Construction of a recombinant single chain antibody recognizing nonreducing terminal mannose residues for use in immunohistochemistry

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SUMMARY **We recently reported characterization of 25 clones isolated from a phage library displaying human scFvs using a neoglycolipid, Man3-DPPE, that was synthesized from mannotriose (Man3) and dipalmitoylphosphatidylethanolamine (DPPE). Of those, 5A3 scFv was successfully expressed and purified as a humanized scFv-Fc form (Sakai *et al.*, Biochemistry 46:253, 2007, Zhang *et al. ibid* 263). To carry out immunohistochemistry (IHC) in human tissues, an HA tag sequence was introduced to the 5A3 scFv-Fc gene and the resulting construct was transfected to murine myeloma NS0 cells. The 5A3 scFv-Fc-HA protein expressed was affinity-purified. Sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing and reducing conditions and enzyme-linked immunosorbent assay confirmed that 5A3 scFv-Fc-HA protein is dimeric and retained the ability to recognize nonreducing terminal mannose residues. IHC staining of non-neoplastic tissues by this recombinant antibody revealed that immunoreactivity was not detected in most of the 16 tissues examined. Exceptions were found in IHC staining of the kidney and pancreas, which demonstrated clear staining of proximal tubules and islets of Langerhans, respectively. These results demonstrated that nonreducing terminal mannose residues are not usually present under normal physiological conditions. This study thus provides a potentially useful tool for examination of nonreducing terminal mannose residues that may become apparent under certain pathophysiological conditions.**

Key Words: scFv-Fc, recombinant antibody, high mannose-specific antibody, IHC, human tissue

Introduction

Since many carbohydrates are self-antigens by nature, an *in vitro* approach such as phage display technology is the best strategy to produce anti-carbohydrate antibodies. The authors recently reported characterization of 25 clones isolated from a phage library displaying human scFvs using a neoglycolipid, Man3-DPPE, that was synthesized from mannotriose (Man3) and dipalmitoylphosphatidylethanolamine (DPPE) (*1*). Of those, 5A3 scFv was successfully expressed and purified as a humanized scFv-Fc form (*2*). During the previous study in which several anti-Man3 scFv-Fc constructs were to be expressed in mammalian cells, most transfected clones did not grow nor survive (*2*), which strongly suggests that antibodies against the Man3-moiety are toxic to mammalian cells. Since Man3 is the core carbohydrate for *N*-glycans, which are essential in many glycoproteins with critical biological

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functions, inhibition of *N*-glycan synthesis by anti-Man3 antibodies could conceivably have a detrimental impact on cell function (*3-5*). Thus, production of antibodies against Man3 epitopes, nonreducing terminal mannose residues in particular, *in vivo* is presumably not possible. Furthermore, such Man3 epitopes are probably not detectable in tissues under normal physiological conditions since nonreducing terminal mannose residues are masked by other carbohydrates such as *N*-acetyl glucose, galactose, and sialic acids when *N*-glycan synthesis is completed. 5A3 scFv-Fc is most likely the only antibody available for evaluation of tissue expression of such Man3 epitopes. To carry out IHC, a HA tag was introduced to 5A3 scFv-Fc so that its specific binding to the epitopes in human tissues could be clearly detected. This study describes construction of a 5A3 scFv-Fc-HA recognizing nonreducing terminal mannose residues for use in IHC.

Materials and Methods

Materials

The cDNA clone of human IgG1 Fc, composed of hinge, CH2, and CH3, was originally from Dr. J. Schlom, Laboratory of Tumor Immunology and Biology, Division of Cancer Biology and Diagnosis, NCI (Bethesda, MD, USA). Plasmid pEE12.4 and a murine myeloma NS0 cell strain were obtained from Lonza Biologics (Slough, UK). The expression vector SFpEE plasmid was as described in previous reports (*2*). EndoFree Plasmid Maxi (Cat. No. 12362) was purchased from QIAGEN Inc. (Valencia, CA, USA). Dulbecco's modified Eagle medium (DMEM, high glucose) was purchased from Invitrogen (Paisley, UK). Goat anti-human IgG (Fc fragment specific) and peroxidase-conjugated rabbit anti-human IgG secondary antibody were from Jackson Immunoresearch Inc. (West Grove, PA, USA). Peroxidase-conjugated rabbit anti-HA antibody was purchased from BETHYL Laboratories, Inc. (Montgomery, TX, USA). A DAB detection kit was purchased from Invitrogen. Protein A-Sepharose was obtained from Bio-Rad (Hercules, CA, USA). Man3-BSA was obtained from Dextra Laboratories (Reading, UK).

Bacterial strains and transformation

Escherichia coli strain JM109 (TaKaRa Shuzo, Kyoto, Japan) was used as a host for all plasmid preparations Plasmid transformations were performed by electroporation using the Gene Pulser Xcell™ Apparatus (Bio-Rad) under conditions of capacitance at 25 μF, a resistor at 200 ohms (pulse controller), and voltage at 1.5 kV. Resulting bacteria were cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.2) containing ampicillin (100 mg/L).

Modification of vector for expression of HA-tag fusion scFv-Fc

HA-tag (YPYDVPDYA) was chosen to be added to the C-terminal of scFv-Fc since this epitope tag is known to be recognized by several commercially available antibodies. Human IgG1 Fc cDNA was amplified from the plasmid SFpEE (*2*) using the forward primer 5' -TGG CGC GCC ATT AAT TAA G-3' and the reverse primer 5'-CCG GAA TTC TCA TCA AGC GTA ATC TGG AAC ATC GTA TGG GTA TTT CCC GGG AGA CAG GGA GA-3' to introduce *Asc*I and *Eco*RI restriction sites (solid underline) and HA-tag coding sequence (dotted underline), respectively, and inserted into the *Asc*I/*Eco*RI linearized expression vector SFpEE. The modified expression vector, designated pSF-HA (Figure 1), was identified by restriction enzyme digestions and verified by DNA sequencing.

Vector construction for expression of 5A3 scFv-Fc-HA

To construct an expression vector, the 5A3 scFv gene prepared by digesting SFpEE/5A3 with AscI and PacI was subcloned into *Asc*I-*Pac*I-digested pSF-HA to generate pSF-HA/5A3 (Figure 1). The sequences of resulting vectors were confirmed by DNA sequencing. The expression vector used for electroporation into mammalian cells was purified by QIAGEN EndoFree Plasmid Maxi and stored in sterile water.

Cell culture, transfection and screening

The murine myeloma NS0 cell line was grown in

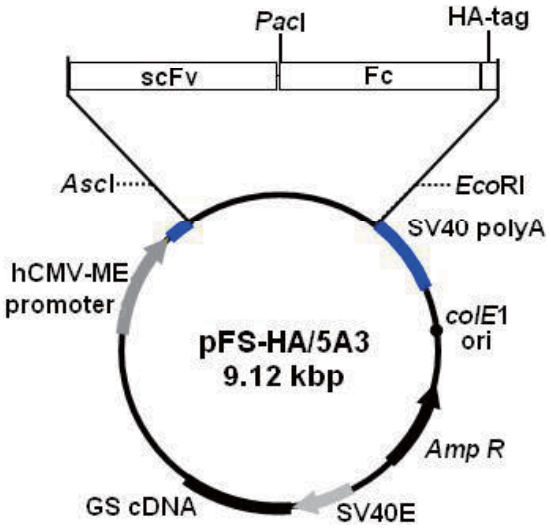

Figure 1. Schematic drawing of the pSF-HA and pSF-HA/5A3 plasmid. GS cDNA, Glutamine synthetase gene; Amp R, Ampicillin resistance gene. The 5A3-scFv gene digested from the plasmid SfpEE/5A3 was inserted into pSF-HA under the control of hCMV-ME promoter as an AscI/PacI fragment. The expressed 5A3 scFv-Fc-HA was expected to be a fusion protein of 491 amino acids with a molecular weight of 54421 that contains a HA-tag at the C-terminus for detection.

DMEM supplemented with 10% FBS and 2 mM glutamine. Selective medium for human Fc-HA-expressing NS0 cells consisted of glutamine-free DMEM, dialyzed FBS, and glutamine synthetase supplement (JRH Biosciences, Lenexa, KS, USA). In a 0.4 cm cuvette, 40 μg of linearized DNA was transfected into 1×10^7 NS0 cells under conditions of 250 volts and 400 μF capacitance. Cells at 1.6×10^5 cell/mL (50 μL/well) were plated in 96-well plates in nonselective media. Twenty-four hours post-electroporation, 150 μL of selective medium were added to each well, and cells were allowed to recover and grow undisturbed for about 3 weeks until discrete surviving colonies appeared.

Supernatants of transfected cells that were able to grow under selective conditions were screened for scFv-Fc-HA protein secretion by sandwich ELISA. Goat anti-human IgG (Fc fragment specific) was diluted in PBS to a final concentration of 10 μg/mL and coated onto 96-well plates, by plating 100 μL/well and incubating at 4°C overnight. After blocking with 3% BSA in PBS, cell culture supernatants from different clones were added and incubated at 37°C for 1 h. After the samples were washed with PBS, horse radish peroxidase (HRP)-conjugated anti-human IgG antibody diluted to 1:20,000 was added to the wells. The wells were washed with PBS and then incubated with 2,2'-azino bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The color produced in the wells of plates was analyzed spectrophotometrically at 415 nm on a microplate reader (Bio-Rad model 680).

Purification of scFv-Fc-HA antibody

One of the clones, A4G12 for 5A3 scFv-Fc-HA, was cultured on a large scale in T175 flasks in selective medium. After culturing for 24-48 h, cell culture supernatants were collected and adjusted to pH 8.0 by adding 1/20 volume 1.0 M Tris (pH 8.0) and then applied to a protein A column (5 mL). The column was washed with 10 column volumes of 100 mM Tris-HCl buffer, pH 8.0. The column was then washed with 100 mM citrate buffer, pH 4.5, to remove bovine IgG derived from FBS (*6*). scFv-Fc-HA was eluted from the column with 100 mM citrate buffer, pH 3.0 (*7*). The eluates were collected in 1.5-mL conical tubes containing 1/10 volume 1 M Tris-HCl, pH 8.0. The purified 5A3 scFv-Fc-HA was dialyzed against PBS and concentrated with Amicon® Ultra (Millipore, Billerica, MA, USA). Protein concentration was measured by BCA assay (Pierce, Rockford, IL, USA) with BSA as a standard according to the manufacturer's protocol.

SDS-PAGE

The purified 5A3 scFv-Fc-HA was analyzed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue (CBB) R-250.

ELISA

Solid-phase ELISA was used to confirm the antigen-binding activity of purified 5A3 scFv-Fc-HA against Man3-BSA. Briefly, the antigen, Man3-BSA was diluted to 5 μg/mL in PBS, and coated onto 96-well ELISA plates (100 μL/well) at 4°C overnight with triplicate samples. After blocking of the sample with 3% BSA/PBS, 5A3 scFv-Fc-HA was added. After 2 h of incubation at room temperature, the bound scFv-Fc-HA was detected with HRP-conjugated rabbit-anti HA antibody (1:25,000 dilution) using ABTS as a substrate. After 15 min of incubation in the dark, the reaction was terminated by adding 100 μL of 2% oxalic acid solution. Absorbance at 415 nm was measured using a plate reader.

Immunohistochemistry

Multi-tumor blocks of human tissues were derived from formalin-fixed, paraffin-embedded archives that had been prepared and stored by the Department of Anatomic Pathology, City of Hope Medical Center (*8*). The study was approved by the IRB of the City of Hope Medical Center. Paraffin sections (4 μm thick) mounted on Probe-on slides (Biotek Solutions; Tucson, AZ, USA) were baked for 1 h at 55°C and then dried overnight at 48°C. They were subsequently deparaffinized in xylene, rehydrated in graduated alcohols to distilled water, and loaded into a Techmate slide holder and placed into 10 mM citrate buffer, pH 6.0. Antigen retrieval was performed by the heat-induced epitope retrieval method (*9*). The slides were steamed in 10 mM citrate buffer for 20 min using a household Black and Decker Steamer (model no. HS90) and then allowed to cool for 5 min. The primary scFv-Fc-HA antibody was used at a concentration of 0.14 mg/mL and incubated overnight in a humid container. The scFv-Fc-HA protein was visualized by reaction with HRP-conjugated rabbit-anti HA antibody (1:300 dilution) followed by a modified ABC technique (Vector Elite Kit; Vector Lab, Burlingame, CA, USA). The slides were lightly counterstained with Mayer's hematoxylin.

Results and Discussion

Production and purification of scFv-Fc-HA protein

The constructed 5A3 scFv-Fc-HA gene was introduced into NS0 cells to produce scFv-Fc-HA protein using the GS/NS0 cell system as previously described (*2*). Of 24 stable clones grown in selective media, Fc-positivity was detected by ELISA in the media of 16 clones (data not shown). The scFv-Fc-HA protein (390 μg) was purified from 240 mL culture medium of an A4G12 clones by protein A-column chromatography. The level of 5A3 scFv-Fc-HA protein expression in the medium

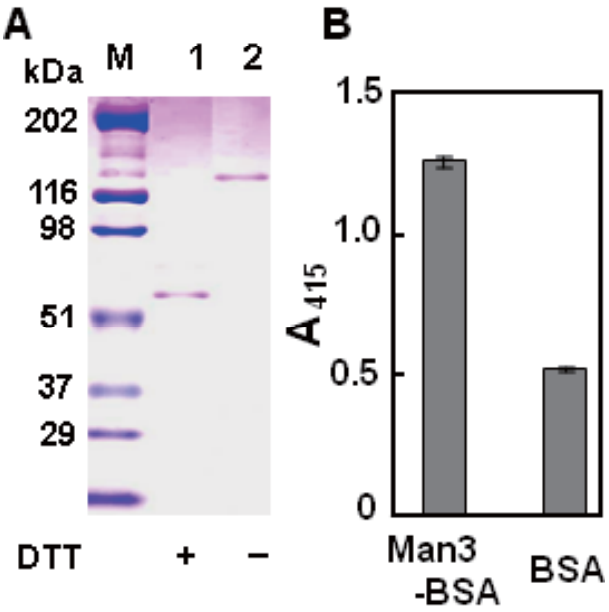

Figure 2. SDS-PAGE and ELISA analyses of the affinity purified 5A3 scFv-Fc-HA protein. (A) CBB staining of a 4-20% acrylamide gel showing 5A3 scFv-Fc-HA under reducing conditions (Lane 1) or under non-reducing conditions (Lane 2), and molecular weight markers (M). (B) Binding of 5A3 scFv-Fc-HA protein to Man3 conjugated with BSA as measure by ELISA. BSA conjugate (0.5 μg/well) was absorbed onto the surface of wells. The purified 5A3 scFv-Fc-HA (3.5 μg/well) was applied to the wells, and the bound 5A3 scFv-Fc-HA was detected with HRP conjugated anti-HA antibody.

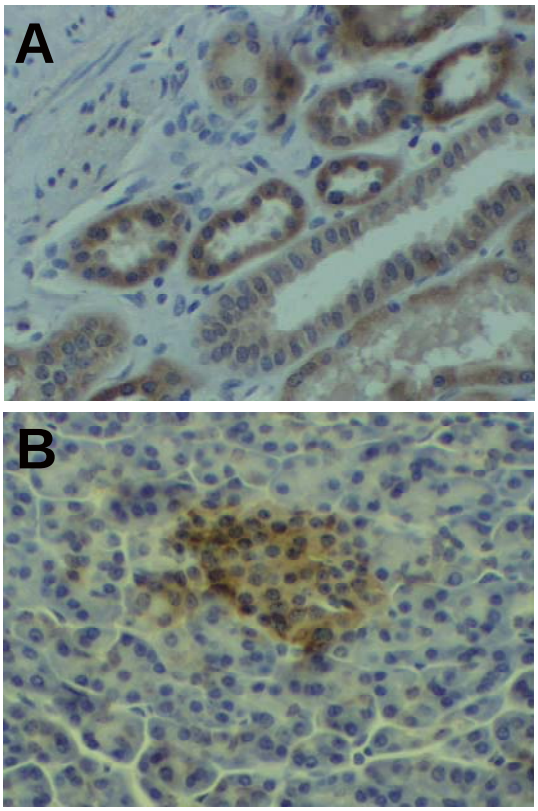

Figure 3. IHC staining for Man3 by the recombinant single chain antibody recognizing nonreducing terminal mannose residues, 5A3 scFv-Fc-HA, in non-neoplastic tissues. A, kidney; B, pancreas.

was thus calculated to be approximately 1.63 μg/mL.

Purity and binding activity of purified 5A3 scFv-Fc-HA protein

The purified 5A3 scFv-Fc-HA protein was analyzed by SDS-PAGE under reducing and nonreducing conditions (Figure 2A lanes 1 and 2, respectively). Since human IgG1 Fc contains a hinge region, the scFv-Fc-HA produced by mammalian cells is expected to form a disulfide-linked dimer. The results clearly show that 5A3 scFv-Fc-HA protein is a dimer consisting of two monomers. The molecular mass of the monomeric 5A3 scFv-Fc-HA as estimated by SDS-PAGE was 55-57 kDa, which is consistent with an expected molecular mass of 54 kDa from an amino acid sequence with a contribution of *N*-glycation.

The 5A3 scFv-Fc-HA is thus a divalent recombinant antibody that has an expected molecular mass of 110.4 kDa. The molecular mass of the dimeric scFv-Fc under nonreducing conditions estimated by SDS-PAGE was not exactly double that of the monomer. This is possibly due to post-translational modifications with *N*-glycation in the Fc regions as well as aberrant mobility caused by a rather high polyacrylamide gel concentration for the dimmer size. Therefore, one can conclude that the S-S bonds between two 5A3 scFv-Fc-HA monomers were correctly formed as expected based on its amino acid sequence.

ELISA analysis of 5A3 scFv-Fc-HA protein against the Man3 antigen

To confirm that the addition of a HA-tag with nine amino acids to the C-terminal of 5A3 scFv-Fc did not disturb its binding activity, the purified 5A3 scFv-Fc-HA protein was subjected to ELISA analysis. The binding of 5A3 scFv-Fc-HA protein to Man3-BSA coated onto plastic plates is shown in Figure 2B. The results confirmed that the purified 5A3 scFv-Fc-HA retained the ability to recognize the Man3 epitope, suggesting that it is suitable for IHC.

IHC staining for nonreducing terminal mannose residues in non-neoplastic tissues by 5A3 scFv-Fc-HA

Multi-tumor blocks of 16 different human tissues containing a total of 70 specimens were examined for immunoreactivity with 5A3 scFv-Fc-HA protein. IHC staining of non-neoplastic tissues carried out with the purified 5A3 scFv-Fc-HA basically revealed no immunoreactivity except that IHC of the kidney and pancreas demonstrated clear staining of proximal tubules (Figure 3A) and islets of Langerhans (Figure 3B), respectively. No immunoreactivity was found in tissue sections including the spleen, uterus, prostate,

ovaries, skin, colon, duodenum, and brain whereas some possible positivity was found in the lung, liver, and testes; this finding requires further examination. These results are consistent with the original prediction that Man3 epitopes are not detectable in tissues under normal physiological conditions as long as *N*-glycation is complete.

In summary, a HA tag was successfully introduced to 5A3 scFv-Fc gene to produce a 5A3 scFv-Fc-HA protein. The purified 5A3 scFv-Fc-HA was dimeric and retained its ability to recognize nonreducing terminal mannose residues. IHC staining for Man3 epitopes in non-neoplastic tissues revealed that immunoreactivity was not detectable in most of the tissues examined, which confirmed that nonreducing terminal mannose residues are not present under normal physiological conditions as predicted. This study thus provided a potentially useful tool for examination of the presence of nonreducing terminal mannose residues that may become apparent under certain pathophysiological conditions. In fact, preliminary IHC studies with 5A3 scFv-Fc-HA detected aberrant expression of nonreducing terminal mannose residues in several cancers. Further investigation is underway.

Acknowledgements

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