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Potential Anti-Cancer Therapeutic Agents: Effects of VHH Antibodies on Mesothelin Binding Affinity in Cancer

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Mesothelin (MSLN) is a glycosyl phosphatidyl inositol-anchored glycoprotein involved in the carcinogenesis and metastasis of some cancers such as ovarian cancer, pancreatic cancer, and mesothelioma. The interaction between cancer antigen 125 (CA125) and MSLN enhances tumor metastasis. MSLNs are highly expressed in cancer and are therefore promising targets for cancer therapy. Variable domains of heavy chain of heavy chain (VHH; also known as nanobodies) monoclonal antibodies (mAbs) from alpacas exhibit unique properties such as high affinity, low immunogenicity, and thermal stability. Herein, we aimed to investigate the effects of anti-MSLN VHH mAbs on MSLN binding affinity. Two anti-MSLN VHH mAb clones (MT1A1 and MT3C2), targeting the extracellular domain of human MSLN, were used. MT1A1 and MT3C2 are specific to human and mouse MSLN. MT1A1 and MT3C2 bound to MSLN are expressed on ovarian, pancreatic, and mesothelioma cancer cells. These antibodies recognize the region (296–390) at the N-terminal of MSLN on the cell surface and relying on their conformation-dependent recognition. MT1A1 and MT3C2 significantly inhibited the MSLN-CA125 interaction. Overall, our results suggest that MT1A1 and MT3C2 are promising anticancer agents.

Key words mesothelin, cancer, single-domain antibodies, antibody engineering, cancer antigen 125

INTRODUCTION

Mesothelin (MSLN), a glycosylphosphatidylinositol anchored cell membrane glycoprotein, encodes a 622-residue (68.1 kDa) precursor protein (pro MSLN).[1] Pro-MSLN is cleaved at 295Arg, by furin protease, resulting in a 296-598 amino acid residue (34.1 kDa) MSLN fragment. MSLN is classified into three regions: region I (residues 296-390; responsible for cell attachment), region II (residues 391-486), and region III (residues 487-598).[2,3] MSLN expression is limited to normal tissues including the pleura, pericardium, peritoneum, and epithelium of the trachea.[4] Additionally, MSLN is expressed in various cancers such as malignant mesothelioma, ovarian cancer, and pancreatic cancer.[5-8] Owing to the differential expression of MSLN in normal and cancer tissues, and the consequent role of MSLN in tumorigenesis, MSLN is a strong candidate for tumor therapy.

Cancer antigen 125 (CA125; also known as MUC16), a high-molecular weight (2,500-5,000 kDa) glycoprotein, is overexpressed in ovarian and pancreatic cancers and is a well-established marker for ovarian cancer.[9] CA125 expression is strongly associated with poor survival in patients with ovarian and pancreatic cancers.[10,11] The binding between MSLN and CA125 enhances the adhesive capacity of metastasizing tumor cells.[12,13] CA125-expressing ovarian cancer cells metastasize to MSLN-expressing mesothelial cells at a high rate. Thus, inhibiting the interaction between CA125 and MSLN could be a useful strategy in preventing tumor metastasis. Therefore, identifying molecules that inhibit the CA125 and MSLN interaction is crucial.

Camelidae species, including alpacas, possess VHH antibodies.[14] These antibodies comprise nearly half of alpaca serum IgG.[15] Alpacas produce dimeric antibodies composing two heavy chains without light chains. The antigen binding domain of these heavy chain antibodies, referred to as variable domain of heavy-chain of heavy-chain (VHH) monoclonal antibodies (mAb), consists of a single immunoglobulin domain, which is generally recognized for its high heat stability and smaller size compared to human immunoglobulins.

In this study, we aimed to investigate two anti-VHH mAbs that target the extracellular domain of MSLN. We characterized anti-MSLN VHH mAbs and demonstrated that these mAbs could inhibit MSLN-CA125 interaction *in vitro.*

MATERIALS AND METHODS

Cells Human fibrosarcoma HT1080 (#JCRB9113) and human pancreatic cancer KP-3L (#JCRB0178.1) cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Human malignant mes-

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othelioma ACC-MESO-4 (#RCB2293) and ovarian cancer OVCAR-3 (#RCB2135) cells were obtained from the RIKEN Bioresource Research Center (Ibaraki, Japan).

The HT1080 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, 100 µg/mL streptomycin sulfate, and 0.1 mM nonessential amino acids. KP-3L, ACC-MESO-4, and OVCAR-3 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin G, and 100 µg/mL streptomycin sulfate. All cells were incubated at 37°C and 5% CO_2. Various stable transfectants (HT1080 cells engineered to express hMSLN (HT1080/hMSLN) and mouse MSLN (mMSLN) (HT1080/mMSLN)) were established by retroviral gene transfer and drug selection as previously described.[16]

VHH mAb and Recombinant MSLN Protein Preparation DNA encoding the N-terminal HHHHHH tag (His tag) region, anti-MSLN VHH gene, and C-terminal DDDDK tag (also known as the FLAG tag) region were obtained via gene synthesis (FASMAC Co. Ltd., Atsugi, Japan). Recombinant plasmids derived from pET-47b were transduced into the *Escherichia. coli* strain BL21 (DE3) (Novagen, Darmstadt, Germany). Recombinant proteins were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries, Osaka, Japan). Protein purification was then performed using the Profinia™ native IMAC purification and buffer kit (Bio-Rad, CA, USA), according to the manufacturer's instructions. MSLN mutant plasmids were prepared by infusion technology using an Infusion HD kit (Takara, Mie, Japan). Gene-encoding human and mouse soluble MSLN fused to a C-terminal Fc tag were cloned into the pSec tag2 vector (Thermo Fisher Scientific, Waltham, MA, USA) between the Ig kappa signal peptide and stop codon. Recombinant proteins were produced using Freestyle 293-F cells (Thermo Fisher Scientific). Human and mMSLN-Fc proteins were purified using Ab capture Extra (ProteNova, Kagawa, Japan), and the buffer was replaced with phosphate buffered saline (PBS) by gel filtration using a Sephadex G-25 column (PD-10, Cytiva, WI, USA).

Flow Cytometry Analysis Cells were detached from culture dishes using 0.05% trypsin and 0.5 mM ethylenediamine tetraacetic acid. Next, the cells were treated with VHH (3.0 µg/mL) in 2% FBS-containing PBS for 60 min at 4°C. Following incubation, the cells were washed with PBS and incubated with anti-his tag mAb (OGHis, MBL, Tokyo Japan) at 0.5 µg/mL in 2% FBS-containing PBS for 60 min at 4°C. The cells were then washed with PBS and incubated with 2 µg/mL (in 2% FBS-containing PBS) AlexaFluor 488 conjugated goat anti mouse IgG (Jackson Immunoresearch, PA, USA) for 30 min at 4°C. Following incubation, the cells were washed with PBS and analyzed using FACSVerse flow cytometer (BD Biosciences) and CytoFLEX SRT system (Beckman Coulter, FL, USA), as previously described.[17]

Surface Plasmon Resonance Analysis Assay To measure the MSLN VHH affinity, we performed an SPR analysis using a Biacore X100 (Cytiva). HBS-EP + buffer (Cytiva) was used as the running buffer. Sensor Chip Protein G (#29179316; Cytiva) was used to immobilize hMSLN-Fc. Anti-MSLN VHH mAb was serially diluted (0.156, 0.313, 0.625, 1.25, 2.50, 5.00, 10.0, 20.0, and 40.0 nM) in the running buffer. The multi-binding cycle was injected sequentially, with increasing concentration, over both the ligand and reference surfaces. The reference surface, an unmodified flow cell, was used to correct for systematic noise and instrumental drift. To determine ka, kd, and KD values, the sensor grams were globally fitted using a 1:1 binding model and analyzed using the Biacore X100 Evaluation Software.

Other Methods Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Immunoblotting and enzyme-linked immunosorbent assay (ELISA) were performed as described previously.[18]

RESULTS

Evaluation of VHH mAbs Targeting Human MSLN in Culture Cell Line We initiated our study by prepared of anti-MSLN VHH mAbs (Clones MT1A1 and MT3C2). Purification proteins were analyzed using SDS-PAGE. Gels contained a single band approximately 20kDa in apparent molecular weight, corresponding to the predicted size (Fig.1).

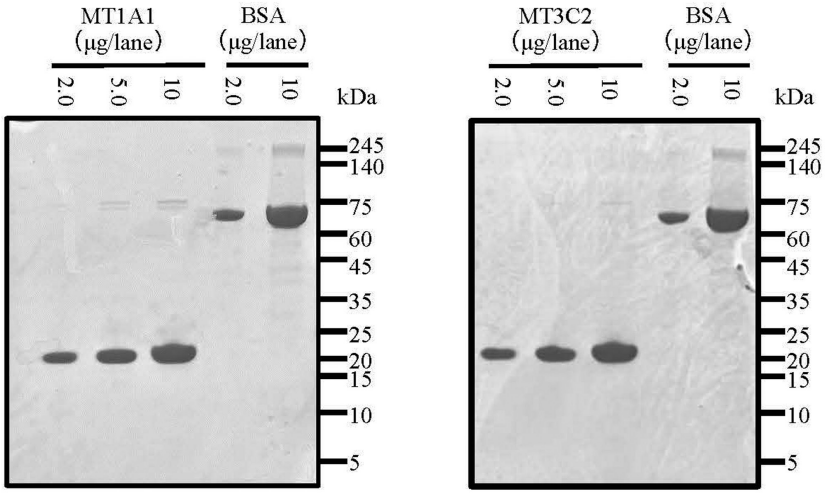

Fig. 1. Preparation of Anti-MSLN-VHH mAbs

Constructed recombinant protein of anti-MSLN VHH mAbs (MT1A1 and MT3C2), and Bovine serum albumin were subjected to 4%–12% gradient SDS-PAGE in the reducing (containing 10% 2-mercaptoethanol) followed by staining with Coomassie Brilliant Blue.

Next, we demonstrated the binding activity of MT1A1 and MT3C2 in a cultured cell line. HT1080, which lacks endogenous MSLN expression, did not show binding to MT1A1 and MT3C2. However, HT1080 cells engineered to express human MSLN (hMSLN) and mouse MSLN (mMSLN) exhibited binding to MT1A1 and MT3C2. Notably, the binding affinity of anti-MSLN VHH mAbs, specifically to MT1A1 and MT3C2 clones, was stronger for hMSLN than for mMSLN. We proceeded to assess the binding capacity of these anti-MSLN VHH mAbs to various cancer cell lines, because MSLN is expressed in different cancer types. Notably, pancreatic cell line KP-3L, mesothelioma cell line ACC-MESO-4, and ovarian cell line OVCAR3 exhibited binding to MT1A1 and MT3C2 (Fig. 2).

Characterization of VHH mAbs Binding Each Extracellular Domain of hMSLN We analyzed anti-MSLN VHH mAbs affinity using the ELISA and SPR (Fig. 3). The ELISA results revealed that anti-MSLN VHH mAbs increased in a dose-dependent manner, with half-maximal effective concentration (EC_{50}) values of 2.57 nM and 0.86 nM for MTA1 and MT3C2, respectively. SPR analysis captured the binding between hMSLN-Fc and VHH mAb. For MT1A1, KD, Ka, and Kd values could not be determined; however, the apparent KD value was 4.19 nM. For MT3C2, KD=0.379 nM, Ka=2.27×10^{6} M^{-1} s^{-1}, and Kd=8.60×10^{-4} s^{-1}. The KD value of MT3C2 was lower than that of MT1A1, indicating that the binding affinity of MT3C2 is stronger than that of MT1A1. Antibodies typically have KD value in the nanomolar range whereas MT3C2 exhibits a sub-picomolar KD value.

Binding Property of Anti-MSLN-VHH mAbs Next, we evaluated anti-MSLN VHH mAb binding to the two MSLN targets using epitope analysis. We prepared hMSLN extracellular domain swap mutants. The three substations were human-to-mouse. Fig. 4A displays expression construction, and Fig. 4B shows the expression analysis using western blotting. All swab mutants were equally expressed. MT1A1 and MT3C2 recognized hMSLN, Xi-2, Xi-3, and mouse but did not bind to Xi-1 (Fig. 4C). These results indicated that MT1A1 and MT3C2 are recognized in the hMSLN region I. Region I is the outermost region responsible for cell attachment; therefore, MT1A1 and MT3C2 may block the binding of MSLN to cancer cells.

Effects of VHH mAbs on MSLN-CA125 Interactions We evaluated whether recombinant hMSLN-Fc and mMSLN-Fc bind to cancer cells and found that hMSLN-Fc and mMSLN-Fc did bind to OVCAR-3 cells but the negative control (hCD68-Fc) did not bind (Fig. 5A). This binding interaction occurred between MSLN and CA125. Next, we determined whether the anti-MSLN VHH mAb could block CA125-MSLN binding. OVCAR-3 cells were treated anti-MSLN VHH mAbs, (MT1A1 or MT3C2) and hMSLN-Fc binding affinity was determined using AlexaFluor 488 conjugated goat anti human IgG-Fc antibody. Anti-MSLN VHH mAbs inhibited hMSLN-Fc binding (Fig. 5B). MT3C2 completely inhibited hMSLN-Fc binding.

DISCUSSION

In the present study, we aimed to investigate the effects of anti-MSLN VHH mAbs on MSLN binding affinity. The robust binding of anti-MSLN VHH mAbs, clones MT1A1 and MT3C2, to intact hMSLN further underscored their strong affinity for the human form over that of the mouse (Figs. 1, 2 and 3). Importantly, MT1A1 and MT3C2 recognize region I domains of MSLN (Fig. 4), hold promise as valuable tools for analyzing mouse models, including xenograft models and biodistribution assays.

MSLN binders are widely used in cancer therapy. Clinical trials are ongoing for scFv, antibody-based approaches (immunotoxin and antibody drug-conjugated), and chimeric antigen receptor CAR-T therapies. MORAb-009 (also known as amatuximab), a mouse-human chimeric monoclonal antibody

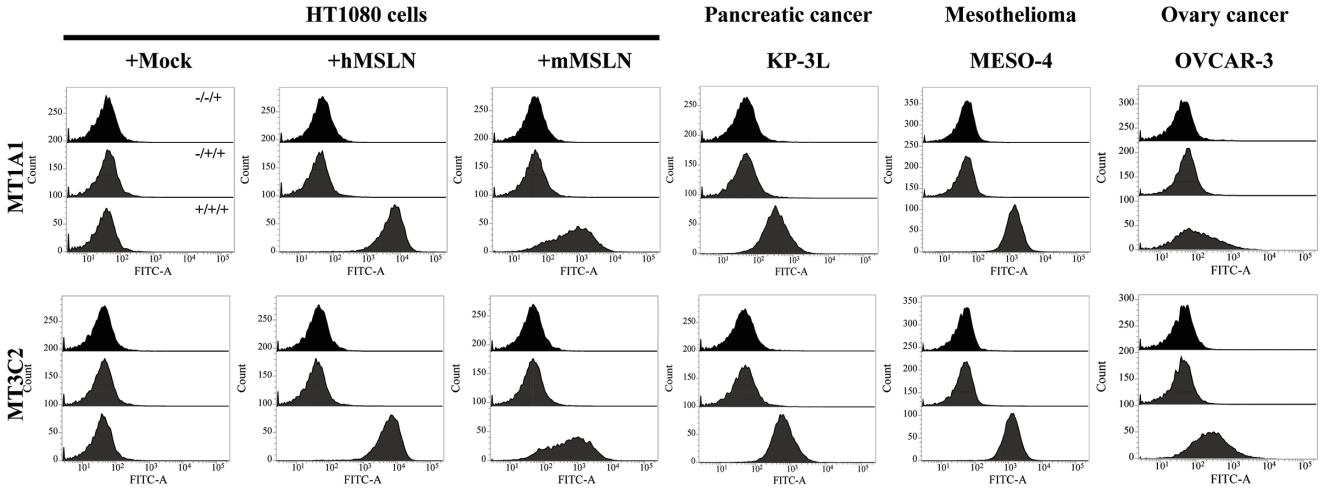

Fig. 2. Binding Specificity of Anti-MSLN VHH mAbs

HT1080 cells (cells without endogenous MSLN expression), HT1080/hMSLN cells (cells stably expressing hMSLN), HT1080/mMSLN cells, and cancer cells were incubated with MT1A1 or MT3C2 at 4 °C for 1 h. The cells were then treated with mouse anti his tag mAb (MBL, Japan) at 4°C for 1 h. Subsequently, the cells were treated with Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) at 4 °C for 30 min and analyzed using flow cytometry. Upper panel: background in the absence of the VHH mAb and anti-His tag antibody (−/−/+). Middle panel: background in absence of VHH mAb (-/+/+). Lower panel: Histograms of VHH mAb-bound cells (+/+/+). MSLN, mesothelin; VHH, variable domain of heavy-chain-only antibody; mAbs, monoclonal antibodies.

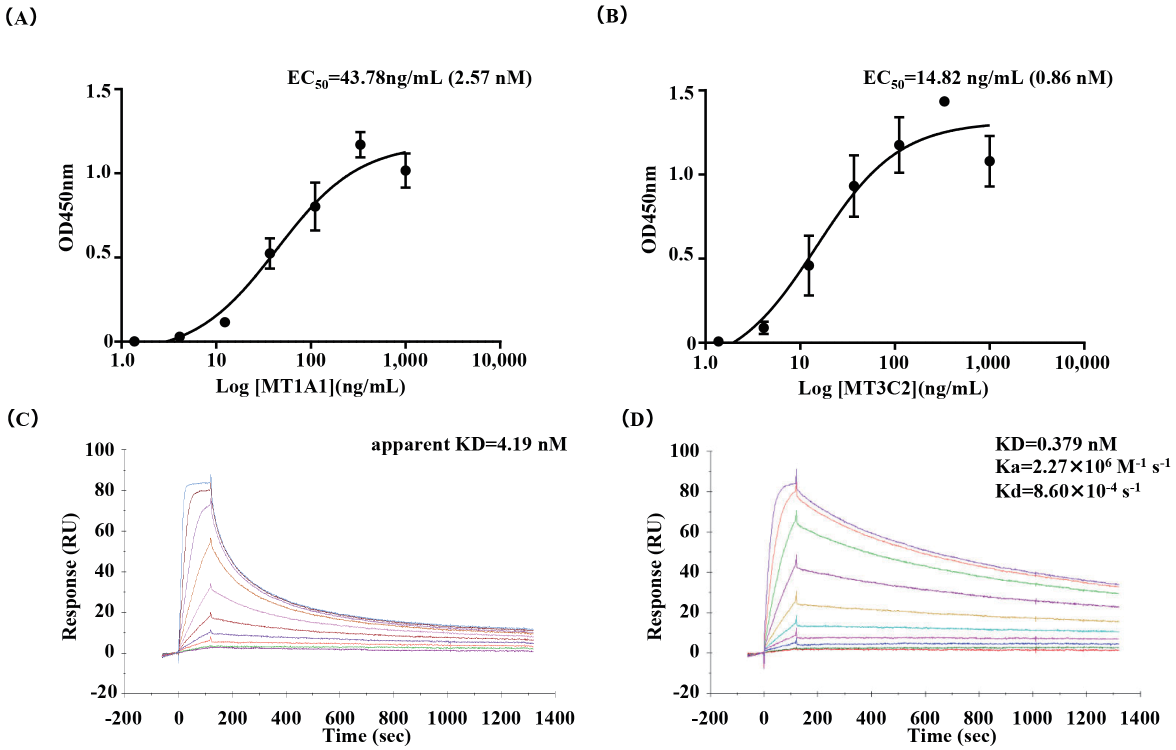

Fig. 3. Binding Affinity of Anti-MSLN VHH mAbs

(A, B) Measurement of MT1A1 or MT3C2 binding to recombinant hMSLN protein using ELISA. 96-well plates were incubated with serial dilutions of each anti-VHH mAb for 2 h at room temperature. Next, plates were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The binding of each antibody to the recombinant MSLN protein was measured as described in the Materials and Methods section. Data are shown as mean ± S.D; (n = 3).

(C, D) Binding affinity was determined using SPR. Multicycle kinetics analysis was simultaneously performed on immobilized hMSLN, with nine injections of analytes (MT1A1 and MT3C2) at 40 nM. Analytes injections lasted for 120 s each and were separated by a 1,200 s dissociation phase. MSLN, mesothelin; VHH, variable domain of heavy-chain-only antibody; mAbs, monoclonal antibodies; ELISA, the enzyme-linked immunosorbent assay; SPR, surface plasmon resonance.

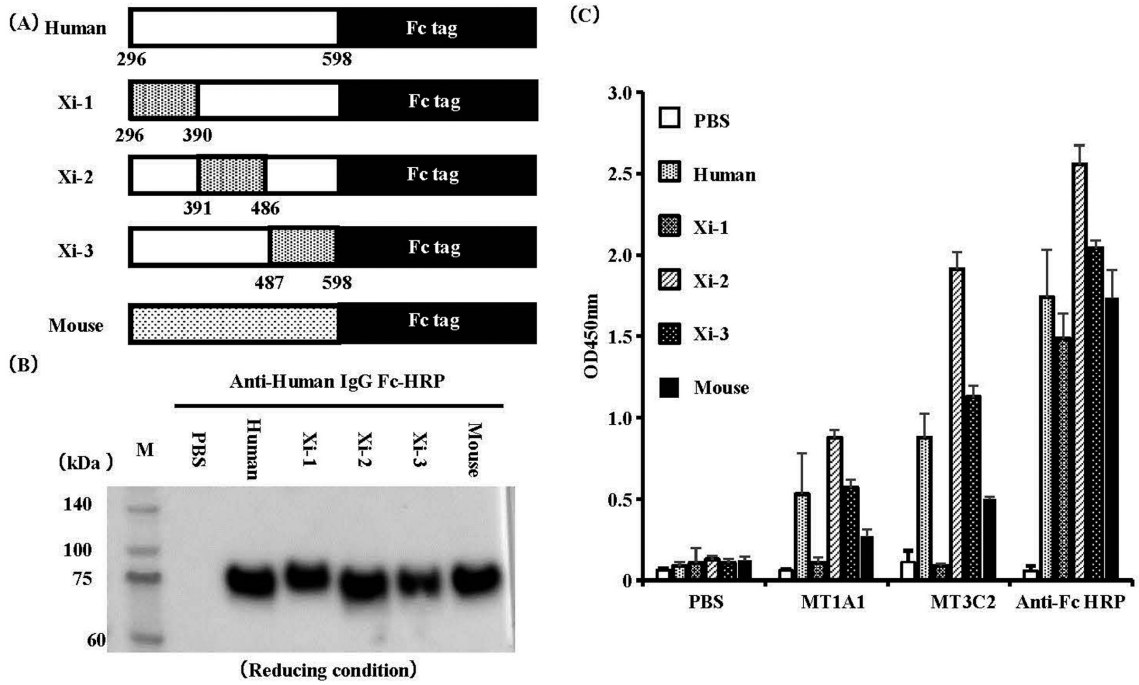

Fig. 4. Epitope Analysis of Anti-MSLN VHH mAbs

(A) Schematic structure of MSLN SWAP mutants. The hMSLN-Fc fusion proteins (hMSLN residues 296-598 and the human IgG4 Fc domain). White: hMSLN, Hatching: mMSLN. Black: IgG4 Fc. (B) Immunoblot analysis. Recombinant MSLN SWAP mutants were subjected to SDS-PAGE and immunoblotted using peroxidase-conjugated goat anti-human IgG (H+L). (C) Epitope analysis was performed using 96-well plates, where each well was coated with swapped mutants. Subsequently, these mutants were incubated with either anti-MSLN VHH mAb (clone MT1A1 or MT3C2) or PBS, followed by incubation with horseradish peroxidase-conjugated anti-His tag mAb (MBL). The binding of the peroxidase-conjugated anti-His tag mAb to the VHH mAb-attached recombinant hMSLN protein was detected. Data are presented as the mean ± SD; (n = 3). MSLN, mesothelin; VHH, variable domain of heavy-chain-only antibody; mAbs, monoclonal antibodies; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline.

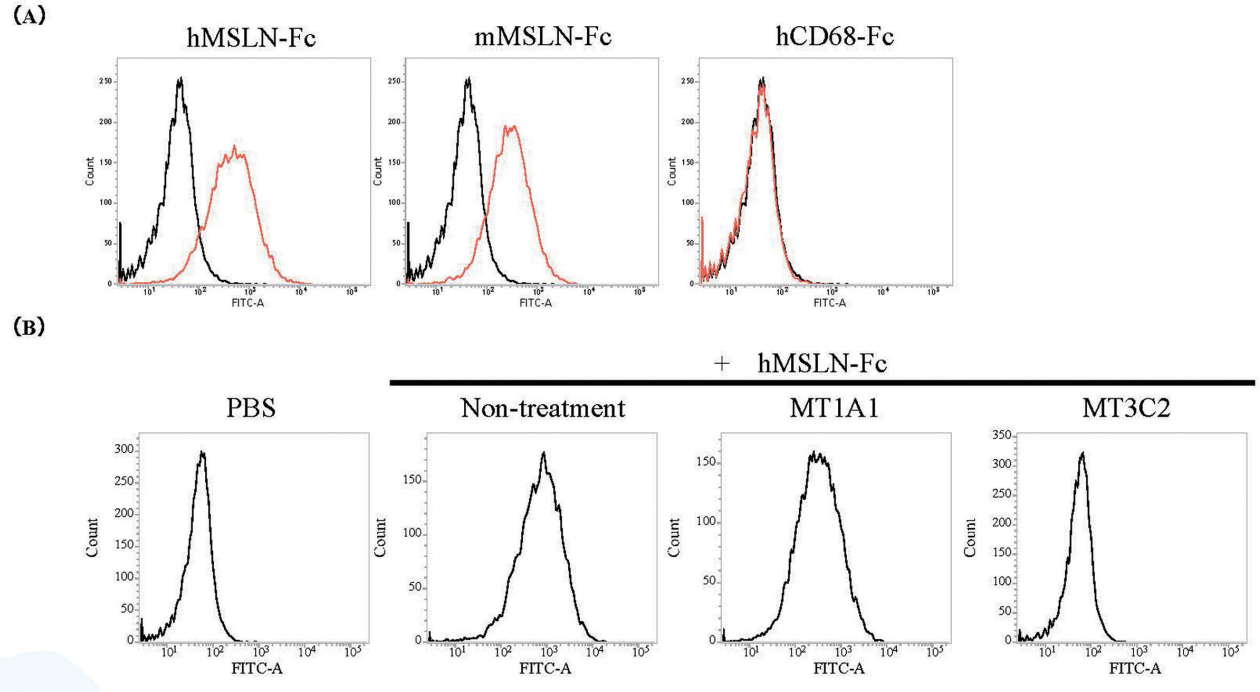

Fig. 5. Inhibition Analysis of Interaction Between MSLN and CA125 on the OVCAR-3 Cell Surface

(A) Ovarian cancer cells, OVCAR-3, were incubated with the full-length extracellular domain of hMSLN-Fc, mMSLN-Fc, and CD68-Fc. Black line: Absence of recombinant Fc protein. Red line: Presence of recombinant Fc protein. (B) Blocking assay. Binding was visualized with a goat anti-human IgG-Fc AlexaFluor488-conjugated secondary antibody using flow cytometry. Light gray shaded plot, secondary antibody only. MSLN, mesothelin, CA125, cancer antigen 125; PBS, phosphate buffered saline.

with selective affinity for MSLN,[19] has been utilized in antibody studies. MORAb-009 blocks MSLN binding to CA125, resulting in the inhibition of hetero type cell adhesion. This action prompted apoptosis in MSLN-expressing tumor cells via antibody-dependent cellular cytotoxicity (ADCC). MORAb-009 exhibited anti-tumor activity alone and in combination with gemcitabine in pancreatic cancer phase II trial (NCT00570713); however, single gemcitabine therapy resulted in a higher patient survival rate. Inadequate results might suggest that ADCC is not fully effective.[20] A sufficient number of immune cells may not have been able to infiltrate the cancer sites. Currently, research is being conducted on cancer drug conjugates and their immunotoxicity. This is considered a useful approach because it does not depend on immune system cells. SS1P is an anti-MSLN immunotoxin composed of anti-MSLN Fv fused to a 38 kDa Pseudomonas exotoxin A fragment (PE38) and has been evaluated in clinical trials.[21] Although there are challenges with half-life along with evident side effects, the combined use of SS1P with l-lysine significantly improved its half-life.[22] MT1A1 and MT3C2 exhibit high affinity, low immunogenicity, and thermal stability because of their nature as VHH mAbs. Notably, MT3C2 has high MSLN binding affinity and blocks CA125-MSLN interactions (Fig. 5). Consistent with this observation, SPR analysis results have suggest that MT3C2 shows higher binding and slower dissociation with MSLN than that with MT1A1. This phenomenon suggests that MT3C2 has more sites that interact with MSLN than with MT1A1. We aim to clarify this through structural analyses in future works.

In this study, we analyzed two anti-MSLN VHH mAbs. These antibodies have a high MSLN affinity and selectivity, and both mAbs can recognize mMSLN. This study is the first to demonstrate the inhibitory effect of anti-MSLN VHH mAbs on the MSLN-CA125 interaction. The anti-MSLN VHH mAbs are expected to contribute to the development of anticancer drugs targeting MSLN through the optimization of binding probes. In our future research, we intend to harness the potential of VHH mAbs by transforming them into immunotoxins, with agents such as bacteria toxins (such as pseudomonas exotoxin and diphtheria toxin). Anti-MSLN VHH mAb MT3C2 is expected to diaplay very strong activity.

In conclusion, we have characterized anti-MSLN VHH mAbs recognizing region I domains of MSLN. To our knowledge, these engineered mAbs are the smallest functional MSLN binders. These smaller MSLN binding probes inhibit MSLN CA125 interaction. Based on their construction, VHH mAbs can be easily modified using genetic engineering to further improve therapeutic potential as anticancer drugs. In addition, the monovalent VHH mAbs against MSLN are expected to be useful tools for analyzing the biological functions of MSLN.

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Conflict of interest The authors declare no conflict of interest.

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