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Characterization of three different single chain antibodies recognizing non-reducing terminal mannose residues expressed in *Escherichia coli* by an inducible T7 expression system

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Ayano Matsumoto-Takasaki[1], Noriyuki Yuasa[1], Daiichi Katagiri[1], Tsubasa Koyama[1], Keiko Sakai[1], Normaiza Zamri[1], Sheryl Phung[2], Shiuan Chen[2], Hiroshi Nakada[3], Munehiro Nakata[1] and Yoko Fujita-Yamaguchi[1,*]

[1]Department of Applied Biochemistry, Tokai University School of Engineering, Kanagawa 259-1292, Japan; [2]Division of Tumor Cell Biology, Beckman Research Institute of the City of Hope, Duarte, CA 91010, USA; and [3]Department of Molecular Bioscience, Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan

*Yoko Fujita-Yamaguchi, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan. Tel: +81-463-58-1211 (ext. 4188), Fax: +81-463-50-2012, email: yamaguch@keyaki.cc.u-tokai.ac.jp

We previously isolated phage antibodies from a phage library displaying human single chain antibodies (scFvs) by screening with a mannotriose (Man3)-bearing lipid. Of four independent scFv genes originally characterized, 5A3 gene products were purified as fusion proteins such as a scFv-human IgG1 Fc form, but stable clones secreting 1A4 and 1G4 scFv-Fc proteins had never been established. Thus, bacterial expression systems were used to purify 1A4 and 1G4 scFv gene products as soluble forms. Purification of 1A4 and 1G4 scFv proteins from inclusion bodies was also carried out together with purification of 5A3 scFv protein in order to compare their Man3-binding abilities. The present studies demonstrated that 1A4 and 1G4 scFv proteins have a higher affinity for Man3 than 5A3 scFv protein, which may determine whether scFv-Fc proteins expressed in mammalian cells are retained in the ER or secreted. Furthermore, the inhibitory effects of anti-Man3 1G4 scFv and anti-Tn antigen scFv proteins on MCF-7 cell growth were evaluated. Despite the fact that no obvious difference was detected in cell growth, microscopic observations revealed inhibition of foci formation in cells grown in the presence of the anti-carbohydrate scFv proteins. This finding provides a basis for the development of cancer therapeutics.

Keywords: anti-mannotriose/single chain Fv/phage display/human antibody/breast cancer cells.

Abbreviations: Ab, antibody; ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); BSA, bovine serum albumin; BSM, bovine salivary gland mucin; CBB, Coomassie brilliant blue; CDR, complementarity determining region; cfu, colony formation unit; Con A, concanavalin A; DAPI, 6-diamidino-2′-phenylindol dihydrochloride; DPPE, dipalmitoylphosphatidylethanolamine; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FBS, fetal bovine serum; GdnHCl, guanidine hydrochloride; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactoside; mAb, monoclonal Ab; Man3, mannotriose [ManR1-6(ManR1-3)Man]; MEM, Minimum Essential Medium; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-Diphenyl-2H Tetrazolium Bromide; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; scFv, single chain variable fragment; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; TBS, Tris-buffered saline; VH, variable region of antibody heavy chain; VL, variable region of antibody light chain.

Production of antibodies against carbohydrate moieties is problematic due to their poor immunogenicity. To overcome this problem, phage display technologies have been used to produce antibodies against various carbohydrate moieties without immunizing animals (*1–3*). Alterations of carbohydrate moieties occur during tumour progression and such aberrant glycosylations are special features of tumour cells (*4–7*). In normal cells, the oligo-mannose core residues are masked by terminal glycosylation. Biosynthesis of core *N*-glycosylation occurs in the ER where a high mannose core is attached to asparagine whereas maturation of *N*-glycosylation occurs in the Golgi apparatus. In contrast, the core mannose residues are exposed on tumour cell surfaces due to aberrant glycosylation (*8*, *9*). Thus, anti-high mannose antibodies, if available, could be developed into cancer diagnostics and/or therapeutics.

We previously reported isolation and characterization of phages screened by a mannotriose (Man3)-bearing lipid as an antigen from a phage library displaying human single chain antibodies (scFvs) (*10*). Four independent scFv genes, 1A4, 1G4, 5C10 and 5A3, were initially selected and characterized as phage antibodies (*10*). Although expression and purification of these scFv genes were not straightforward, 5A3 and 5C10 scFv proteins were expressed and purified as a glutathione-*S*-transferase-scFv fusion protein in *E. coli*. The 5A3 gene was also successfully expressed

and purified as a scFv-human IgG_1 Fc form in mouse myeloma NS0 cells whereas NS0 or CHO stable clones expressing 1A4 and 1G4 scFv-Fc proteins had never been established (*11*). In addition, a sharp decline in 5A3 scFv-Fc protein production was observed during early passages of the stable NS0 cell culture (*12*). Recent studies using Cos-7 cells and transient expression systems have suggested that 1A4 and 1G4 scFv-Fc proteins were synthesized but retained in the ER, which may have impaired cell growth (*12*). To avoid such severe problems associated with the mammalian expression systems, in the current study, pET bacterial expression systems were used to produce and purify anti-Man3 1A4 and 1G4 scFv proteins as well as 5A3 scFv protein to compare their Man3-binding abilities. These studies suggested that 1A4 and 1G4 scFv proteins have a higher affinity for Man3 than previously characterized 5A3 scFv protein, and that the difference in their affinities may determine whether anit-Man3 scFv-Fc proteins are retained in the ER and accumulated or secreted.

Furthermore, the inhibitory effects of anti-Man3 1G4 scFv and anti-Tn antigen scFv proteins on MCF-7 breast cancer cell growth were evaluated. Despite the fact that no obvious difference was detected in cell growth as judged by MTT assays, microscopic observations revealed foci development in cells grown for 6–10 days in the absence of antibodies or in the presence of a control IgG_3 while inhibition of foci formation in cells grown in the presence of the anti-carbohydrate scFv proteins was clearly observed. Although the inhibitory effects of 1G4 scFv protein on focus formation represent a preliminary result, this finding provides support for the development of cancer therapeutics with anti-Man3 scFv proteins.

Materials and Methods

Materials

Helper phage M13KO7, horseradish peroxidase (HRP)-labelled anti-M13, Ni^{2+}-Sepharose, Ni^{2+}-Sepharose 6 Fast Flow, CM3 Sensorchip, amine coupling kit and isopropyl β-D-thiogalactoside (IPTG) were purchased from GE Healthcare Biosciences (Piscataway, NJ, USA). The human breast carcinoma MCF7 cell line was obtained from American Type Culture Collection. 4′, 6-diamidino-2′-phenylindol dihydrochloride (DAPI) and 2,2′ azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were from Roche Diagnostics (Mannheim, Germany). SDS–polyacrylamide gradient gel, EZWestBlue and pre-stained molecular marker reagent were from ATTO Ltd (Tokyo, Japan). Mannotriose- and lactose-conjugated BSA were from Dextra Laboratories (Reading, UK). *Escherichia coli* BL21(DE3), lysozyme, Benzonase and pET22b(+) expression vector were purchased from Novagen (Germany). *Escherichia coli* JM109 was from Takara Bio Inc (Shiga, Japan). Tween-20, bovine serum albumin (BSA), Trizma base, polyethylene glycol 8000 (PEG), concanavalin A (Con A), kanamycin, imidazole, monoclonal anti-vinculin antibody, FITC labelled anti-rabbit IgG, mouse IgG_3, guanidine hydrochloride (GdnHCl), L-glutathione oxidized form (GSSG), ribonuclease B (RNase B), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H tetrazolium bromide (MTT), lysozyme and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) were purchased from Sigma-Aldrich (St Louis, MO, USA). Biotin-labelled Con A was obtained from J-OIL MILLS (Tokyo, Japan). His-probe was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). HRP-conjugated anti-His-tag antibody was obtained from Abcam (Cambridge, UK). BSA-conjugated mannotriose (Man3-BSA) was purchased from Glyco Tech (Gaithersburg, MD, USA). B-PER and His Probe-HRP were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Carbenicillin, dithiothreitol (DTT), glycerol, polyoxyethylene, octylphenyl ether (NP-40), chloramphenicol, tetracycline, L(+) arginine and L-glutathione (reduced form) (GSH) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other purchased reagents were of the highest grade. Antibodies used in this study included MLS128 with affinity for three consecutive Tn-antigen clusters (*13*), MLS128 scFv protein (Yuasa, N., Ogawa, H., Koizumi, T., Tsukamoto, K., Matsumoto-Takasaki, A., Asanuma, H., Nakada, H., and Fujita-Yamaguchi, Y., manuscript in preparation), phages displaying 1H7 scFv protein against insulin-like growth factor-I receptor (IGFIR) as previously prepared (*14*), and 1H7 scFv-Fc (*15*).

Cell culture

MCF-7 cells were cultured in Improved MEM containing 5% FBS and 11.25 nM insulin (Sigma-Aldrich). MCF 7 aro cells stably expressing human aromatase (*16*) were maintained in T-75 or T-25 flasks (SARSTEDT; Numbrecht Germany) in Minimum Essential Medium containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin (10 IU/ml)/streptomycin (10 μg/ml) and 100 μg/ml G418 in the presence of Phenol Red. For MTT and focus assays, the medium was changed to the MEM containing charcoal–dextran-treated foetal bovine serum (Omega Scientific, Tarzana, CA, USA). Cells were incubated in this medium for 3 days before use in assays.

Preparation of phages displaying human scFvs (phage antibodies)

The phages displaying anti-M3 scFvs that were isolated were prepared as previously described (*10*). Briefly, phages displaying 1A4, 1G4 and 5A3 proteins were amplified in *E. coli* XL1-Blue co-infected with helper phages. The resulting phage particles were concentrated by PEG-precipitation, and dissolved in TBS containing 0.5% BSA. The phage solutions were treated with benzonase, followed by centrifugation and filtration to remove pellets as described (*17*). The resulting supernatants containing phages were titrated (*17*) and used in binding assays.

Construction of expression vectors

1A4, 1G4 and 5A3 scFv genes were amplified by PCR from phagemids encoding respective scFv genes as templates using Fw and Rv primers as listed in Table I 1A4, 1G4 and 5A3 scFv genes were ligated into EcoRI and HindIII-digested pET22b to construct pET/1A4, pET/1G4 and pET/5A3 expression vectors (Fig. 2). *Escherichia coli* JM109 was used as a host for all plasmid preparations whereas *E. coli* BL21 (DE3) was used for expression of scFv proteins. The scFv sequences of pET22b /1A4, 1G4 and 5A3 were confirmed by DNA sequencing.

Table I. Primers used in PCR for construction pET expression vectors.

Primers	Sequences[a]
1A4 Forward	5′-ATCGATCGA*GAATTC*GCAGGTGCAGCTGCAGCAG-3′ (EcoR I)
1A4 Reverse	5′-CATACGTAT*AAGCTT*GCCTTTGATTTCCTACCTTGGT-3′ (Hind III)
1G4 Forward	5′-ATCATGTC*GAATTC*GCAGGTGCAGCTGGTG-3′ (EcoR I)
1G4 Reverse	5′-ATCATACG*AAGCTT*GCCTTTAATCTCCAGTCGTGT-3′ (Hind III)
5A3 Forward	5′-CGATGC*GAATTC*CCAGGTGCAGCTGCAGCAGT-3′ (EcoR I)
5A3 Reverse	5′-CGATGC*AAGCTT*TAGGACGGTCAGCTTGGTCC-3′ (Hind III)

[a]Underlined and italic sequences are restriction sites for enzymes indicated parenthesis.

Purification of 1A4 and 1G4 scFv proteins from periplasmic and cytoplasmic fractions of E. coli cells

Escherichia coli BL21 (DE3) cells transformed with pET22b (+)/1A4 or 1G4 expression vector were inoculated into 50 ml of LB medium containing ampicillin (50 μg/ml) and cultured at 37°C with shaking at 250 rpm. After the culture's optical density at 600 nm reached 0.3–0.5, the expression of scFv proteins was induced by the addition of 250 μM IPTG and culturing overnight at 20°C with shaking at 250 rpm. Periplasmic and cytoplasmic extracts were prepared from bacterial cells as described previously (*18*). Briefly, bacterial cells were collected by centrifugation at 7,500 *g* for 30 min and cell pellets were suspended in 1 ml of 0.2 M Tris–HCl, pH 8.0, containing 0.5 mM EDTA and 0.5 M sucrose (TES). This was followed by adding 1.5 ml of ice-cold 5-fold diluted TES and incubating the cells on ice for 30 min. Pellets were removed by centrifugation at 10,000 *g* for 40 min at 4°C. The supernatants were recovered as periplasmic extracts. Pellets were suspended in 2 ml of non-ionic detergent BugBuster (Novagen) containing a protease inhibitor cocktail (Sigma-Aldrich; P8340) and lysozyme (Novagen) and then rotated for 1 h at room temperature. After centrifugation at 10,000 *g* for 50 min at 4°C, supernatants were recovered as cytosolic extracts. The periplasmic extracts were treated with 0.1% NP-40. Soluble scFv proteins from the periplasmic and cytoplasmic extracts were purified by Ni^{2+}-Sepharose affinity chromatography as described (*18*).

Preparation of inclusion bodies from E. coli cells overexpressing scFv proteins

Escherichia coli BL21(DE3) cells transformed with pET22b (+)/1A4, 1G4 or 5A3 expression vector were inoculated into 80 ml of LB medium containing 50 μg/ml carbenicillin. After cells were grown to OD_{600} of ~0.5 by incubation at 37°C with shaking, expression of scFv proteins was induced by the addition of 1 mM IPTG into the medium. After incubation for 4 h, bacterial cells were harvested by centrifugation at 2,900 *g* for 30 min, and then stored at −30°C for future experiments.

Inclusion bodies were prepared from the bacterial cells by suspension in B-per Bacterial Protein Extraction Reagent (Pierce; Thermo Scientific Life Science, Rockford, IL, USA) according to the manufacture's protocol. Briefly, 2.5 ml of the reagent containing 500 μg of lysozyme and 63 U of benzonase per 1 g of wet cells were added to bacterial cells. After gentle shaking for 10 min, pellets were collected by centrifugation at 14,500 *g* for 20 min, and then suspended in 15 ml of 10-fold diluted B-per reagent. After gentle shaking for 10 min, pellets were then collected as inclusion bodies by centrifugation at 14,500 *g* for 20 min.

Purification and refolding of scFv proteins from inclusion bodies

Inclusion bodies were solubilized in 20 mM sodium phosphate buffer, pH 7.4, containing 3.5 M GdnHCl and 0.2 M NaCl (3.5 M GdnHCl buffer), and subjected to centrifugation at 14,500 *g* for 30 min. Ni^{2+}-Sepharose gels (0.5 ml) were added into a test tube containing the supernatant. After the tube was inverted overnight at 25°C, the Ni^{2+}-Sepharose gels were packed into a column. The column was washed thoroughly with 3.5 M GdnHCl buffer containing 40 mM imidazole. ScFv proteins were then eluted with 3.5 M GdnHCl buffer containing 500 mM imidazole and 5 mM DTT.

Refolding of denatured scFv proteins was carried out with some modifications according to the stepwise dialysis method previously described (*19*). The scFv proteins purified in the presence of 3.5 M GdnHCl were re-folded by stepwise dialyses as follows. The concentrations of the unfolded scFv proteins were adjusted to 3.3 μM in 3.5 M GdnHCl buffer. The affinity-purified denatured scFv protein in 20 ml was dialysed against 300 ml of 50 mM Tris–HCl buffer, pH 8.0, containing 0.2 M NaCl and 1 mM EDTA (dialysis buffer) in the presence of 2 M GdnHCl, and then (i) 80 ml of the dialysis buffer containing 1 M GdnHCl, 0.4 M L-arginine, 1 mM GSSG and 2 mM GSH; (ii) 80 ml of the dialysis buffer containing 0.5 M Gdn-HCl, 0.4 M L-arginine, 1 mM GSSG and 2 mM GSH and (iii) 300 ml of PBS (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl).

SDS–PAGE and western blotting

The purified scFv proteins were analysed by SDS–PAGE (5–20% acrylamide gel) (ATTO) under reducing conditions. For immunoblotting, the proteins on the SDS–PAGE gel were transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with PBS containing 3% BSA for 2 h at 37°C. After it was washed with PBS, the membrane was incubated with HRP-conjugated His-probe at room temperature for 1 h. After the membranes was washed with PBS containing 0.2% Triton X-100 and PBS five times each, scFv proteins were visualized by colour development using EzWestBlue (ATTO).

ELISA using lipid- or protein-based carbohydrate probes

Various versions of ELISA were used to evaluate binding of both phage antibodies and purified scFv proteins to carbohydrate residues. Wells of a 96-well plate were coated with Man3-dipalmitoylphosphatidylethanolamine (DPPE) or Man3- or lactose-conjugated BSA (Lac-BSA) as described previously (*10*, *11*). Briefly, the carbohydrate probe-coated wells (0.5 μg/well) were blocked with 150 μl/well of TBS containing 3% BSA by incubation at 37°C for 2 h. Control plates were prepared as above without carbohydrate probes. Fifty microlitre of phage suspensions (5×10^{10} cfu of phages) or scFv proteins were added to the wells and incubated at 37°C for 2 h. The wells were washed 10 times with 200 μl of TBS. Bound phage antibodies or scFv proteins were detected by incubation with HRP-labelled anti-M13 antibody or HRP-conjugated anti-His-tag antibody, respectively. The bound phage antibodies or scFv proteins were detected by colour development using a 100 μl ABTS solution (Roche Diagnostics, Basel, Switzerland). Absorbance at 415 nm was measured with a plate reader (Bio-Rad, Hercules, CA, USA).

Alternatively, RNase B, containing high mannose type of *N*-glycans (*20*), was used as a natural antigen. Wells were coated with ribonuclease B (2.5 μg /well). After the wells were blocked with PBS containing 3% BSA, 100 μl of refolded cFv proteins were added. The bound scFv proteins were detected with HRP-conjugated anti-His tag antibody using ABTS solution as described above.

Detection of 1A4, 1G4 or 5A3 phage antibody binding to MCF-7 breast cancer cells

MCF-7 cells cultured in 96-well plastic plates (1×10^4 cells/well) for 16 h were used to measure the binding activities of phage antibodies. Fifty microlitre of 10^8–10^{10} cfu/ml of phages displaying 1A4, 1G4 or 5A3 scFv protein, phages displaying anti-IGF-I receptor scFv protein derived from 1H7 mAb (*14*) as a positive control, and helper phages as a negative control were added to the wells to observe binding ability to MCF-7 cells. Following 16 h incubation at 4°C, phages bound to cells were detected by incubation with HRP-labelled anti-M13 antibody at 37°C for 1 h. After cells were washed 10 times with 200 μl TBS, colour development and measurement were carried out as described above.

Detection of 1A4 or 1G4 scFv protein binding to MCF-7 breast cancer cells by fluorescence microscopy

Breast cancer MCF-7 cells were grown on 10-well glass slides (Matsumi Glass, Osaka, Japan) that had been coated with collagen, 50 μl/ well of freshly prepared cells (suspended in culture medium: 5×10^5 cells/ml) were seeded and cultured for 18 h. Attached cells were fixed with a Zamboni solution (phosphate-buffered 4% paraformaldehyde with picric acid) for 15 min. Cells fixed in wells were washed quickly with 50 μl/well of PBS and then treated with PBS containing 0.1% of Triton X-100 for 5 min to permeabilize cells. After cells were washed with PBS and blocked with 3% BSA for 2 h, cells in wells were washed with PBS, and then incubated with 50 μl of purified scFv proteins (10 μg/ml) or biotin-labelled Con A (2 μg/ml) for 18 h at 4°C. After cells were washed with PBS, the cells were incubated with anti-vinculin antibody for 1 h at room temperature and washed with PBS. ScFv proteins-bound cells were immunostained with anti-His-tag antibody by incubation at room temperature for 1 h, followed by washing with PBS and incubation with Cy3-labelled anti-mouse IgG. Biotin-labelled Con A was visualized by incubation with Rhodamine-labelled Streptavidin. Anti-vinculin antibody was detected by incubation with FITC-labelled anti-rabbit IgG. After the cells were stained with 1 μg/ml DAPI for 5 min, slides were washed with PBS three times and mounted with PBS/30% glycerol containing DABCO solution (Wako). Binding of 1A4 and 1G4 scFv proteins, and Con A was examined using Keyence fluorescence microscopy (BZ9000; Keyence, Osaka, Japan).

Determination of affinity constants by surface plasmon resonance

Real-time measurement of the binding of scFv proteins to Man3-BSA was analysed by surface plasmon resonance (SPR). All SPR analyses were carried out at 25°C on a Biacore 3000 biosensor (GE Healthcare, Uppsala, Sweden). Man3-BSA was immobilized on CM3 sensor chips using the amine coupling kit supplied by the manufacturer. Binding of scFv proteins to carbohydrate moieties immobilized on the surface of the sensor chips was monitored at a flow rate of 20 µl/min. The rates of association (k_a) and dissociation (k_d) for scFv proteins were determined by a monovalent analyte model using BIA evaluation version 3.0 software. The dissociation constant (K_D) was derived by dividing k_d by k_a.

Effects of anti-carbohydrate scFv proteins and antibodies on breast cancer cell proliferation

Monolayer cell-growth was measured using the MTT assay (*21*). MCF-7 aro cells were seeded in 96-well plates with 2.5×10^4 cells/well in 0.2 ml of the regular medium. After 24 h, cells were fed with 0.2 ml of the basal medium containing 1 nM testosterone in the absence or presence of MLS128, IgG_3, 1H7, MLS128 scFv or 1G4 scFv. The cells were fed with media containing antibodies or scFvs on Days 4 and 7. On Days 1, 4, 7 and 10, 100 µl of 1% MTT (10 mg/ml of MTT in the culture medium) were added to each well. After incubation for 1 h at 37°C, cells were dissolved by adding 200 µl of DMSO. The absorbance was measured at 570 nm using a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Since previous research has indicated that confluent monolayer MCF-7 cells develop multi-cellular nodules, termed foci and that disruption of the terminal sugars on membrane receptors by β-galactosidase results in the inhibition of focus development (*22*), a focus assay was performed as described (*22*, *23*). MCF-7 aro cells were prepared and treated with antibodies or scFv proteins as above. On Day 10, cells were fixed with 10% formalin. After drying, cells were stained with 1% Rhodamine B in distilled water (200 µl/well) and rinsed with tap water until most of the stain was removed from the monolayer. Stained cells in each well were observed under a microscope (Eclipse TE2000-S, Nikon). The stained foci that were readily distinguishable from the monolayer were quantified by measuring fluorescence intensity using the SpectraMax M5 Microplate Reader, set at a bottom read with 550 excitation and 590 emission filters. Results were recoded in relative fluorescence units.

Results

Comparison of binding characteristics of three anti-Man3 phage clones

Binding characteristics of phage clones, 1A4 and 1G4, to Man3-BSA or control Lac-BSA were determined by ELISA to further characterize those phage clones, which had been screened from a phage library using Man3-DPPE as described previously (*10*). Both phage clones displaying 1A4 and 1G4 scFv proteins bound to the Man3-BSA but did not bind to Lac-BSA or BSA (Fig. 1A), suggesting that phage displayed 1A4 and 1G4 scFv proteins bound to the Man3 epitope. Since breast cancer MCF-7 cells display mannose-rich glycans on the cell surface (*24*), ELISA was carried out using MCF-7 cells as natural Man3 targets. Phages displaying anti-IGFIR 1H7 scFv protein (*14*) were used as a positive control since MCF-7 cells express a large amount of IGFIR (*25*). Phage concentrations ranging from 10^9 to 10^{11} cfu/ml were used to compare the binding ability of each phage clone to MCF-7 cells. The results clearly demonstrated that anti-IGFIR 1H7 scFv displaying phages bound to MCF-7 cells at the highest level (Fig. 1B). Among three anti-Man3 phages, 1A4 phage clone had the highest, nearly as high level of binding, followed closed by the 1H7 phage clone, whereas 1G4 and 5A3 phages had medium- and low levels of binding, respectively. The binding of M13 helper phage to MCF-7 cells was negligible (Fig. 1B). These results indicated that 1H7 and anti-Man3 phages bound to IGFIR and Man3-like epitopes, respectively, which are expressed on the surface of MCF-7 cells.

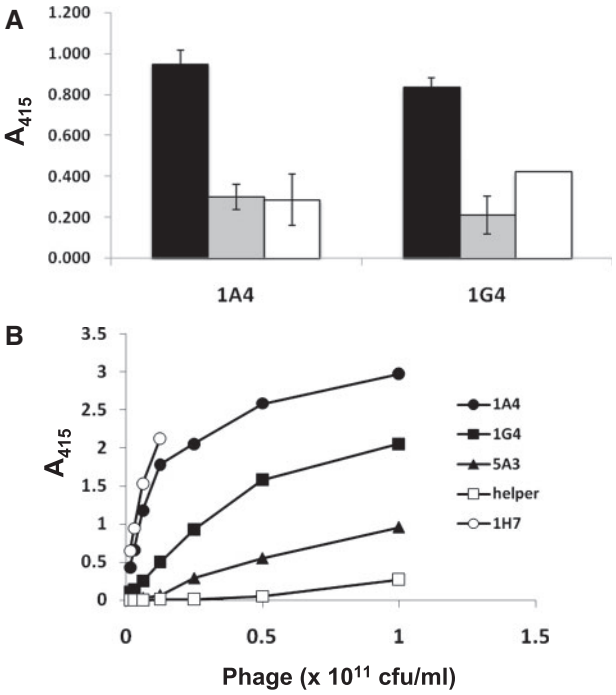

Fig. 1 Binding abilities of phage antibodies displaying 1A4, 1G4 or 5A3 scFv. (A) Binding of 1A4 or 1G4 scFv protein-displaying phages to Man3-BSA (closed column), lactose-BSA (shaded column) and BSA (open column). (B) Binding of scFv protein-displaying phages to MCF-7 breast cancer cells. MCF-7 cells fixed in wells of 96-well plates were incubated at 37°C for 2 h with 1A4, 1G4, 5A3 or 1H7 scFv protein-displaying phages and helper phage at indicated concentrations. Bound phage antibodies were detected by incubation with HRP-labelled anti-M13 antibody followed by colour development as described in the 'Materials and Methods' section.

Purification of soluble scFv proteins expressed in E. coli *cells*

Since the levels of scFv protein expression from phagemid-transformed *E. coli* cells were too low to purify for further characterization (*10*), various expression systems have been utilized, resulting not only in some successes but also leading to problems as described previously (*11*, *12*). In this study, scFv genes of 1A4, 1G4 and 5A3 were subcloned into the pET-22 b(+) vector as illustrated in Fig. 2, in order to express scFv proteins in *E. coli*. The pET expression system allows a high level T7 RNA polymerase-based gene expression in *E. coli* BL21 (DE3). The constructs contained the pelB leader sequence at the amino-terminus and the His-tag sequence at the carboxyl-terminus, thus allowing accumulation of expressed proteins in the periplasm, and detection or purification of the expressed protein, respectively.

Conditions for induction of 1A4 and 1G4 scFv gene expression by IPTG were optimized to express scFv

proteins in the periplasm. Periplasmic fractions were then prepared from IPTG-induced *E. coli* BL21(DE3) cells under the conditions described in the 'Materials and Methods' section, and subjected to Ni^{2+}-Sepharose affinity chromatography to purify 1A4 and 1G4 scFv proteins. The passed-through fractions and eluates were analysed by SDS–PAGE and western blotting (Fig. 3). CBB-stained gels revealed protein bands with a molecular mass of ~30 kDa (Fig. 3A, lanes 2 and 4, respectively), and the proteins in question were recognized by His probe-HRP (Fig. 3B, lanes 2 and 4). These results indicated that 1A4 and 1G4 scFv proteins were purified from periplasmic extracts to ~80 and 60% homogeneity, respectively, by Ni^{2+}-Sepharose affinity chromatography. The scFv proteins were also purified from the cytosolic fractions by Ni^{2+}-Sepharose affinity chromatography. As summarized in Table II, significant amounts of both 1A4 and 1G4 scFv proteins were purified from cytosolic fractions (Table II). Binding activity of the periplasm-derived scFv proteins to Man3-DPPE examined by ELISA indicated that 1A4 and 1G4 scFv proteins had binding affinity for Man3 (Fig. 3B). The cytoplasm-derived scFv proteins, however, did not exhibit significant binding activity (data not shown). Binding kinetics of the periplasm-derived scFv proteins to Man3-BSA were next determined by SPR. K_D of 1.1×10^{-7} M was calculated from sensorgrams obtained for binding of 1A4 scFv protein to Man3-BSA (data not shown). Similar SPR experiments on 1G4 scFv protein, however, failed to yield sensorgrams.

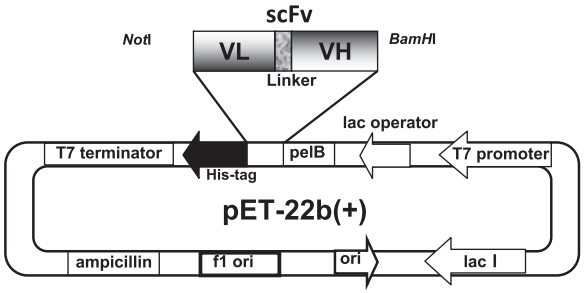

Fig. 2 Schematic presentation of the bacterial scFv expression vector used in this study. The pET expression system allows a high level T7 RNA polymerase-based gene expression in *E. coli*. BL21(DE3). The construct contains the pelB leader and His-tag sequence at the amino-terminus and the carboxyl-terminus of scFv proteins, respectively.

Immunofluorescent staining of cancer cells by scFv proteins

To evaluate the binding ability of anti-Man3 scFv proteins to glycoproteins expressed in cancer cells, binding of the periplasm-derived 1A4 and 1G4 scFv proteins as well as Con A as a positive control to MCF-7 cells

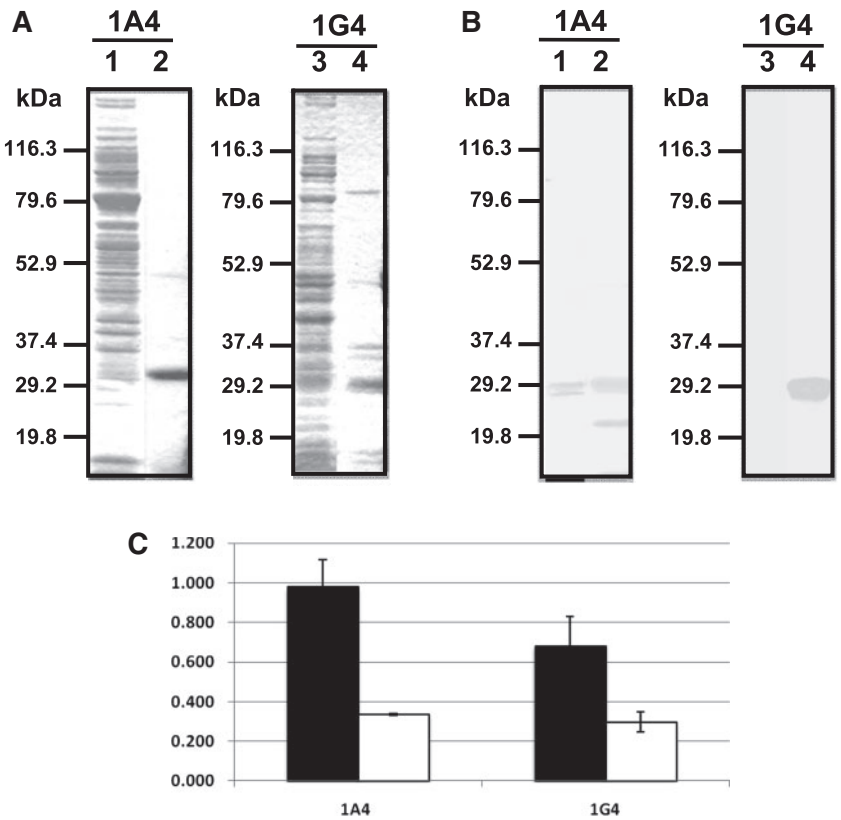

Fig. 3 SDS–PAGE, immunoblotting and binding analyses of 1A4 and 1G4 scFv proteins prepared from periplasmic fractions. (A) CBB-stained 4–20% acrylamide gels. Samples included are passed-through fractions (lanes 1 and 3) and 400 mM imidazole-eluates from a Ni^{2+}-Sepharose column (lanes 2 and 4). (B) Immunoblotting of the SDS–PAGE gels shown in A with His-probe. (C) Binding of the 400 mM imidazole-eluates containing 1A4 and 1G4 scFv proteins to Man3-DPPE (closed columns) or DPPE (open columns) determined by ELISA.

Table II. Summary of two purifications of anti-Man3 scFv proteins expressed in *E. coli*.

Clone[a]	1A4		1G4	
Origin of scFv proteins	**Periplasm**	**Cytosol**	**Periplasm**	**Cytosol**
Eluates from Ni^{2+}-Sepharose chromatography (μg)	120	420	76	576

Clone[b]	1A4	1G4	5A3
GdnHCl solubilized fraction (mg)	15.1	11.9	17.3
Eluates from Ni^{2+}-Sepharose chromatography (μg) (%)	756 (100)	660 (100)	528 (100)
Soluble scFv proteins after refolding (μg) (%)	612 (81)	586 (89)	271 (51)

[a]Soluble scFv proteins prepared from periplasmic and cytosolic fractions starting from 100 ml cultures. [b]Purification and refolding of anti-Man scFv proteins from inclusion bodies prepared from 40 ml culture.

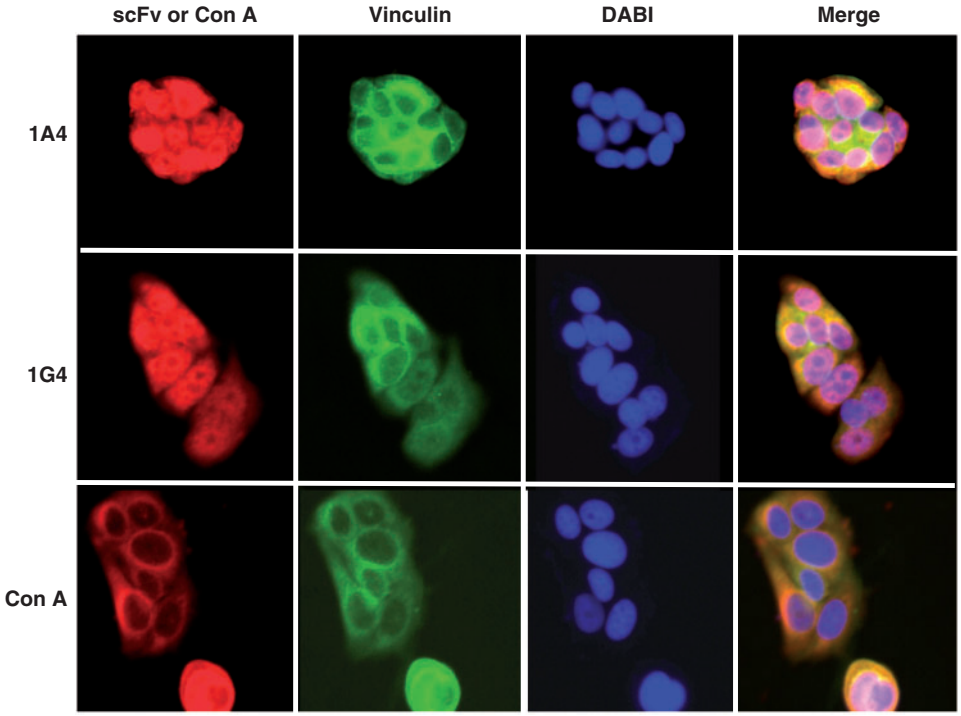

Fig. 4 MCF-7 breast cancer cells immunostained with 1A4 and 1G4 scFv proteins in comparison to those stained with Con A. 1A4 or 1G4 scFv protein-bound MCF-7 cells were immunostained with anti-His-tag antibody, followed by incubation with Cy3-labelled anti-mouse IgG (red). Biotin-labelled Con A was visualized by incubation with Rhodamine-labelled Streptavidin (red). Anti-vinculin antibody was detected by incubation with FITC-labelled anti-rabbit IgG (green). Nuclei were stained with DAPI (blue). All three colours are merged, as shown in the far-right column (merge).

were analysed by immunofluorescence microscopy. Vinculin was used to stain cytoplasmic faces of both cell–cell and cell extracellular matrix adherens type junctions. As seen in Fig. 4, the staining of MCF-7 cells with Con A matched areas stained with vinculin, but interestingly both 1A4 and 1G4 scFv proteins bound not only to cell surface-cytoplasmic areas where vinculin bound to but also to nuclei. The results are in fact consistent with the earlier observation that binding of 1A4 and 1G4 phages to MCF-7 cells was partially inhibited in the presence of Con A as measured by ELISA (data not shown). These results does not, however, prove their Man3-specificity, but indicated that 1A4 and 1G4 scFv proteins bound to natural yet unidentified epitopes expressed in MCF-7 cells.

Purification and refolding of three scFv proteins from inclusion bodies

Generally speaking, purification of soluble forms of scFv proteins which are usually folded correctly and thus expected to have binding activity would be preferable. The results as described above, however, revealed that the yields of active scFv proteins purified from soluble fractions were too low to be practical. As a next step, abundant 1A4, 1G4 and 5A3 scFv proteins were recovered in inclusion bodies, which were then subjected to purification and refolding processes. Inclusion bodies were isolated from IPTG-induced *E. coli* BL21(DE3) cells transformed with pET22b (+)/1A4, 1G4 or 5A3 expression vector. Most of the insoluble proteins recovered were scFv proteins as detected by CBB staining and Western blotting with

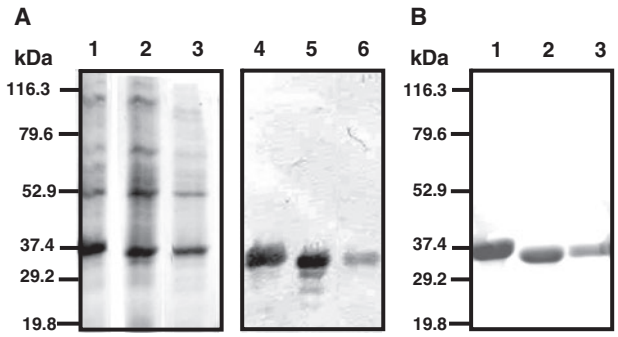

Fig. 5 SDS–PAGE and Western blotting analyses of 1A4, 1G4, and 5A3 scFv proteins purified from inclusion bodies by Ni^{2+}-Sepharose chromatography followed by refolding. (A) SDS–PAGE on 4–20% acrylamide gel under reducing condition was carried out to analyse inclusion bodies prepared from IPTG-induced *E. coli* BL21 cells transformed with pET/1A4, 1G4 or 5A3 vector. Shown are the CBB-stained SDS–PAGE gel (lanes 1, 2 and 3) and its western blot (lanes 4, 5 and 6) for 1A4, 1G4 and 5A3 scFv proteins, respectively. (B) SDS–PAGE of soluble fractions prepared by stepwise dialysis from purified 1A4, 1G4 and 5A3scFv proteins in the presence of 3.5 M Gdn-HCl. CBB-stained bands for the purified and refolded 1A4, 1G4 and 5A3scFv proteins are seen in lanes 1, 2 and 3, respectively.

Table III. Kinetic parameters of 1G4, 1A4 and 5A3 scFv proteins binding to Man3-BSA.

Antibodies	k_a ($M^{-1}s^{-1}$)	k_d(s^{-1})	K_D (M)
1A4 scFv	1.6×10^2	3.9×10^{-4}	2.5×10^{-6}
1G4 scFv	2.2×10^2	4.0×10^{-4}	1.8×10^{-6}
5A3sc Fv	1.9×10^2	7.1×10^{-4}	3.8×10^{-6}

Kinetic parameters were determined from data shown in Fig. 6A by a monovalent analyte model using BIAevaluation version 3.0 software.

HRP-conjugated anti-His tag antibody (Fig. 5A). Inclusion bodies were solubilized in 3.5 M Gdn-HCl, from which scFv proteins were purified by Ni^{2+}-Sepharose chromatography in the presence of 3.5 M Gdn-HCl. A typical purification result is shown in Table II. The purified scFv proteins were refolded by the stepwise dialysis method, which resulted in 612, 586 and 271 μg of soluble 1A4, 1G4 and 5A3 scFv proteins, respectively, from 40 ml of culture (Table II). The resulting soluble scFv proteins were apparently homogenous as judged by SDS–PAGE and CBB staining (Fig. 5B). The sFv proteins moved on the SDS–PAGE gel according to their theoretical molecular masses of 34.0, 32.1 and 33.7 kDa for 1A4, 1G4 and 5A3 scFv proteins, respectively.

Binding activity of the purified scFv proteins

The three scFv proteins that had been purified and refolded were subjected to SPR analyses to readily compare their affinity constants against Man3-BSA. Kinetic parameters for 1A4, 1G4 and 5A3 scFv proteins were determined (Table III) from the sensorgrams shown in Fig. 6A. The results indicated that K_D values for 1A4 and 1G4 scFv proteins are smaller than that of 5A3 scFv protein. Binding ability of the three scFv proteins to RNase B, a natural glycoprotein, was next compared by ELISA. As seen in Fig. 6B, 1A4 and 1G4 scFv proteins have higher binding activities for RNase B than 5A3 scFv protein, confirming the results obtained by SPR.

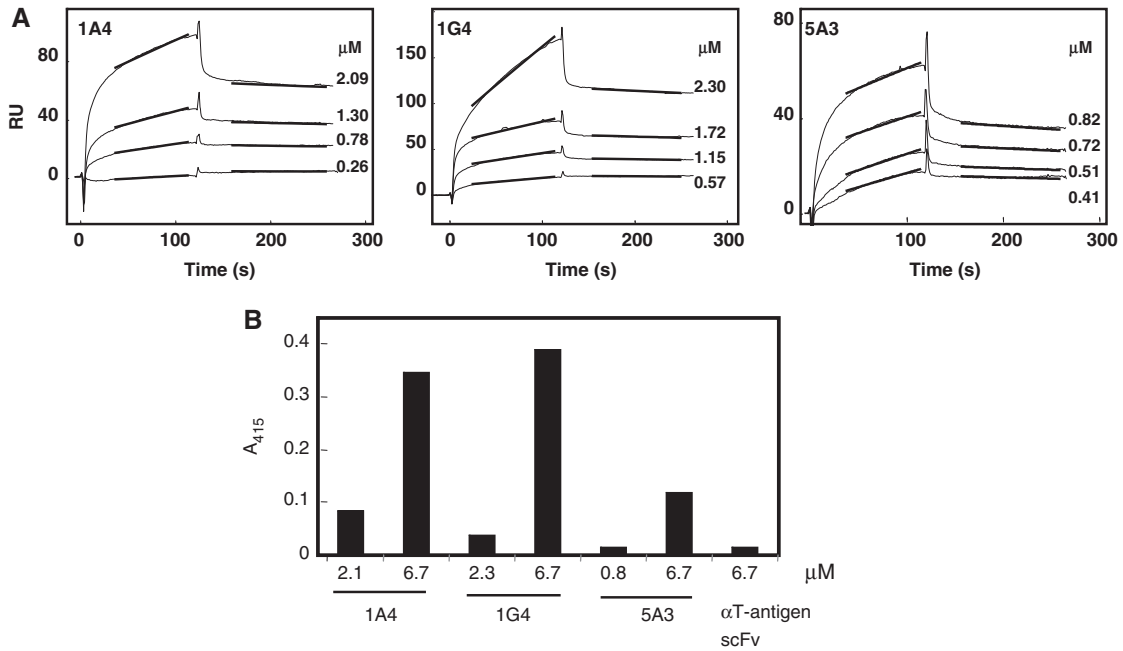

Fig. 6 Binding activity of purified and refolded 1A4, 1G4, or 5A3 scFv proteins as determined by SPR and ELISA. (A) Sensorgrams of the binding of antibodies, 1A4, 1G4 or 5A3 scFv proteins to the immobilized Man3-BSA. Different concentrations of each scFv protein as indicated were analysed on the same Man3-BSA-immobilized CM3 sensorchip. Kinetic parameters derived from these experiments are tabulated (Table III). (B) Binding of 1A4, 1G4 and 5A3 scFv proteins to RNase B as determined by ELISA. The scFv proteins at indicated concentrations were added to RNase B (2.5 μg/well)-coated wells. Bound scFv proteins were then detected with HRP-conjugated anti-His-tag antibody. The activity shown represents the relative absorbance after subtraction of the absorbance obtained with the BSA control.

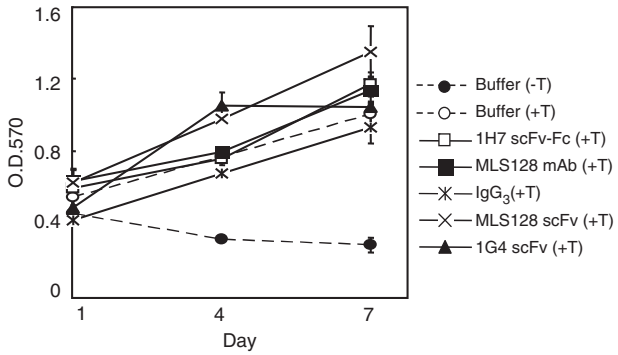

Fig. 7 Effects of various forms of antibodies and anti-Man3 scFv protein on breast cancer cell growth. MCF-7 aro cells cultured in 96-well plates were fed with 0.2 ml of the basal medium, Buffer (+T), containing 1 nM testosterone in the absence or presence of MLS128, IgG3 used as a control antibody for MLS128, 1H7 used as a non-anti-carbohydrate antibody control, MLS128 scFv or 1G4 scFv as described in the 'Materials and Methods' section. The cells were fed with the medium containing antibodies or scFvs on Days 4 and day 7. On Days 1, 4 and 7, cell growth was measured by MTT assays. Buffer (−T) is MCF-7 aro cell growth in the medium without testosterone, showing no cell growth.

Effects of anti-carbohydrate scFv proteins and control antibodies on breast cancer cell growth and focus development

As seen in Figs 1B and 4, 1G4 and 1A4 scFv proteins clearly bound to the Man3-like epitopes expressed on the MCF-7 cells. Table III and Fig. 6 suggested that purified1G4 and 1A4 scFv proteins have similar affinities for Man3 epitopes presented by Man3-BSA and RNase B. To further evaluate those anti-carbohydrate antibodies as potential cancer therapeutics, the effects of 1G4 scFv protein on MCF-7 proliferation were examined along with those of other control anti-carbohydrate antibodies such as anti-Tn antigen MLS128 mAb (*13*) and MLS128 scFv (Yuasa, N. *et al*., manuscript in preparation), and anti-IGFIR 1H7 scFv-Fc (*15*). IgG_3 was used as a negative control antibody in the experiments. In the following studies, MCF-7 aro cells were used, so that instead of oestrogen, all media used contained 1 μM testosterone which is converted by aromatase to oestrogen (*16*). Like MCF-7 cells which require oestrogen for growth, MCF-7 aro cell growth requires testosterone as seen in Fig. 7; Buffer (−T) versus Buffer (+T). The tested antibodies did not appear to have any effect on the monolayer cell growth of MCF-7 aro cells for up to 7 days of culturing as determined by MTT assays (Fig. 7). Extended culture of MCF-7 cells in the presence of oestrogen is known to lead to focus development (*22*, *23*). In fact, a phase-microscope revealed focus development on Days 6 and 7 with the control (corresponding to 'Buffer + testosterone' in Fig. 7) whereas MCF-7 aro cells remained flat in the presence of anti-carbohydrate antibodies or scFv proteins. To compare focus formation in cells cultured with or without antibodies, foci were stained by Rhodamine B according to the method previously reported (*23*). This staining revealed foci as pink islands as seen in Fig. 8A (a). Addition of 1 μM IgG3 did not affect focus formation [Fig. 8A (b)]. In contrast, pink islands were not observed after cells were incubated with 500 nM or 1 μM 1G4 scFv protein for 10 days [Fig. 8A (g and h, respectively)], suggesting that inhibition of foci development had taken place. Anti-Tn antigen MLS128 mAb and its scFv protein also inhibited focus formation [Fig. 8A (e and f, respectively)]. Anti-IGFIR 1H7 scFv-Fc treatment resulted in no focus formation as well, but the appearance of the cells differed [Fig. 8A (c and d)], which may have been reflected by the higher fluorescence intensity observed when foci were quantified as described below [see Table IV, Experiment 3 and Fig. 8B (b)]. To quantify the degree of focus development, the fluorescent intensity in wells was recorded based on the assumption that the intensity of fluorescence should roughly correlate to the number of foci in the wells. Two preliminary experiments and one experiment in triplicate were carried out. Those results are summarized in Table IV as Experiments 1 and 2, and Experiment 3, respectively. All of the experiments revealed a dose-dependent inhibition of focus formation in the presence of anti-carbohydrate antibodies. The average ± SD ($n = 3$) from experiment 3 is shown in Fig. 8B. As described above, the fluorescence intensity for 1H7 scFv-Fc-treated cells was as high as that of cells not treated with antibody [Fig. 8A (a)] although focus development was not observed [Fig. 8A (c and d)]. MTT assays of the same sets prepared for foci formation measurements were performed in parallel with Experiments 1 and 2. Although foci formation was inhibited in the presence of anti-carbohydrate antibodies (Table IV, Experiments 1 and 2), the total live cell count remained roughly the same for the control and antibody-treated cells (Fig. 8C).

Discussion

The aim of this study was to characterize three phage clones screened by Man3-DPPE from a phage library displaying human scFvs as was previously reported (*10*). Of four independent scFv genes thus far characterized, 5A3 clone was the first to be expressed as a scFv-Fc form in NS0 cells and was successfully purified and characterized, whereas NS0 or CHO stable clones expressing 1A4 and 1G4 scFv-Fc proteins had never been established (*11*). Recent studies revealed that 1A4 and 1G4 scFv-Fc proteins were synthesized but accumulated in the ER (*12*). A possible interpretation is that 1A4 and 1G4 scFv-Fc proteins expressed in mammalian cells capture *N*-glycosylated proteins that are essential for cell survival. These results strongly suggest that mammalian cells are not suitable for expression of such anti-carbohydrate antibodies, and that optimization of bacterial expression systems may be necessary to purify anti-Man3 scFv gene products. In this study, 1A4 and 1G4 scFvs were expressed as soluble forms as well as inclusion bodies in *E. coli* cells for evaluation of purification and characterization of those scFv proteins.

Since scFv proteins expressed by the pET-22 b(+) expression vectors contain the pelB signal sequence of 3.4 kDa at N-termini, they are presumably transported to the periplasm where proper folding is

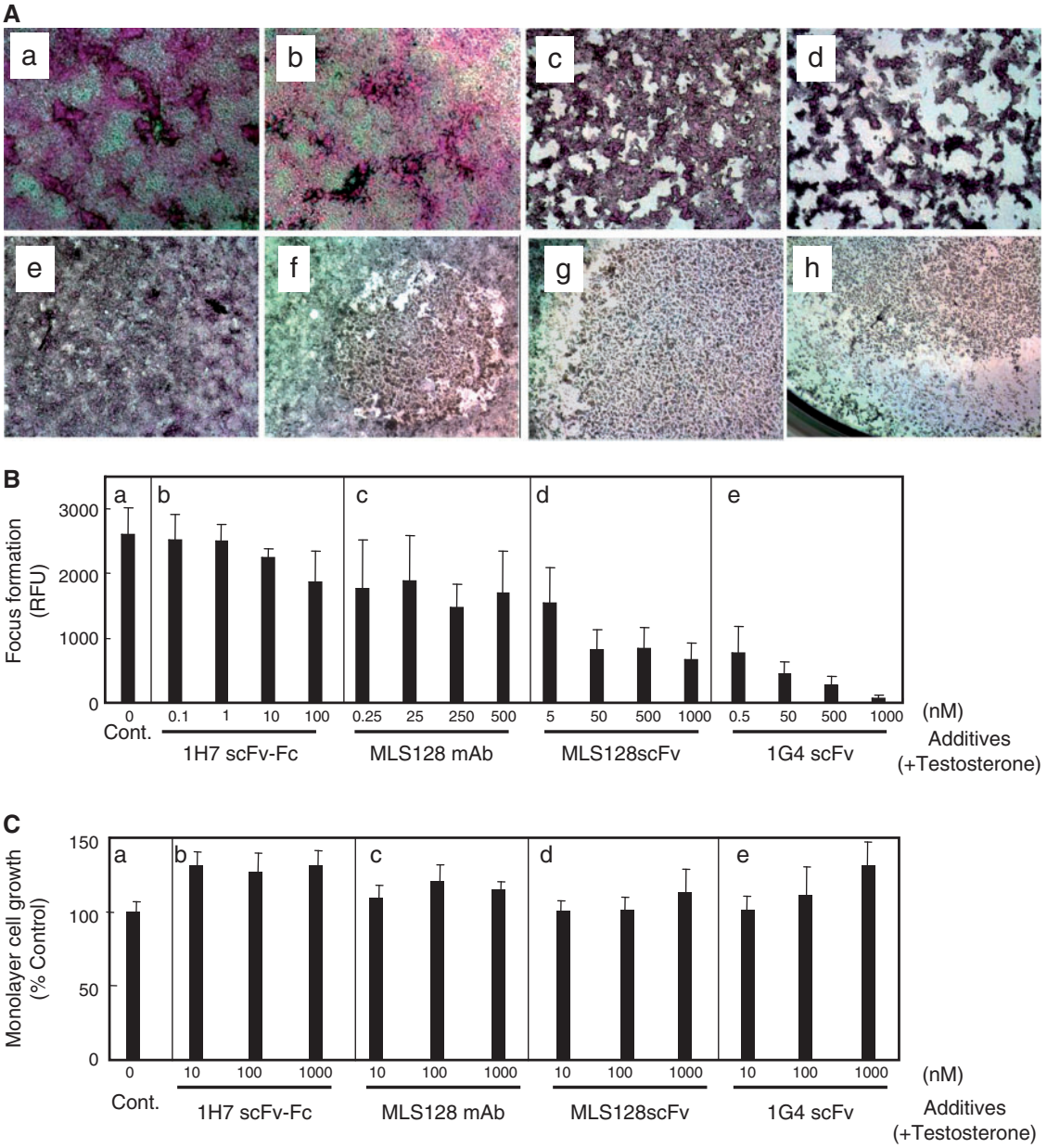

Fig. 8 Effects of anti-carbohydrate scFv proteins and control antibodies on foci development in breast cancer cells. (A) Microscopic observation of foci development in breast cancer cells in the presence or absence of antibodies or scFv proteins. The MCF-7 aro cells in wells were observed under a microscope (Eclipse TE 200-S, Nikon) after staining with Rhodamine B on day 10. All of the media contained 1 nM testosterone. Additives to the basal medium were: (a) none, (b) 1 μM IgG_3 used as a control antibody for MLS128, (c and d) 100 nM and 1 μM 1H7 scFv-Fc, respectively, used as non-anti-carbohydrate antibody controls, (e) 1 μM MLS128mAb, (f) 1 μM MLS128 scFv protein, (g and h) 500 nM and 1 μM 1G4 scFv protein, respectively. (B) Quantification of foci formation in MCF-7 aro cells cultured for 10 days in the presence or absence of various forms of antibodies. MCF-7 aro cells were grown as described in A in the absence (control) or presence of 1H7 scFv-Fc, MLS128 mAb, MLS128 scFv protein or 1G4 scFv protein. On Day 10, foci were stained with Rhodamine B. The fluorescence intensity of stained foci was measured at a bottom read with 550 excitation and 590 emission filters as described in the 'Materials and Methods' section. Shown are average ± SD of three independent experiments (Table IV, Experiment 3): (a) Control (no additives) or (b–e) treated with antibodies at four indicated concentrations: (b) 1H7 scFv-Fc, (c) MLS128mAb, (d) MLS128 scFv protein and (e) 1G4 scFv protein. (C) Cell growth assessment as measured by MTT assays of MCF-7 aro cells cultured for 10 days in the presence or absence of various forms of antibodies. MCF-7 aro cells were grown as described in A and B in the absence (control) or presence of 1H7 scFv-Fc, MLS128 mAb, MLS128 scFv protein or 1G4 scFv protein at indicated concentrations. On Day 10, MTT assays were performed. Shown are the results of two experiments which were carried out along with focus assays in Experiments 1 and 2 (Table IV).

supposed to take place. Both 1A4 and 1G4 proteins partially purified from the periplasm fractions had a molecular mass of ~30 kDa, which indicated that the signal sequence was removed from the scFv proteins. Such 1A4 and 1G4 scFv preparations were shown to have binding affinities for Man3-DPPE as judged by ELISA. Further, K_D of 1.1×10^{-7} M was calculated for the binding of 1A4 scFv protein to Man3-BSA by SPR analyses. ELISA and SPR results indicated that 1G4 scFv protein appears to have a lower binding activity for Man3 than 1A4 scFv protein. Although this research was somewhat successful in preparing soluble forms of 1A4 and 1G4 scFv proteins from the periplasm, there were difficulties in purifying sufficient

Table IV. Fluorescence intensity after staining cells and foci with Rhodamine B.

Experimental number	Additives concentration and FU values										
	None (FU)[a]	IgG3		1H7 scFv-Fc		MLS128 mAb		M LS128 scFv		1G4 scFv	
		(n M)	(FU)	(nM)	(FU)	(nM)	(FU)	(nM)	(FU)	(nM)	(FU)
1	1,381 ± 56	100	1,423 ± 449	100	486 ± 65	100	1,240 ± 136	100	799 ± 190	100	884 ± 192
		1,000	1,218 ± 155	1,000	390 ± 107	1,000	653 ± 73	1,000	655 ± 240	1,000	427 ± 155
2	2,004 ± 13	100	2,099 ± 556	100	1,284 ± 384	100	664 ± 129	100	685 ± 250	100	741 ± 120
		1,000	1,715 ± 797	1,000	1,267 ± 336	1,000	914 ± 159	1,000	677 ± 121	1,000	603 ± 119
3	2,198 ± 337		ND	0.1	2,529 ± 376	0.25	1,774 ± 103	5	1,540 ± 163	0.5	776 ± 178
				1	2,505 ± 179	25	1,881 ± 274	50	892 ± 319	50	453 ± 109
				10	2,241 ± 117	250	1,471 ± 281	500	848 ± 243	500	269 ± 70
				100	1,868 ± 443	500	1,704 ± 276	1,000	664 ± 213	1,000	73 ± 27

[a]FU, The stained foci that were readily distinguishable from the monolayer were quantified by measuring fluorescence intensity using the SpectraMax M5 Microplate Reader, set at a bottom read with 550 excitation and 590 emission filters. Results are recorded in relative fluorescence units. ND, Not determined.

amounts of the scFv proteins displaying activity. The expression of antibodies in *E. coli* in the periplasm is generally preferred due to the periplasm's oxidizing environment for disulphide formation and involvement of fewer proteases. In the cytoplasm, however, reducing conditions prevent the formation of correct disulphide linkages, and proteolytic degradation is more likely to occur (*26*, *27*). The current results are consistent with these previous findings that soluble scFv proteins are often not fully active.

scFv proteins were also purified from inclusion bodies. In bacterial cells, recombinant proteins usually fail to fold properly and accumulate as refractive, insoluble particles called inclusion bodies. Inclusion bodies are considered to be formed by unspecific hydrophobic interactions between irregularly deposited polypeptides. Those protein aggregates might provide a good reservoir of alternative conformational states, from which sufficient amounts of functional scFv proteins may be purified if highly efficient refolding of denatured scFv proteins can be achieved. In this study, a stepwise dialysis method adapted from the published methods (*19*) was used to refold 1A4, 1G4 and 5A3 scFv proteins that had been affinity purified as denatured proteins in the presence of 3.5 M Gdn-HCl. The scFv proteins recovered in PBS after use of the stepwise dialysis method were pure, as judged by SDS–PAGE, with expected molecular masses for the signal peptide-scFv protein-His-tag sequence (Fig. 5B). The yields for purified and refolded 1A4, 1G4 and 5A3 scFv proteins were 15.3, 14.7 and 6.8 mg/l culture, respectively. ELISA (Fig. 6B) and SPR (Fig. 6A and Table IV) analyses revealed that 1G4 scFv protein appears to have the highest binding activity, followed by 1A4 scFv protein, and that the 5A3 scFv protein has the lowest affinity for Man3 epitopes. These results are consistent with the working hypothesis that 1A4 and 1G4 scFv-Fc proteins having higher affinity for high mannose moieties are retained in ER while 5A3 scFv-Fc protein avoids from retention in the ER and is therefore secreted, when expressed in mammalian cells (*12*).

Since high-mannose moieties are abundantly expressed in MCF-7 cells (*22*, *24*), MCF-7 cells were used as natural target antigens for anti-Man3 scFv proteins in this study. All three phage antibodies displaying 1A4, 1G4 and 5A3 scFv proteins bound to the MCF-7 cell surface (Fig. 1B). Immunofluorescence staining of MCF-cells revealed that both 1A4 and 1G4 scFv proteins bound to the epitopes not only on cell surface but also those in the nucleus, as indicated by purple nuclei when staining colours were merged (Fig. 4). These results differ vastly from those with Con A. These results confirmed that 1A4 and 1G4 scFv proteins recognize natural glycoproteins. Further analyses will be needed, however, to identify the target glycoproteins with Man3-like epitopes in the nucleus and cell surface.

Previouse observation indicated that 1H7 scFv-Fc binds to IGFIRs on the MCF-7 cell surface, causing down-regulation of IGFIR and inhibition of anchorage-dependent cell growth but not inhibiting monolayer cell growth (*28*). Similarly, inhibitory effects of anti-Man3 1G4 scFv protein on MCF-7 cell growth were evaluated. As controls, other anti-carbohydrate antibodies such as anti-Tn antigen mAb MLS128, MLS128 scFv protein, and 1H7 scFv-Fc were included in the experiments. As described in the 'Materials and Methods, and Results' sections, MCF-7 aro cells (*16*) were used in these studies for practical reasons since the experiments were conducted in conjunction with aromatase inhibitor projects. Interestingly, monolayer cell growth was not inhibited by the addition of anti-carbohydrate antibodies or 1H7 scFv-Fc as judged by MTT assays (Fig. 7). In fact, the number of live cells remained the same for up to 10 days of culturing when assayed by the MTT method (Fig. 8C). Despite no obvious difference in cell growth as judged by MTT assays, microscopic observations revealed foci development only in cells grown in the absence of antibodies or in the presence of a control IgG_3. This observation led to the next sets of experiments in which the cells were fixed on Day 10 and stained with Rhodamine B. The results shown in Fig. 8A revealed significant inhibition of foci formation especially with cells grown in the presence of scFv proteins [Fig. 8B (f–h)] compared tp those grown in the presence of scFv-Fc and mAb [Fig. 8B (c–e)].

Quantitation of foci in wells confirmed this trend as summarized in Table IV and Fig. 8B. More effective inhibition by anti-Man3 and Tn-antigen scFv proteins may be due to their small molecular masses which make them more accessible to the cell surface epitopes. The inhibitory effects of 1G4 scFv protein on focus formation agree with a previous study that found that α-mannosidase treatment inhibited development of foci in MCF-7 cell culture in the presence of 0.1 M 17β-estradiol (*22*). Although the results shown in this study are preliminary, they have provided a good evidence for use of anti-Man3 scFv proteins in the developing cancer therapeutics.

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Conflict of interest

None declared.

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