Large Molecule Therapeutics

A Human Single-Domain Antibody Elicits Potent Antitumor Activity by Targeting an Epitope in Mesothelin Close to the Cancer Cell Surface

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Abstract

Monoclonal antibodies against mesothelin are being evaluated for the treatment of mesothelioma and multiple forms of cancers, and show great promise for clinical development for solid cancers. Antibodies against mesothelin have been shown to act via immunotoxin-based inhibition of tumor growth and induction of antibody-dependent cell-mediated cytotoxicity (ADCC). However, complement-dependent cytotoxicity (CDC), considered an important additional mechanism of therapeutic antibodies against tumors, is inactive for such antibodies. Here, we used phage display antibody engineering technology and synthetic peptide screening to identify SD1, a human single-domain antibody to mesothelin. SD1 recognizes a conformational epitope at the C-terminal end (residues 539–588) of mesothelin close to the cell surface. To investigate SD1 as a potential therapeutic agent, we generated a recombinant human Fc (SD1-hFc) fusion protein. Interestingly, the SD1-hFc protein exhibits strong CDC activity, in addition to ADCC, against mesothelin-expressing tumor cells. Furthermore, it causes growth inhibition of human tumor xenografts in nude mice as a single agent. SD1 is the first human single-domain antibody targeting mesothelin-expressing tumors, shows potential as a cancer therapeutic candidate, and may improve current antibody therapy targeting mesothelin-expressing tumors.

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Introduction

Mesothelin has been suggested as a therapeutic target because it is highly expressed in malignant mesotheliomas (1, 2) and other solid tumors such as cholangiocarcinoma, ovarian cancer, lung adenocarcinoma, and breast cancer (3–5). The mesothelin (MSLN) gene encodes approximately 71 kDa precursor protein that is processed to approximately 31 kDa N-terminal protein and approximately 40 kDa C-terminal membrane-bound mature mesothelin (3). A number of antimesothelin monoclonal antibodies (mAb) have been developed. The mAb drugs, SS1P immunotoxin and MORAb-009 (also known as amatuximab), are currently being evaluated in clinical trials (3, 4). SS1P is an immunotoxin consisting of a murine anti-mesothelin Fv fused to a truncated Pseudomonas exotoxin that mediates cell killing (6). MORAb-009, a chimeric (mouse/human) antibody based on the murine SS1 Fv, elicits antibody-dependent cell-mediated cytotoxicity (ADCC) on mesothelin-bearing tumor cells (7). Recently, we and our collaborators generated 2 fully human mAbs (HN1 and m912) that recognize mesothelin (8, 9). HN1 recognizes an epitope overlapping the SS1 site in mesothelin, indicating that HN1 can be developed as a fully human version of SS1 Fv-based mAbs (such as MORAb-009).

In our previous report, we proposed 3 distinct domains in cell surface mature mesothelin (10): Regions I (residues 296–390), II (residues 391–486), and III (residue 487–598; Fig. 1A). We experimentally established a minimum recognition sequence (named IAB; residues 296–359) in Region I for the binding of mucin MUC16/CA125. However, despite the fact that several mesothelin mAbs are now available, none have shown complement-dependent cytotoxicity (CDC) against tumor cells.

CDC has been suggested as an important additional mechanism for cancer therapeutic antibodies (11). The first approved mAb for cancer therapy, rituximab, is
partially dependent on CDC for its antitumor activity (12, 13). It has been suggested that CDC may occur when the antibody-binding site is close to the cell membrane (14). As evidence, ofatumumab, which binds much closer to the cell membrane of CD20 than rituximab, also has much higher CDC activity (14). However, a new anti-CD20 mAb (obinutuzumab or GA101) exhibits strong inhibition of cell growth in addition to ADCC, but no CDC (15, 16). Almost all of the existing mesothelin mAbs recognize Region I, the N-terminal end of cell-surface mesothelin presumed to be located far from the cell membrane (10; Fig. 1A). ADCC is the only mechanism that has been found to contribute to the activity of known antimesothelin mAbs. Therefore, we hypothesize that a more desirable antimesothelin mAb will be capable of causing additional antitumor activity (i.e., CDC, direct inhibition of tumor cell growth) as well as ADCC by targeting novel epitopes. To this end, antibodies recognizing a domain in mesothelin beyond Region I must be made and tested.

To generate antibodies with potential CDC against tumors, we surmised that they should bind Region III of mesothelin close to the cell surface as ofatumumab does. However, such mAbs have been challenging to generate because this region is poorly immunogenic. Our recent study using rabbit hybridoma technology produced around 8,000 individual clones immunized by a full-length mesothelin protein. Ninety-six percent of all positive clones were Region I-binders (like HN1 and SS1/
MORAb-009). Only 3 were Region III binders. None bound the C-terminal end of mesothelin. This finding was consistent with our previous mouse hybridoma screening in which almost all high-affinity binders bound Region I (17). Given that standard hybridoma technology failed to produce antibodies specific for the desired C-terminal end of mesothelin, we used phage display technology to identify new antimesothelin human mAbs. Epitopes close to the cell surface may be occluded and difficult to access by full-size immunoglobulin G (IgG) antibodies and large fragments such as Fab's. Therefore, we used a phage display library of smaller binders, human single-domain (VH) antibodies displayed on phage, and panned it against a peptide corresponding to the C-terminal end of mesothelin. After isolating the SD1 human antibody domain, we converted it to a human Fc fusion protein (SD1-hFc) for analysis. The SD1-hFc protein shows strong antitumor activity against tumor cells in vitro and inhibits xenograft tumor growth in nude mice, suggesting use for potential antibody therapeutics that could improve current mesothelin-targeted cancer therapy.

Materials and Methods

Cell culture

Human cholangiocarcinoma (CCA) lines (KMBC, MzChA-1, and HuCCT-1) were obtained from Gregory J. Gores (Mayo Clinic, Rochester, MN; ref. 18). A431 (epidermal carcinoma), OVCAR3 (ovarian), and NCI-H226 (mesothelioma) were obtained from American Type Culture Collection. EKVX (human non–small cell lung cancer; NSCLC) and OVCAR-8 (ovarian cancer) were obtained from National Cancer Institute (NCI, Bethesda, MD; Development Therapeutics Program). L55 (NSCLC) was provided by Steven M. Albelda (University of Pennsylvania, Philadelphia, PA). All tumor cell lines were grown as described (8, 18). A431/H9 is a transfected A431 cell line stably expressing human mesothelin (19). The HEK-293F cell line (Invitrogen) was grown in FreeStyle serum-free medium (Invitrogen). All cell lines were passaged only a few times (<1 month) after thawing of initial frozen stocks, which were generated right after obtaining cell lines, to reduce the total number of passages to less than 15. All cell lines were tested and authenticated by morphology and growth rate and were Mycoplasma free.

Screening an engineered human antibody domain library

An engineered human (VH) antibody domain library named m81 showed an estimated diversity of $2.5 \times 10^{10}$ (20). The C-terminal mesothelin peptide consisting of 50 amino acids (NH2–VQKLLGPVHEGLKAERHRPVRDVWILRQDLDTLGLQGIFNGYLV–COOH) was synthesized (GenScript). Full-length human mesothelin protein (MSLN) was prepared as described (10). The phage library was subjected to 4 rounds of panning on full-length human mesothelin (rabbit Fc-mesothelin fusion protein, rFc-MSLN) or the C-terminal mesothelin peptide coated on Nunc 96-well ELISA plates (Maxisorb, Nunc/Thermo Fisher Scientific) according to our lab protocol (21, 22). Randomly picked 384 phage clones at the end of each round of panning were analyzed for antigen binding by phage ELISA.

Production of an SD1-hFc fusion protein

The VH region encoding the SD1 human antibody domain fused with human IgG1 Fc and FLAG/His tag was PCR amplified with 2 primers (forward: 5’-GTC ATC ACA ACT TCG ATA TCG CGG TGC AGC GGT GCA GTC TGG GGG AGG CTT GGT A-3’; reverse: 5’-GAA GTT GTG ATG ACT CCG GAG CCC TTA TCG TCA TCG TCC TTG TAG TCG CCG TGG-3’). The PCR product was inserted into the EcoRV and BspEl sites (underlined) of the vector, pVRC8400 (provided by Gary J. Nabel, the National Institute of Allergy and Infectious Diseases, Bethesda, MD; refs. 23, 24). The final plasmid (named pMH148) was transfected into HEK-293F cells and the protein was purified using protein A column (GE healthcare). We established a stable cell line by transfecting HEK-293F (Invitrogen) cells with pMH148. The stable line produced the SD1-hFc fusion protein with a high-expression level (>70 mg/L) in culture supernatant.

Immunoprecipitation and Western blot analysis

Cell lysate (1.5 mg) was incubated with 50 μg of SD1 or an irrelevant single-domain hFc fusion protein in 500 μL of radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling) and rotated overnight at 4 °C. Thirty microliters of protein A beads were added (Sigma) and rotated at 4 °C for 2 hours. Beads were spun down and washed with RIPA buffer. Immune complexes were released from the beads after 5 minutes of boiling in 100 μL of 2× loading buffer. Western blot analysis was conducted following a lab protocol (18).

ELISA

Direct ELISA and affinity measurement—direct binding and affinity of SD1-hFc were evaluated on the ELISA plates coated with mesothelin peptide, human mesothelin, or mouse mesothelin (mMSLN) following the procedures described previously (10).

Competition ELISA—various amounts of mesothelin peptide, an irrelevant 50 amino acid peptide, the HN1 human mAb, or the SS1P immunotoxin (kindly provided by Ira Pastan, NCI, Bethesda, MD) were mixed with 5 μg/mL of SD1-hFc and incubated at room temperature for 1 hour. The mixture was then transferred to an ELISA plate coated with 5 μg/mL of human mesothelin protein and incubated at room temperature for an additional hour following the procedure described above.

Flow cytometry

To determine the binding of the SD1 antibody to cell surface–associated mesothelin, flow cytometric analysis was conducted according to our standard protocol (18). The average number of mesothelin sites per cell was
measured on a FACS Calibur (BD Biosciences) using BD Quantibrite PE beads (BD Biosciences).

To investigate whether SD1 inhibits the binding of mesothelin to MUC16, 10 μg/mL of rFc-MSLN protein were preincubated with different concentrations of SD1 or SS1P, and then incubated with MUC16-positive ovarian cancer cells (OVCAR3). Binding of rFc-MSLN to cell surface-associated MUC16 on OVCAR3 cells was analyzed following a flow cytometry protocol (10).

Clq and antimesothelin binding assays were conducted following an established protocol (14, 25). Briefly, A431/H9 or NCI-H226 cells were suspended at 1 x 10^6 cells/mL and incubated with different concentrations of SD1-hFc, control human IgG, or the HN1 human IgG on ice for 1 hour. After washing, cells were incubated with 20 μg/mL purified Clq (Complement Technologies) at 37°C for 0.5 hour. The cells were washed again and then incubated with fluorescent isothiocyanate (FITC)-labeled sheep anti-human Clq mAb (AbD Serotec) for 0.5 hour on ice. At the end of the incubation, cells were washed and analyzed on a FACS Calibur.

**ADCC and CDC**

ADCC assay was conducted by using human peripheral blood mononuclear cells (PBMC, purified human natural killer (NK) cells (26), and mouse NK cells (27), and measured by an LDH kit (Roche) according to a standard protocol (8). Human NK cells were isolated from the peripheral blood of healthy donors using the NK cells isolation kit (Miltenyi Biotec). Mouse NK cells were purified following a protocol as previously described (27). The purity of mouse NK cells was determined by an anti-CD49b conjugated with FITC (eBioscience) on a FACS Calibur.

CDC activity was measured by lactate dehydrogenase (LDH)-releasing assay according to previous reports (14). Normal human sera were provided by the Department of Transfusion Medicine (NIH Clinical Center, Bethesda, MD), and mouse serum was freshly drawn before use. Percentage of specific lysis was calculated according to the following formula: % lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release) x 100.

**Xenograft antitumor testing in mice**

Animal testing evaluating SD1-hFc was conducted using A431/H9 xenografts in nude mice following a well-established NCI protocol (7, 28). Four- to six-week-old female athymic nude mice (NCI-Frederick Animal Production Area, Frederick, MD) were housed in microisolation cages during the course of the experiment. The animal protocol (LMB-059) was approved and mice were maintained as per Institutional guidelines of the NIH. Three million A431/H9 cells were inoculated subcutaneously into the right flank of the mice. Tumor dimensions were determined using calipers, and the tumor volume (mm^3) was calculated by the formula: length x (width)^2 x 0.5. Treatment was initiated when tumors reached approximately 70 mm^3 in size. The different treatment regimens included: PBS and SD1-hFc (50 mg/kg) via intravenous injection on days 7, 9, 11, 14, 17, and 20 after tumor inoculation. Mice were sacrificed when tumors reached more than 1,000 mm^3.

**Results**

**Discovery and production of the SD1 human antibody**

To find a new antimesothelin mAb targeting a site close to the cell surface, we designed a C-terminal mesothelin peptide for screening and used a library of small-size binders (VHs). Using the big-PI Predictor program, we predicted that the glycosylphosphatidylinositol cleavage site was Ser598 (3, 4, 10). We designed a peptide (residues 539-588; NH2-VQKLLGLPVEGLKAEERHRPVRDWIL-RQRQDDDLTGLQGLQGIPNGYLV-COOH), which is 10 amino acids away from the glycosylphosphatidylinositol cleavage site of mesothelin (Fig. 1A) for phage panning with an engineered human antibody domain phage display library (Supplementary Table S1; ref. 20). After the fourth round of phanning, phage titer was significantly increased (Fig. 1B), and more than 95% of clones were peptide binders. Phage clone SD1 was selected for further analysis because it bound not only the peptide but also full-length mesothelin (Fig. 1C). In comparison, we screened the same single-domain antibody phage library on full-length human mesothelin protein and found 5 different VH sequences. The clone SD2 sequence was found in 90% of all phage clones (total 384) analyzed at the end of the last round, indicating that SD2 was highly enriched. By ELISA, SD2 was specific for full-length human mesothelin, but not the peptide (Fig. 1D). Because our aim was to isolate an antibody that binds to a site close to the cell surface, SD2 did not meet our criteria. We decided to choose SD1 for further analysis.

To investigate SD1 as a potential therapeutic, we converted it into a clinically relevant hFc fusion protein (Fig. 2A). For large-scale production, we established a stable cell line with a high expression level (>70 mg/L) in the culture supernatant of HEK-293F cells, and the purity of SD1-hFc protein was more than 95%.

**The SD1 human antibody binds cancer cell surface-associated mesothelin**

To analyze the binding properties of SD1 to mesothelin protein in cancer cells, we conducted Western blot analysis and pull-down assays using cancer cell lysates. Initial Western blot analysis of various cancer cell lysates using SD1-hFc could not detect a mesothelin band under reducing conditions even in high mesothelin-expressing cell lines such as A431/H9 and NCI-H226 (data not shown), indicating that SD1-hFc did not recognize denatured mesothelin protein. By conducting pull-down assays to detect endogenous mesothelin proteins in solution (Fig. 2B), SD1-hFc successfully pulled down mature mesothelin protein from 3 different cancer cell lines (A431/H9, NCI-H226, and KMBC). The molecular weight of mature
mesothelin (~40 kDa) was consistent with previous studies (18, 29). In ELISA assays, SD1-hFc bound both full-length human mesothelin protein and peptide (Fig. 3A) and did not bind full-length mouse mesothelin protein, bovine serum albumin, or other irrelevant proteins. As expected, SS1P and HN1 bind only full-length human mesothelin, and not the C-terminal peptide.

To evaluate whether SD1-hFc recognizes the C-terminal end, we preincubated SD1-hFc, HN1, or SS1P with peptide (residues 539–588) and tested binding of the antibody–peptide mixture to human mesothelin coated on an ELISA plate. Competition ELISAs (Fig. 3B) showed that the C-terminal peptide blocked the binding of SD1-hFc, not SS1P or HN1, to full-length human mesothelin. We also measured the kinetics of SD1 binding using full-length mesothelin protein and the C-terminal peptide. SD1-hFc binds to human mesothelin protein with dissociation equilibrium constants and Scatchard plots were determined by using Prism (version 3.02) for Windows (GraphPad software; ref. 10). To determine whether SD1 blocks the binding of mesothelin to MUC16, we preincubated SD1 or SS1 with mesothelin, added the mixture to MUC16-positive OVCAR3 cells, and then conducted flow cytometry analysis. We found that unlike SS1, SD1 did not inhibit the mesothelin–MUC16 interaction (Fig. 3E and F) indicating that the SD1-binding site does not overlap the SS1 site.

To analyze whether SD1 is suitable for cancer therapy, we determined whether SD1-hFc binds native mesothelin molecules on human tumor cells. We conducted flow cytometric analysis on a panel of mesothelin-expressing cancer cells and experimentally measured the average number of mesothelin sites per cell using the Quantibrite fluorescence quantitation system (Supplementary Table S2). SD1-hFc binds A431/H9, but not A431, indicating that binding on cell surface–associated mesothelin is highly specific (Fig. 4). We tested the binding of SD1-hFc on a panel of native human tumor cell lines. SD1-hFc strongly bound human ovarian cancer (OVCAR-8), mesothelioma (NCI-H226), and cholangiocarcinoma cell lines (KMBC). It moderately bound CCA cell line (Mz-ChA-1) and weakly bound human NSCLC cell lines (EKVX and L55). It did not bind to cholangiocarcinoma cells, HuCCT1, which is a mesothelin-negative line (18). SD1 recognizes a conformational epitope of native mesothelin close to the cancer cell surface and binds cell surface–associated native mesothelin proteins with high affinity and excellent specificity.

**Antitumor activity of SD1-hFc: CDC and ADCC**

To evaluate the antitumor activity of SD1-hFc against cancer cells, we tested the cytotoxic activity in A431/H9 and NCI-H226 cell models in the presence of human serum as a source of complement. A human VH single-domain antibody isolated from the same phage library specific for an irrelevant tumor cell surface antigen was used as a control in the present study. In CDC assays, SD1-hFc exerted potent CDC activity by killing 40% of A431/H9 (Fig. 5A) and more than 30% of NCI-H226 mesothelioma cell lines (Fig. 5B) and showed no activity on the mesothelin-negative A431 cell line (data not shown). In contrast, the control single-domain antibody and HN1 showed no activity at the same concentrations, indicating that targeting to the C-terminal epitope of SD1 is capable of inducing CDC. A previous study showed that MORAb-009 showed no significant CDC activity against the tumor cells (7). To analyze the role of complements in the antitumor activity of SD1-hFc, we used flow cytometry to determine C1q binding to cancer cells reacted with anti-mesothelin human mAbs following a well-established protocol for characterization of rituximab, ofatumumab, and other anti-CD20 therapeutic mAbs (14, 25). As shown in Fig. 5C and D, the C1q complement bound to A431/H9 or NCI-H226 cells in the presence of SD1-hFc. However, no C1q binding was found in the presence of HN1 or an irrelevant human single-domain antibody control. Moreover, the binding of C1q to cancer cells is associated with the binding of SD1-hFc in a dose–response manner. These results show that the C-terminal end binder SD1-hFc, and not the N-terminal end binder HN1, can recruit C1q to the
A Human Single-Domain Antibody to Mesothelin

Figure 3. Binding properties of SD1-hFc. A, direct ELISA. MSLN, full-length human mesothelin protein; mMSLN, mouse mesothelin. B, competition ELISA. C and D, the dissociation equilibrium $K_D$ of SD1-hFc to the human mesothelin protein was 13.58 nmol/L (C) and 16.08 nmol/L for the peptide (D). E and F, recombinant mesothelin protein bound to MUC16 in the presence of SD1.

mesothelin-expressing cell surface. The HN1 human IgG has an extra CH1 domain in its Fc portion. To determine whether the CH1 domain in the HN1 IgG may interfere with its potential CDC activity on tumor cells, we generated a new HN1 single-chain Fv(scFv)-Fc fusion protein by fusing the HN1 scFv to the same Fc sequence used in SD1-hFc (Supplementary Fig. S3A). When comparing SD1-hFc with HN1(scFv)-hFc in CDC assays, we found that HN1(scFv)-hFc did not show any CDC activity (Supplementary Fig. S3B and S3C). Therefore, it is unlikely that the CDC activity observed with SD1 treatment was related to its shorter Fc portion.

In addition to CDC activity, we tested ADCC activity of SD1-hFc against tumor cells. High levels of cytotoxicity were found using SD1-hFc with human PBMCs at different concentrations or purified human NK cells with various ratios between effector cells and target cells (E:T). SD1 exhibited significant ADCC activity by killing more than 40% against A431/H9 cells (Fig. 5E) and more than 30% of NCI-H226 mesothelioma cells (Fig. 5F). No activity was found on mesothelin-negative A431 cells (data not shown). NK cells played an important role in SD1-mediated ADCC. Using purified human NK cells, SD1 killed almost 20% cells even with the lower E:T ratio of 5:1 (Fig. 5G and H). Taken together, SD1-hFc has strong CDC and ADCC antitumor activity against mesothelin-expressing tumor cells in vitro.

Antitumor activity in mice

To evaluate the antitumor activity of SD1-hFc in vivo, we used immunodeficient mice bearing tumor xenografts following an established protocol used to evaluate MORAb-009 in preclinical studies (7). Athymic nude mice bearing A431/H9 tumors were treated with 50 mg/kg of SD1-hFc (Fig. 6A). The number of mesothelin sites in A431/H9 is comparable with that of malignant mesothelioma cells endogenously expressing mesothelin, and their implantation in mice consistently results in
aggressive tumor growth. Twenty days after inoculation of tumor cells, the average tumor size in mice treated with SD1-hFc alone was significantly reduced (average 300 mm³) compared with the control group (average 1,000 mm³), showing that SD1-hFc is very active as a single agent.

To evaluate SD1-induced CDC in mice, we examined CDC activity using mouse sera and found that SD1-hFc killed 11% of A431/H9 cells in the presence of 30% mouse serum freshly drawn from nude mice (Fig. 6B), indicating that mouse complement was 10-fold less active than human complement (Fig. 5) for SD1-hFc, consistent with previous studies involving hFc (12). SD1 was able to induce ADCC with purified mouse NK cells and killed mesothelin-positive cancer cells (H9) with different E:T ratios (Fig. 6C). The purity of mouse NK cells was around 50% (Fig. 6D). Taken together, SD1-hFc caused growth inhibition of tumor xenografts in nude mice in vivo. Our results suggest that the SD1 single-domain antibody represents a new class of antimesothelin mAbs and is a promising therapeutic candidate for novel mesothelin-targeted therapy.

Discussion

In the present study, we used phage display to develop an engineered antibody domain, called SD1, recognizing an epitope at the C-terminus of mesothelin. This epitope does not overlap with any previously developed mesothelin therapeutic antibodies in preclinical or clinical development. SD1-hFc shows strong CDC and ADCC activity against mesothelin-expressing cancer cells in vitro and causes growth inhibition of human tumor xenografts in nude mice in vivo. Our results suggest that the SD1 single-domain antibody represents a new class of antimesothelin mAbs and is a promising therapeutic candidate for novel mesothelin-targeted therapy.

We isolated the SD1 domain by phage panning on a C-terminal 50-residue peptide of mesothelin and showed that the antibody binds native mesothelin proteins in cancer cells. This region has never been accessed by any known antimesothelin antibodies. Our data show that CDC triggered by SD1-hFc depends on the specific new epitope because the HN1 human IgG (specific for the N-terminus of mesothelin, Region I, far from the cell surface) does not exhibit CDC activity and cannot recruit C1q to cancer cells. Furthermore, the HN1(scFv)-hFc that contains the same Fc sequence of SD1-hFc does not exhibit CDC activity. We also tested SD1 and HN1 in cell proliferation assays and found that none of these antibodies exhibited direct inhibition of tumor cell growth (data not shown).
shown). It remains uncertain whether any antimesothelin antibodies can directly inhibit tumor cell proliferation. Taken together, our results indicate that CDC likely contributes to the antitumor activity of SD1.

MORAb-009 is the only antimesothelin antibody currently being evaluated in clinical trials (30–33) and should be compared with SD1. MORAb-009 is a mouse/human chimeric IgG antibody containing mouse Fv with
improved affinity (34), whereas SD1 is a fully human VH single-domain antibody isolated from a naïve human antibody phage library. SD1 recognizes the C-terminus of cell surface–associated mesothelin, whereas MORAb-009 and other existing antibodies including HN1 recognize Region I. SD1 exhibited strong CDC activity, whereas a previous study indicated that MORAb-009 did not (7). Interestingly, SD1 caused 70% growth inhibition of tumor xenografts in nude mice as a single agent, whereas MORAb-009 showed about 40% growth inhibition (7). Future studies are necessary to directly compare SD1 with MORAb-009 in both in vitro and in vivo assays.

Human single domain antibodies are attractive candidates for cancer therapy. However, human VHs are typically prone to aggregation (35). In the present study, we fused the SD1 VH to CH2 and CH3 of human IgG1 and produced SD1-hFc as a dimeric IgG-like protein in mammalian HEK-293F cells. We also produced recombinant immunotoxins based on SD1 and showed it could inhibit proliferation of mesothelin-positive tumor cells in a dose-dependent manner (Supplementary Table S2 and Supplementary Fig. S1). All recombinant proteins were properly folded for in vitro and in vivo assays. Currently, SD1 and SD2 are the only 2 single-domain antibodies targeting mesothelin that have been identified. To determine whether the SS1 VH alone in MORAb-009 is functional, we also constructed VH single-domain molecules based on SS1 as well as HN1. We expressed and compared the VH domains of SD1, SS1, and HN1 using mammalian cell display technology (36). Only SD1 VH, and not the VH domain of SS1 or HN1, bound mesothelin (Supplementary Fig. S2).

We noticed the discrepancy between calculated and actual molecular weights of the SD1-hFc and HN1 (scFv)-hFc on SDS-PAGE gels (Supplementary Fig. S3). Similar discrepancies were previously described in other studies (37–40). We ran commercially available human IgG proteins on a SDS-PAGE gel (Supplementary Fig. S3D) and found a molecular weight of a dominant human IgG band of 250 kDa, instead of the 150 kDa theoretical value. This discrepancy may not be rare in human IgG or Fc fusion molecules although the precise mechanism is unclear. To evaluate whether N-glycosylation on hFc may affect the actual molecular weight, we treated hFc fusion proteins with PNGase F and found that enzymatic
deglycosylation only modestly reduced molecular masses (Supplementary Fig. S3E and S3F). This discrepancy may be due to the Y-shaped structure of IgG molecules, which is distinct from other globular proteins (40).

Although our in vivo animal testing using a xenograft tumor model in nude mice showed strong antitumor activity of SD1-hFc, it would be interesting to conduct complement depletion studies to further validate the role of CDC in vivo. Future studies may further improve the antitumor activity of SD1 by affinity maturation of the Fv portion (21) and by enhancing ADCC via glycoengineering of the Fc portion (15, 16), or by combining the SD1 antibody therapy with chemotherapy (7).

In summary, we generated the first human single-domain antibody against mesothelin-expressing tumors and showed it has strong antitumor activity in vitro and in vivo by targeting an epitope close to the cancer cell surface via CDC as well as ADCC. Such a binding site has not been accessed by any known antimesothelin antibodies. Development of the SD1 human antibody may lead to novel antibody therapies targeting mesotheloma and other mesothelin-expressing tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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