01\times10^{7}$
scFv (lot 2)	$3.00\pm0.05\times10^{5}$	$1.20\pm0.01\times10^{-2}$	$2.48\pm0.01\times10^{7}$

The k_{assos}, k_{diss} and K_A values were determined from seven sensorgrams using antibody at different concentrations between 1.37–1000 n*M*.

in ELISA, but MHA and FACS demonstrated that it has selective reactivity for cells expressing truncated EGFR. The present study of 3C10 scFv revealed a quite similar reactivity to that of the whole antibody. The positive staining of a transfectant and a glioblastoma specimen expressing type III deletion-mutant EGFR is evidence of reactivity with a conformational epitope at the fusion junction. Competition MHA assay demonstrated that the ratio between Pep3 and control peptides required to show 50% inhibition of the reactivity of 3C10 scFv to ERM5 cells was 6 to 11, which showed that 3C10 scFv still retained a good specificity to the native epitope, although it binds severalfold more weakly than the parental 3C10. Biacore analysis showed that the binding constant of 3C10 scFv to immobilized Pep3 peptide (K_A) was $2.45–2.48\times10^{7}$ M^{-1}, showing a similar affinity to that of MR1 (scFv) for purified mutant EGFR protein, for which the K_A is 4.3×10^{7} M^{-1}.[22] Preliminary studies on the *in vivo* distribution of 99m-Tc labeled 3C10 whole antibody demonstrated a tumor/blood ratio of over 25 at 24 h after intravenous injection into nude mice bearing the mouse ERM5 transfectant expressing type III mutant EGFR. Studies are now in progress to compare the results with those for 3C10 scFv, with the ultimate aim of clinical application to glioblastoma patients.

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