Development of engineered antibodies specific for the Müllerian inhibiting substance type II receptor: a promising candidate for targeted therapy of ovarian cancer

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Abstract

The Müllerian inhibiting substance type II receptor (MISIIR) is involved in Müllerian duct regression as part of the development of the male reproductive system. In adult females, MISIIR is present on ovarian surface epithelium and is frequently expressed on human epithelial ovarian cancer cells. Müllerian inhibiting substance has been found to be capable of inhibiting the growth of primary human ovarian cancer cells derived from ascites and ovarian cancer cell lines. This suggested to us that MISIIR could be an attractive target for antibody-based tumor targeting and growth inhibition strategies. Here, we describe the production of recombinant human MISIIR extracellular domain-human immunoglobulin Fc domain fusion proteins and their use as targets for the selection of MISIIR-specific human single-chain variable fragments (scFv) molecules from a human nonimmune scFv phage display library. The binding kinetics of the resulting anti-MISIIR scFv clones were characterized and two were employed as the basis for the construction of bivalent scFv:Fc antibody-based molecules. Both bound specifically to human ovarian carcinoma cells in flow cytometry assays and cross-reacted with mouse MISIIR. These results indicate that antibody-based constructs may provide a highly specific means of targeting MISIIR on human ovarian carcinoma cells for the purpose of diagnosing and treating this disease. [Mol Cancer Ther 2006;5(8):2096 – 105]

Introduction

Human ovarian cancer is frequently diagnosed at an advanced stage as its early symptoms are generally minor and easy to ignore. Although early diagnosis is associated with improved survival, advanced stage ovarian cancer is generally associated with a poor prognosis (1–3). Current therapeutic modalities including surgery, chemotherapy, radiotherapy, and immunotherapy are often inadequate to overcome the disease. Early detection and new treatment strategies are clearly needed.

In males, Müllerian inhibiting substance (MIS), or anti-Müllerian hormone, is produced in fetal and postnatal testes. MIS primarily binds to the Müllerian inhibiting substance type II receptor (MISIIR) and triggers the regression of the Müllerian ducts, the anlagen of the uterus, fallopian tubes, and vagina (reviewed in refs. 4, 5). In contrast, MIS is undetectable in females until low levels appear in the circulation in adolescence, thus allowing the development of reproductive organs. *In situ* hybridization has revealed that MISIIR mRNA is developmentally expressed in the urogenital ridges of both sexes, in the epithelium and mesenchyme of the Mullerian duct, on the coelomic epithelium, and in the tubules of embryonic gonads (6–8). The most common human ovarian cancers, derived from coelomic epithelium, resemble the fetal Mullerian structures that form in the embryo by invagination of the coelomic epithelium and regress in the male embryo under the influence of MIS (9–11). Recently, it has been observed that human MISIIR is expressed in a large percentage of both ovarian cancer cell lines and cells isolated from the ascites fluid of patients with ovarian cancer, and that the binding of MIS to MISIIR on these cells can trigger apoptosis (12). Recombinant human MIS was recently reported to inhibit the *in vitro* and *in vivo* growth of mouse ovarian carcinoma (MOVCAR) cells (13). Clinical trials to assess the therapeutic potential of MIS in patients with ovarian cancer are currently under way. However, the production of large quantities of recombinant MIS has proven to be difficult (11). We, and others (14), hypothesized that antibodies could be generated that bind specifically to MISIIR. Ideally, such antibodies would mimic the function of the MIS ligand and trigger the regression of ovarian tumors (15). The highly tissue-specific expression pattern of MISIIR also suggests that it would be an attractive target for immunoconjugates containing

agents with properties that facilitate the detection or treatment of ovarian cancer.

Here, we describe our efforts to develop MISIIR-specific antibodies by expressing two fusion proteins comprised of the human MISIIR extracellular domain (ECD) and the human IgG_1 Fc domain (Fc), with the MISIIR ECD on either the carboxyl or NH_2 terminus, and using them as targets for the selection of anti-MISIIR single-chain variable fragments (scFv) from a large nonimmune human scFv phage display library. The resulting scFvs were characterized for the ability to specifically bind to MISIIR, and the two most promising clones were engineered into scFv:Fc ("midibody") fusion molecules for *in vitro* evaluation.

Materials and Methods

Expression and Purification of MISIIR ECD + Fc Fusions

cDNA encoding MISIIR ECD was obtained by PCR from a human testes cDNA library (Human Testes PCR-Ready cDNA, Ambion, Inc., Austin, TX). The gene encoding MISIIR ECD without the signal portion was amplified using primers that did not include the first 17 amino acids but incorporated the *Hin*dIII restriction site (MISIIR ECD-Afor) and the *Kpn*I restriction site (MISIIR ECD-Brev; all primer sequences are provided in Table 1). The human IgG_1 Fc domain gene was isolated from the G1pBR322

plasmid (Biogen Idec, Cambridge, MA). The MISIIR ECD gene was then fused with the human IgG_1 Fc (hinge-C_H2-C_H3) in two orientations, either 5'- or 3'- of the Fc gene and the resulting gene was cloned into pSecTag2/Hygro (version B; Invitrogen, Inc., Carlsbad, CA) to produce two fusion proteins. The sequences of every clone described here were confirmed by DNA sequencing.

The IgG-Hinge-Afor forward and the IgG-CH3-Arev reverse primers were used to amplify the human IgG_1 Fc gene for use in the fusion with MISIIR ECD at the NH_2 terminus of Fc (designated MISIIR_ECD:Fc). *Kpn*I and *Xho*I restriction sites were incorporated, respectively, into the above PCR primers along with an additional nucleotide ("C" preceding *Xho*I site, as underlined in primer IgG-CH3-Arev, to keep the myc and his6 tag sequences in frame) for cloning of the Fc gene into the vector pSecTag2/MISIIR_ECD, yielding the final construct pSecTag2/MISIIR_ECD/Fc.

The gene encoding MISIIR ECD at the COOH terminus followed by the TEV cleavage site and Fc (designated Fc:TEV:MISIIR_ECD TEV) was constructed similarly employing the IgG-Hinge-Bfor and IgG-CH3-Brev primers that incorporated *Asc*I and *Hin*dIII restriction sites into the Fc-coding segment. MISIIR ECD was PCR-amplified from the pSecTag2/MISIIR_ECD construct using the MISIIR_ECD-Cfor and MISIIR_ECD-Drev primers which incorporated *Bam*HI and *Xho*I sites as well as a FLAG tag on the NH_2 terminus. The two coding genes were inserted sequentially into pSecTag2/Hygro to create pSecTag2/Fc/MISIIR_ECD. A synthetic linker containing *Hin*dIII site, Gly4Ser linker, the seven amino acid TEV cleavage site (16), and the *Bam*HI site was inserted between the Fc domain and the MISIIR ECD using the TEVsen and TEVas primers. The TEV cleavage site was added to provide additional spacing between the MISIIR ECD and Fc and establish a straightforward mechanism for future isolation of pure MISIIR ECD. The two oligos were heated and annealed (95°C, 5 minutes, air-cooled to room temperature for 20 minutes) to facilitate ligation into the pSecTag2 Fc/MISIIR ECD construct which was previously digested (37°C, 2 hours) with *Bam*HI and *Hin*dIII and purified (QiaQuick PCR purification kit; Qiagen, Valencia, CA), resulting in the final construct, pSecTag2/Fc/TEV/MISIIR ECD.

Both the pSecTag2/MISIIR_ECD/Fc and the pSecTag2/Fc/TEV/MISIIR ECD constructs were transiently transfected into COS-7 cells (American Type Culture Collection, Manassas, VA) using FuGene6 (Roche, Inc., Indianapolis, IN) to validate protein expression and secretion. Positive constructs were then linearized with *Ssp*I and used to stably transfect HEK 293 cells (American Type Culture Collection). Successfully transfected colonies were selected by the addition of 250 μg/mL of hygromycin B (Invitrogen) to the DMEM 36 to 48 hours after transfection. Subclones that produced the highest levels of secreted MISIIR fusion protein were identified and cultured in DMEM containing 10% "low IgG" fetal bovine serum (Invitrogen) to facilitate purification. Cells were grown at 37°C in a humidified incubator in air with 5% CO_2.

Table 1.   Primers employed

Primer name	
MISIIR ECD-Afor	5'-GGG AAG CTT CCC CCA AAC AGG CGA ACC TGT GTG-3'
MISIIR ECD-Brev	5'-GGG GGT ACC GGA CTC ACC TGG GGC AGC CTG G-3'
IgG-Hinge-Afor	5'-GGG GGT ACC GTT GAG CCC AAA TCT TGT GAC-3'
IgG-CH3-Arev	5'-GGC CTC GAG C̲TT TAC CCG GAG ACA GGG AGA G-3'
IgG-Hinge-Bfor	5'-GGC GCG CCG TTG AGC CCA AAT CTT GTG-3'
IgG-CH3-Brev	5'-GGG AAG CTT TTT ACG CGG AGA CAG GGA GAG-3'
MISIIR_ECD-Cfor	5'-GGG GAT CCG ACT ACA AAG ACG ATG ACG ACA AGC CCC CAA ACA GGC GAA CC-3'
MISIIR_ECD-Drev	5'-GGG CTC GAG TCA GGA CTC ACC TGG GGC AGC CTG G-3'
TEVsen	5'-AGC TTG GGG GAG GGG GAA GCG AGA ACC TGT ACT TCC AGG GTG-3'
TEVas	5'-GAT CCA CCC TGG AAG TAC AGG TTC TCG CTT CCC CCT CCC CCA
LW743	5'-GAA ATA CCT ATT GCC TAC G<3') and LW744 (5'>CTT ATT AGC GTT TGC CAT T-3'
LW744	5'-CTT ATT AGC GTT TGC CAT T-3'

The protein fraction was concentrated from the tissue culture supernatant by ammonium sulfate precipitation (at 0.4 g/mL of supernatant), pelleted (10,000 rpm, 4°C, 30 minutes), resuspended in PBS (1:10 volume), and dialyzed at 4°C for 24 hours in three 4,000-mL volumes of PBS. The dialyzed protein was then isolated using an immobilized protein-A strategy (Pierce, Rockford, IL) following the manufacturer's instructions. Each fraction was analyzed by reduced SDS-PAGE and those containing protein bands of the appropriate size were concentrated and further purified by high-performance liquid chromatography (HPLC) on Superdex200 and Superdex75 size exclusion chromatography columns (Pharmacia, New York, NY) rigged in tandem. Concentration was measured by reading the samples at A280 on a spectrometer (Beckman model DU530).

Subtraction Library Panning

Anti-MISIIR scFv-phage were isolated from a human nonimmune scFv phage display library (approximate size of 1.6×10^{10} independent transformants). Prior to panning, MISIIR ECD:Fc was coated onto a Maxisorp-Immunotube (NUNC, Denmark) at a concentration of 20 μg/mL in coating buffer (BupH carbonate-bicarbonate buffer; Pierce) at 4°C, overnight. The coated tube was washed thrice in PBS and blocked with 4% MPBS (nonfat powdered milk dissolved in PBS) at 37°C for 2 hours. Phage library stock (100 μL; 1.3×10^{13} pfu/mL) was added to 4 mL of 2% MPBS containing 100 μg/mL of purified human IgG_1 Fc domain (produced in our laboratory) to block the isolation of Fc domain–specific clones. The mixture was incubated at room temperature for 30 minutes, transferred to the coated Immunotube, and incubated with rotation for another 2 to 3 hours at room temperature. The tube was washed 10 times with PBST (PBS, 0.1% Tween 20) and 10 times with PBS and bound phage were then eluted by the addition of 1 mL of fresh 100 mmol/L triethylamine (Sigma, St. Louis, MO) and immediately neutralized with 0.5 mL (1/2 volume) of 1 mol/L Tris (pH 7.4). Ten microliters of log-phase ($0.3-0.5$ OD_{600}) TG1 *Escherichia coli* (Stratagene, La Jolla, CA) was infected with 750 μL of the elutate. The infected *E. coli* were concentrated to a volume of 1 mL. A 1 μL aliquot was removed and serially diluted to determine the titer. The remaining infected *E. coli* were plated on a 25 mm 2×YT-AG plates (2×YT containing 1% glucose, 100 μg/mL ampicillin) overnight at 37°C, 6 mL of 2×YT-AG was added and the *E. coli* were removed by scraping. One hundred microliters of the bacterial slurry was used as the source for the next round of phage panning and amplification. A total of three rounds of selection were done as described above except that the stringency of the selection procedure was increased in the second and third rounds by reducing the quantity of coated MISIIR ECD:Fc to 10 μg/mL, and increasing the wash cycles to 20 times with PBST followed by 20 times with PBS.

ELISA Screening

After three rounds of selection, random colonies were screened for the presence of anti-MISIIR ECD scFv by monophage ELISA, as described (17). Briefly, colonies were picked from the 2×YT-AG plates and inoculated into individual wells of a "Master" 96-well plate (Nunclon Surface, NUNC) containing 180 μL of 2×YT-AG medium. The plates were grown overnight at 37°C, 180 rpm, and 10% of the volume of each well was transferred into an identically prepared "Working" 96-well plate. The plate was incubated for 1 hour at 37°C, 180 rpm and KO7 helper phage (10^{11} pfu/mL; New England Biolabs, Inc., Ipswich, MA) were then added to each well to facilitate the expression of scFv/phage. The plate was spun at 1,800 × g for 5 minutes and supernatants were removed and evaluated by ELISA as described (17) for the presence of anti-MISIIR scFv/phage using 96-well plates (NUNC Maxisorp) coated with MISIIR ECD:Fc fusion, Fc:TEV:MISIIR ECD fusion, and human Fc. Clones were considered to be positive for MISIIR ECD if their absorbance values in the MISIIR ECD:Fc or Fc:TEV:MISIIR ECD plates were ≥2.5 times the background absorbance, and they failed to bind to the human IgG Fc control plate.

PCR Fingerprint and scFv Expression

Positive clones were picked from the "Master" plate and checked for scFv inserts by PCR using the LW743 and LW744 primers. Unique colonies were identified using DNA fingerprinting with *Bst*NI digestion of PCR amplification (18) and automated sequencing. To facilitate large-scale scFv production, the scFv-coding segments were cut using the *Nco*I and *Not*I restriction enzymes (New England Biolabs), and cloned into expression vector pUC119mycHis as described (19). Soluble scFv were expressed in *E. coli* TG1, isolated from the periplasmic space and purified by Ni-NTA agarose affinity chromatography and HPLC on a Superdex75 column (Pharmacia) as previously described (20). The size and integrity of the resulting scFv were assayed by 12% SDS-PAGE.

Construction of scFv:Fc Midibody

Based on BIAcore and flow cytometry studies described below, two scFv clones were selected for the construction of scFv:Fc fusion midibodies. The genes encoding scFv GM-7 and GM-17 were isolated from the pUC119mychis vector using the *Sfi*I and *Not*I restriction enzymes (New England Biolabs) and ligated into pHingestuffer plasmid (21), engineered for the creation of scFv:Fc midibody by fusing scFv with the human IgG_1 hinge, C_H2 and C_H3 domains. The resulting constructs were initially transiently expressed in COS cells (as above) to show their ability to produce scFv:Fc midibody and then linearized with *Ssp*I (New England Biolabs) for stable transfection into HEK 293 cells (as described above). Colonies were selected using 1,000 μg/mL G418 antibiotic and grown in triple Nunc flasks (Fisher, Pittsburgh, PA) in DMEM containing 10% low IgG fetal bovine serum (Invitrogen) as above. The scFv:Fc midibody was purified from pooled supernatants using Protein-A, as described above. The size and integrity of the resulting scFv:Fc midibodies were assayed by 9% SDS-PAGE.

BIAcore Affinity Determination

The binding properties of the expressed scFv molecules were examined by surface plasmon resonance (SPR) using a BIAcore 1000 instrument (BIAcore, Piscataway, NJ). SPR was initially used to determine the specificity of the scFv

molecules for recombinant MISIIR ECD. Approximately 5,000 resonance units of MISIIR ECD:Fc, Fc:TEV:MISIIR ECD, or purified IgG Fc domain were each covalently coupled to carboxymethylated dextran matrix CM-5 sensor chips (BIAcore, Pharmacia) using the amine coupling method provided by the manufacturer. The expressed scFv molecules (1 μmol/L in PBS buffer) were passed over the three flow cells on a BIAcore 1000 Instrument (BIAcore, Pharmacia) and the resulting response units were recorded. The scFv clones that exhibited binding to both fusions (MISIIR ECD fused with IgG Fc) but failed to bind to human IgG Fc domain were further evaluated by determining their affinity. For the kinetic (affinity) analysis serial dilutions of each scFv (ranging from 2 to 6,000 nmol/L) were passed over MISIIR ECD:Fc immobilized CM-5 chip (400–600 response units) and a control (Fc immobilized) flow cell which provided a source of background subtraction for each concentration. The resulting data was analyzed with BIAEvaluation 3.2 software (BIAcore, Pharmacia) using the 1:1 (Langmuir) binding algorithm to determine the affinity constants. The affinities of the scFv:Fc midibody molecules were examined similarly; monovalent affinities were determined with the bivalent analyte algorithm and functional affinities were determined with the 1:1 (Langmuir) algorithm. In these studies, the χ^2 value was always <10% of R_{max}.

Flow Analysis of scFv and their Bivalent Derivatives

The specificity of the scFv and scFv:Fc molecules for MISIIR was confirmed in flow cytometry studies done with cell lines that naturally or artificially express MISIIR. Human IGROV-1 (naturally MISIIR "+") and human MISIIR transfected OVCAR-8 (22) human ovarian cancer cells were a kind gift from Dr. Patricia Donahoe (Massachusetts General Hospital). A human endometrial cell line, AN3 CA (American Type Culture Collection), expressing native, functional MISIIR (23), and a mouse ovarian cancer cell line, MOVCAR1 (a kind gift from Drs. Denise Connally and Thomas Hamilton of the Fox Chase Cancer Center, Philadelphia, PA) expressing murine MISIIR were employed. The COS-7 cell line that lacks MISIIR was used as a negative control. Cells (2×10^5/line) were harvested from tissue culture flasks with Hanks/EDTA, washed in 2 mL of fluorescence-activated cell sorting buffer (1% bovine serum albumin in PBS, 0.02% NaN_3), centrifuged for 5 minutes at $500 \times g$ and resuspended in 100 μL of fluorescence-activated cell sorting buffer containing 1 to 10 μL (1 mg/mL) of anti-MISIIR scFv or scFv:Fc midibody. The cells were then incubated on ice for 45 minutes, washed in fluorescence-activated cell sorting buffer, centrifuged as above and resuspended in 100 μL of fluorescence-activated cell sorting buffer containing the appropriate diluted secondary antibody. As the scFvs contain a 6×His tag, 2.5 μL (0.2 mg/mL) of anti-His antibody-Alexa488 conjugate (Penta-His Alexa Fluor 488, Qiagen) was applied to each sample. To detect the scFv:Fc fusions, 0.3 μL of FITC-conjugated goat anti-human IgG (γ and light chain) (1.4 mg/mL, Biosource, Camarillo, CA) was applied to each sample. Controls included unstained cells and secondary antibodies

alone for each cell line. Binding was detected by flow cytometry using a FACScan instrument (Becton Dickinson, Mountain View, CA) and the resulting data was acquired and analyzed using the CellQuest Pro software program (Becton Dickinson).

Results

MISIIR ECD:Fc Fusion Proteins

The MISIIR ECD:Fc and the Fc:TEV:MISIIR ECD fusion proteins were expressed from transfected HEK cells growing in monolayer cultures over a span of 12 weeks. Following purification on a protein-A column and HPLC size exclusion chromatography, the proteins exhibited a high level of purity as determined by SDS-PAGE run under reducing conditions (Fig. 1). Both fusions migrated on the gel with apparent molecular weights of ~50 to 60 kDa. As the predicted molecular weights of the monomers is ~42 to 45 kDa based on the protein sequence, this suggested that the expressed proteins were glycosylated as expected (4). The final yield of each ECD fusion protein was ~1 mg of purified protein per liter of tissue culture supernatant. Following their purification, the proteins were dialyzed into PBS containing 10% glycerol and 1 mL aliquots (~1 mg/mL) were stored at −70°C.

Subtraction Biopanning

Three rounds of selection were done to isolate anti-MISIIR scFv clones from a nonimmune human scFv phage display library. Following the final round of selection, clones from 192 distinct colonies were assayed by ELISA in plates coated with MISIIR ECD:Fc, Fc:TEV:MISIIR, or human IgG Fc domain. Thirty-four clones were identified that yielded a positive signal, plates coated with either of
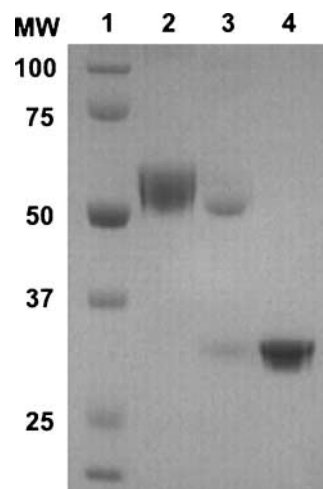

Figure 1. SDS-PAGE analysis of purified MISIIR-ECD:Fc fusions. Both MISIIR ECD/Fc fusions were expressed from stably transfected HEK 293 cells. The proteins ran under reducing conditions on a PAGE gel as monomers with approximate molecular weights in the range of 50 to 60 kDa. *Lane 1,* molecular weight standards (kDa); *lane 2,* purified MISIIR ECD:Fc NH2-terminal fusion; *lane 3,* Fc:TEV:MISIIR ECD COOH-terminal fusion (note partial degradation of the fusion resulting in a fragment at the size of an Fc domain); *lane 4,* purified human IgG1 Fc domain.

the MISIIR ECD fusion proteins but were negative in the plates coated with IgG Fc (data not shown). All of these clones were found to contain scFv-coding genes in PCR, amplifying with the LW743 and LW744 flanking primers (data not shown). DNA fingerprinting further categorized these clones into nine distinct groups (Fig. 2), seven of which were determined from sequencing to be associated with full-length open reading frame for scFv (sequences not shown). These scFv genes were cloned into expression vector pUC119mycHis for expression.

Binding Determination on BIAcore

The seven expressed scFvs eluted from an HPLC size exclusion column as a single major peak associated with the expected size of a scFv monomer, with a minor shoulder (1–5%) of the expected size of noncovalent scFv dimers present in some of the clones (data not shown). For each scFv, only the HPLC fractions associated with the ''scFv monomer'' peak were collected and used in the studies described below. Each of the expressed scFv molecules migrated on SDS-PAGE gels under reducing conditions as a single band of the expected size of ~28 kDa (data not shown). scFv yields at the conclusion of the purification and quality control steps ranged from 0.13 to 2.5 mg/L for the shake flask expressions. The specificity of the expressed scFv molecules for MISIIR was evaluated by SPR on the BIAcore using chips coated with MISIIR ECD:Fc, Fc:TEV:-MISIIR ECD, or IgG Fc domain alone as described above. Based on an analysis of response units, all clones revealed positive binding to the MISIIR ECD:Fc-coated chips. This result was expected as this fusion protein was employed in the panning procedure. Interestingly, three of the seven clones did not reveal significant binding to MISIIR in the second orientation (Fc:TEV:MISIIR ECD), suggesting either that these clones were specific for epitopes that were not exposed on both fusion proteins or that they bound to unique epitopes created by the fusion junction between the MISIIR and the Fc domains. Of the four clones that bound to both MISIIR fusion proteins, two clones (GM-7 and GM-29) exhibited a similar degree of binding to both fusion proteins, and the other two clones (GM-23 and GM-17) exhibited a large decrease in binding to the Fc:TEV:MISIIR ECD as compared with the MISIIR ECD:Fc fusion protein. The observed differences in binding to the two orientations of MISIIR ECD on the fusion proteins could be due to a number of factors including potential conformational differences in the structures of the MISIIR ECD in the two constructs or partial steric hindrance imposed by the Fc domain on the scFv molecules target epitopes when the Fc domain is located on the NH_2 terminus of MISIIR.

scFv:Fc Midibody Construction and Expression

The GM-7 and GM-17 scFv molecules were selected for cloning into the scFv:Fc midibody format to increase their avidity. Both the GM-7 and GM-17 scFv:Fc midibodies were stably transfected into HEK293 cells, expressed, and purified as described above. Yields of ~1 mg/L of supernatant were achieved from expressions of both scFv:Fc midibodies done in tissue culture flasks. Both scFv:Fc midibodies eluted from the HPLC size exclusion

column with the fractions associated with the expected molecular weights of ~110 kDa (data not shown) and ran on SDS-PAGE gels under reducing conditions as 55 kDa bands (Fig. 3). Both the GM-7 and GM-17 scFv:Fc midibodies exhibited specific binding to MISIIR ECD on the BIAcore (Fig. 3).

Affinity Determination

The affinities of the scFv and scFv:Fc midibody forms of both GM-7 and GM-17 for the MISIIR ECD were evaluated by SPR on the BIAcore. In order to provide the greatest available surface area of MISIIR ECD for binding, the MISIIR ECD:Fc was employed in these studies. The results of the affinity determinations are provided in Table 2. The GM-17 scFv exhibited a moderate affinity of 3.8×10^{-8} mol/L for the MISIIR fusion protein. However, the GM-7 scFv was found to be highly unstable at 4°C, thereby preventing the performance of rigorous affinity studies. In contrast, both the GM-7 and GM-17 scFv:Fc midibodies were stable for at least 1 month at 4°C. The GM-17 scFv:Fc exhibited a monovalent affinity of 1.6×10^{-8} mol/L which was very similar to that of the GM-17 scFv. Formation of the divalent molecule increased the functional affinity (avidity) to 6.5×10^{-9} mol/L. The GM-7 scFv:Fc exhibited a monovalent affinity of 5.4×10^{-9} mol/L and a functional affinity of 1.7×10^{-9} mol/L.

Flow Cytometry Studies

As the antibodies in this study were isolated and validated using recombinant MISIIR/Fc fusion proteins, it was necessary to verify their ability to recognize native MISIIR ECD. When assayed in flow cytometry studies, saturating concentrations of both the GM-7 and the GM-17 scFv molecules were undetectable on the surface of all four cell lines (IGROV-1, AN3Ca, OVCAR-8 stably transfected
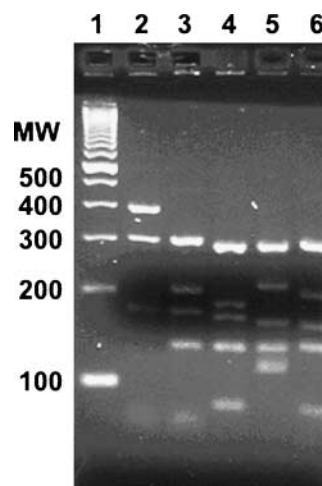

Figure 2. scFv-DNA fingerprinting of monophage ELISA-positive clones. Following ELISA screenings, positive clones were evaluated by PCR fingerprinting to identify potentially unique clones through the presence of distinct restriction digest patterns. *Lane 1,* 100 bp DNA ladder (Nucleic Acid Markers, Invitrogen), sizes indicated on the left (in bp); *lanes 2* to *6,* representative scFv clones with unique restriction digest patterns.
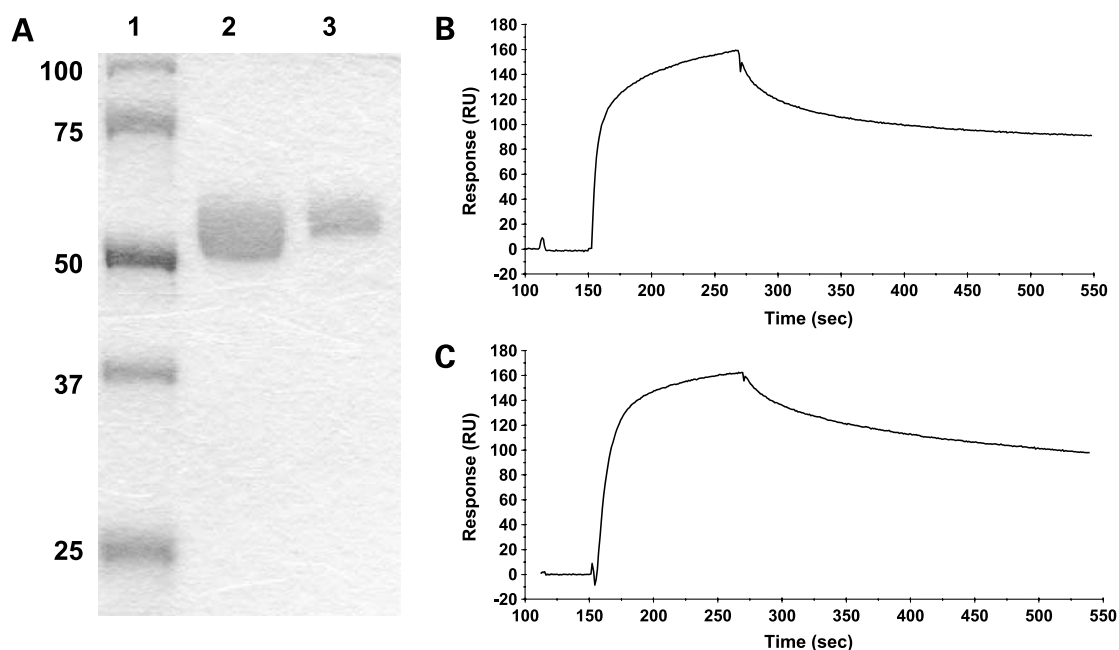
Figure 3. SDS-PAGE and SPR analysis of GM-7 and GM-17 scFv:Fc molecules. Purified GM-7 scFv:Fc and GM-17 scFv:Fc were analyzed by 9% reduced SDS-PAGE (**A**). Molecular weight standards with sizes in kDa indicated (*far left lane*). GM-7 scFv:Fc (*lane 1*) and GM-17 scFv:Fc (*lane 2*). The binding of GM-7 scFv:Fc (**B**) and GM-17 scFv:Fc (**C**) to MISIIR ECD was evaluated by SPR on a BIAcore instrument using a chip coated with MISIIR ECD.

with MISIIR, and MOVCAR-1; data not shown) when an anti-His6 antibody-Alexa488 conjugate was used as a secondary reagent. However, it was possible to detect the binding of both scFv clones by flow cytometry if the anti-His6 antibody-Alexa488 conjugate was preincubated with the scFv molecules to create divalent complexes prior to their application to the target cells. This observation provided the impetus for the creation of the scFv:Fc clones.

In contrast to the results obtained with the scFv forms of the molecules, flow cytometry analysis of the higher avidity GM-7 and GM-17 scFv:Fc midibodies revealed detectable binding to all four cell lines that expressed full-length MISIIR, including modest binding to the MOVCAR-1 cells that express the murine form of MISIIR (Fig. 4). No binding was seen to COS-7 cells that lack MISIIR. As the MISIIR antigen is not expressed at a very high level on tumor cells, the signal in these studies was amplified

through the use of a polyclonal secondary antibody. In these assays, an irrelevant control scFv:Fc midibody specific for murine fibroblast-activating protein (a gift from Dr. Jonathan Cheng of the Fox Chase Cancer Center, Philadelphia, PA) was not detected on any of the four cell lines (data not shown).

Discussion

Here, we describe the production of recombinant MISIIR/Fc domain fusion proteins, their use in the isolation of anti-MISIIR scFv molecules, the generation of divalent anti-MISIIR scFv:Fc molecules and the characterization of their *in vitro* binding properties. MISIIR is a very attractive target for antibody-based detection and treatment of ovarian cancer. Unlike most tumor-associated antigens, it is expressed in a highly restricted manner. During

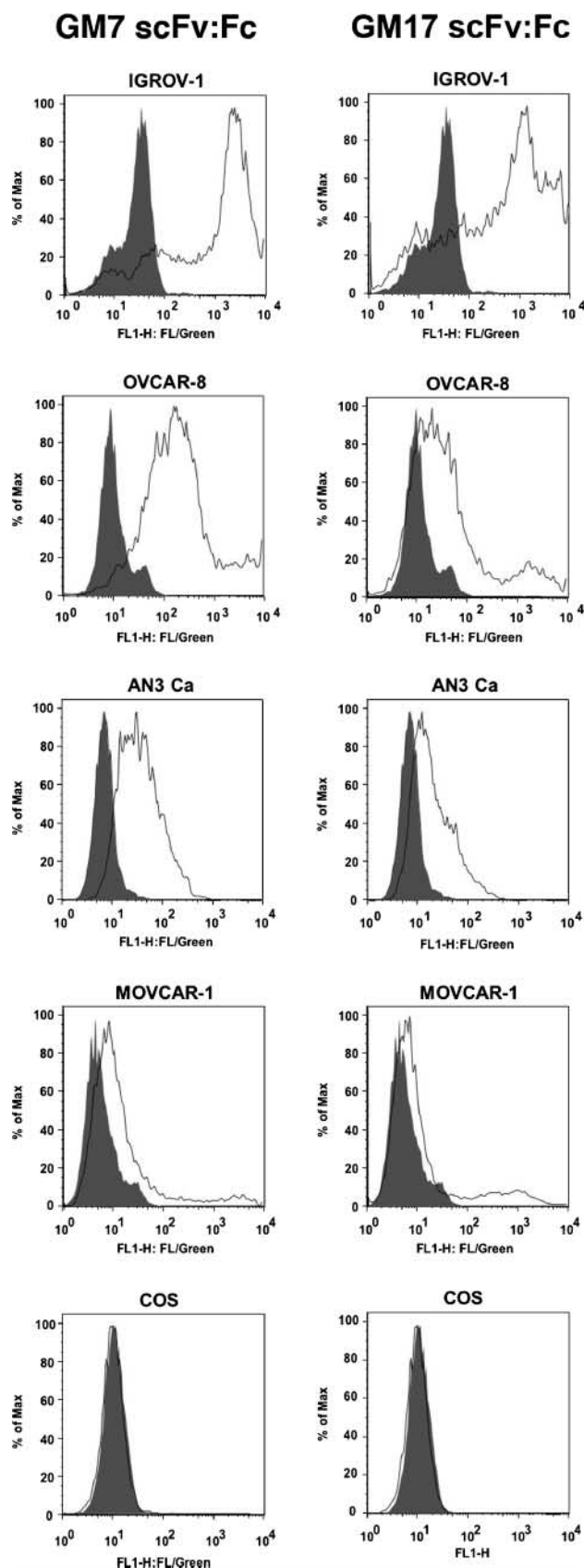**Table 2.** SPR measurements of monovalent binding affinity for MISIIR ECD:Fc

Name	Ka (1/ms)	Kd (1/s)	χ^2	KD (mol/L)	Functional KD (mol/L)
GM-7 scFv	n/a*	n/a	n/a	n/a	—
GM-7 scFv:Fc	9.47×10^4	5.14×10^{-4}	10.9 †	5.4×10^{-9}	1.7×10^{-9}
GM-17 scFv	8.57×10^4	3.30×10^{-3}	3.9 ‡	3.8×10^{-8}	—
GM-17 scFv:Fc	6.23×10^4	1.01×10^{-3}	9.1 ‡	1.6×10^{-8}	6.5×10^{-9}

NOTE: Affinities were determined by SPR as described in the text. Functional (bivalent) affinities are provided for both scFv:Fc molecules.
*Not achieved due to instability.
† <10% of R_{max}.
‡ <5% of R_{max}.

Figure 4. Flow cytometric analysis of anti-MISIIR ECD scFv:Fc molecules. The ability of the GM-7 scFv:Fc (*left*) and GM-17 scFv:Fc (*right*) midibodies to bind to cell lines expressing or lacking MISIIR was evaluated by flow cytometry. The human IGROV-1 and OVCAR-8 (stably transfected with MISIIR) ovarian cancer cell lines, the human AN3Ca endometrial carcinoma cell line, and the mouse MOVCAR-1 cancer cell line all exhibited binding by both scFv:Fc molecules. COS cells lacking MISIIR displayed no binding of either scFv:Fc molecule. Secondary antibody controls are presented as solid dark peaks. The shift associated with the binding of the anti-MISIIR ECD scFv:Fc clones is indicated by the unfilled lines.

embryogenesis, it is present on the mesenchymal cells surrounding the Müllerian ducts and follicular structures of fetal gonads (24), whereas in adults, it is present on tubular, Sertoli and Leydig cells of the testis, and granulosa cells of the ovary (8). In embryogenesis, it plays a major role in the regression of the Müllerian duct, which is one of the first steps in the organogenesis of the male genital tract (reviewed in ref. 25). Its natural ligand, MIS or anti-Müllerian hormone, belongs to the transforming growth factor-β family (26) and acts in a restricted manner on the reproductive organs (11, 27). MISIIR is a member of the transforming growth factor-β receptor family and its expression on the cell surface is down-regulated as a result of ligand-induced receptor internalization (28). The molecular mechanisms underlying signaling through MISIIR and its role in Müllerian duct regression have been studied by a number of groups. Following the binding of MIS to MISIIR, the MIS type I receptor is recruited into the ligand-receptor complex and MIS type I receptor becomes phosphorylated. MIS type I receptor activation, in turn, triggers the phosphorylation of downstream SMADS 1, 5, and 8, which interact with SMAD4. After translocation into the nucleus, the signaling complex either directly or in association with other transcriptional factors, regulates gene expression (29, 30), ultimately leading to regression of the Müllerian duct (31, 32), dissolution of the extracellular matrix (33), and apoptosis (34). The role of MISIIR receptor signaling in development has been studied in transgenic mice that constitutively overexpress MIS. The presence of high levels of MIS in female mice who usually would not express the ligand resulted in Müllerian duct regression during the embryogenic stage of development and complete ablation of ovaries in adults (35).

The potential value of MISIIR as an effective target for antibody-based cancer therapy results from its selective presence on a number of gynecologic malignancies. Functional MISIIR has been reported to be widely expressed on granulosa cell tumors (36), primary ovarian tumors, ovarian cancer cells isolated from ascites, and banked ovarian cancer cell lines (12), thereby making it a promising target for the treatment of ovarian cancer. Ovarian cancer cell lines and primary human ovarian cancer cells from the ascites of patients apoptose following exposure to MIS in a manner resembling that of the fetal Müllerian structures from which they were ultimately derived (37, 38). Recombinant human MIS has also been found to inhibit the growth of ovarian cancer xenografts

growing in immunodeficient mice (39). MISIIR expression has also been detected in breast cancer cell lines (40) and prostate cancer cell lines (41). These cells are also reported to respond to treatment with MIS. Taken together, these studies underscore the potential utility of MISIIR as a therapeutic target.

Based on the reasons described above, we decided to develop anti-MISIIR antibodies for the treatment of ovarian cancer. Antibodies have been successfully exploited to target numerous tumor-associated receptors including epidermal growth factor receptor (42, 43), HER2/neu (44, 45), and CD20 (46) for cancer immunotherapy. Antitumor antibodies have been associated with significant clinical responses when used as single agents (47) and even greater responses when employed in combination with existing therapeutic strategies (48). Although MISIIR is an attractive target, it has not been effectively exploited in cancer therapy. Chicken (8) and rabbit polyclonal antibodies (6) have been generated against a peptide sequence present on the extracellular juxtamembrane domain of the rat type II receptor, which is highly homologous (84.69% homology in nucleic acid) with human MISIIR (6, 49). Mouse monoclonal antibodies have been developed (14) against recombinant human MISIIR ECD expressed in *E. coli* (50). However, these monoclonal antibodies would not be suitable for clinical applications due to their immunogenicity. To the best of our knowledge, the scFv-based molecules reported here are the first human antibodies specific for MISIIR.

Recombinant human MISIIR ECD was cloned for use as a target to isolate reactive scFv clones from a nonimmune human phage display library. Attempts to express the MISIIR ECD protein from transiently transfected mammalian cells failed to yield the desired molecule. Accordingly, we decided to produce MISIIR ECD as a fusion protein with human IgG$_1$ Fc domain which has been widely employed to aid in the expression and secretion of a wide variety of poorly expressing proteins (51, 52). Although it is easier to pan libraries using a pure antigen as a target, selections can be successfully done against fusion proteins if care is taken to rule out the targeting of the carrier protein or unique sequences created at the fusion junction. To address the former concern, we used a competition (subtraction) strategy in which the scFv phage display library aliquot was premixed with a large excess (400 μg) of human IgG$_1$ Fc domain and incubated for 30 minutes at room temperature prior to transferring the phage display library to the immunotubes that had been precoated with a significantly smaller quantity (20 μg/mL) of the MISIIR/Fc fusion protein. In this manner, a large portion of the anti-Fc scFv clones in the library would be precomplexed with Fc, preventing their association with the fusion protein. The latter concern was addressed by creating two different fusion proteins, one with MISIIR at the NH$_2$ terminus of the IgG$_1$ Fc domain, and the other with MISIIR at the COOH terminus of the IgG$_1$ Fc domain. As MISIIR ECD is naturally located at the NH$_2$ terminus of the intact receptor, selections were done using the MISIIR ECD:Fc fusion

protein, which has a similar orientation. This was expected to reduce the potential for steric hindrance imposed by the presence of the Fc domain of the fusion protein on the binding of scFv clones to the region of the ECD around the ligand binding site. A reactive clone was then considered to be specific for MISIIR ECD if it bound to both orientations of the fusion protein but not to the Fc domain alone in a monophage ELISA and by SPR analysis on the BIAcore instrument. The SPR analysis was also used to provide an initial indication of binding strength. By using this strategy, we were able to isolate several scFv clones with the desired specificity for MISIIR ECD. Undoubtedly, it is likely that additional positive clones were discarded as our selection criteria required the ability to bind to both fusion protein orientations and the Fc domain could have obscured different MISIIR epitopes on the two fusion proteins.

Seven unique scFv clones were identified that bound to both forms of the MISIIR FC fusion proteins in ELISA assays. All seven exhibited binding by SPR to MISIIR:Fc (the orientation used in the selection procedure) immobilized on sensor chips. When the clones were assayed for their ability to bind to Fc:TEV:MISIIR ECD (the orientation not used in the selection procedure), a significant decrease in binding strength was observed for four of the seven clones, suggesting weaker or negative binding. Based on these observations, we postulated that three of the scFv clones (GM-7, GM-23, and GM-29) recognized epitopes that were structurally independent of the Fc domain. Affinity determination studies on the BIAcore revealed that the GM-17 scFv bound to MISIIR ECD with an affinity of 3.8 × 10^{-8} mol/L. Attempts to determine the affinity of the GM-7 scFv revealed that this clone was unstable when stored at 4°C, precluding measurement of an accurate affinity.

Both the GM-7 and GM-17 clones were assayed by flow cytometry for their ability to bind to cell lines known to express MISIIR. The IGROV-1 human ovarian carcinoma cell line and the AN3 Ca human endometrial carcinoma cell line that naturally express moderately high levels of human MISIIR (23) were used for this purpose. In addition, we also examined the ability of the clones to recognize OVCAR-8 cells that were stably transfected with full-length human MISIIR and murine MOVCAR-1 epithelial ovarian tumor cells isolated from ovarian tumors that spontaneously developed in tgMISIIRTAg transgenic mice that express the SV40 T antigen under control of the MISIIR promoter (53). Both of these cell lines are reported to undergo apoptosis in response to stimulation with MIS.

Although the scFv forms of the molecules exhibited significant binding by SPR, binding of both the GM-7 and GM-17 scFv molecules was not detectable when assayed by flow cytometry. This observation was consistent with our experience with other moderately low-affinity scFvs specific for a variety of cell surface antigens and may also reflect the poor stability of the GM-7 scFv (data not shown). As low affinity can be overcome by increasing the valance of the interaction, we employed an antibody-based dimerization strategy similar to that initially reported by Wang et al. in which an anti-FLAG tag monoclonal antibody was

used to increase the avidity of a FLAG-tagged scFv (54). In this case, anti-His6 monoclonal antibody was added to the anti-MISIIR scFv molecules at a 1:2 molar ratio to preform immune complexes prior to their addition to the cells. Using this strategy, both the GM-7 scFv and the GM-17 scFv revealed positive shifts in the mean fluorescence intensity in the flow cytometry assays. This observation served to both validate the specificity of the anti-MISIIR scFv molecules and indicate that substantial improvements could be achieved in targeting if the scFv were engineered into a format capable of divalent binding.

The scFv:Fc structure was selected over other dimeric scFv-based molecules (e.g., diabody or minibody) as it incorporates a fully functional human IgG Fc domain that is capable of mediating an antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity and prolonged retention in circulation due to interactions with FcRn (55). *In vitro* characterization of the two scFv:Fc midibody molecules revealed that both were capable of specific binding to MISIIR ECD. Interestingly, re-engineering the GM-7 into an scFv:Fc molecule significantly increased its stability at $4°C$, such that it was still intact and functional for at least 30 days allowing its affinity to be accurately measured. These experiments revealed that GM-7 scFv:Fc had a monovalent affinity of 5.4×10^{-9} mol/L and a functional affinity of 1.7×10^{-9} mol/L, representing an ~3-fold improvement. A 6-fold increase in functional affinity accompanied the conversion of the GM-17 scFv to the scFv:Fc format; 3.8×10^{-8} versus 6.5×10^{-9} mol/L, for the monovalent and divalent forms, respectively. Both the GM-7 and the GM-17 scFv:Fc molecules were observed to bind to MISIIR-positive cells by flow cytometry. In these assays, the presence of an intact Fc domain provided a target for signal amplification using polyclonal anti-human Fc domain antibodies. Interestingly, we observed that both scFv:Fc molecules bound to a significant but modest degree to the mouse MOVCAR-1 cell line that expresses the murine form of MISIIR. Although this is not completely surprising, as human and mouse (and rat) MISIIR have ~80% of amino acids in common in their ECDs (6), this cross-reactivity could form the basis for the clinically relevant preclinical evaluation of these anti-MISIIR antibodies in transgenic mice that spontaneously form murine MISIIR-positive ovarian tumors (53).

The limited success of current treatments for ovarian cancer has resulted in a critical need for new therapeutic strategies. Antitumor antibodies targeting unique and functional ovarian cancer–associated antigens offer an attractive alternative. Such antibodies could directly alter the growth properties of MISIIR-positive tumor cells in a manner similar to MIS ligand–mediated regression of the Mullerian duct during fetal development, or by redirecting components of the immune system to elicit antibody-dependent cell-mediated cytotoxicity or complement-mediated cytotoxicity (56). As the latter two mechanisms are dependent on the presence of a functional Fc domain, the scFv:Fc molecules described here could be ideal candidates for this approach. Alternatively, engineered

anti-MISIIR antibody fragments could serve as effective vehicles for the selective delivery of either cytotoxic agents (e.g., radioisotopes, catalytic toxins, or drugs) for the treatment of these tumors or diagnostic radionuclides for tumor detection via positron emission tomography or gamma camera imaging (57). We are currently evaluating the scFv:Fc molecules described here for the ability to directly induce growth inhibitory effects and indirectly inhibit tumor cell growth by targeting cytotoxic agents to ovarian tumor cell lines that express MISIIR. In conclusion, we have described the production of two recombinant human MISIIR-ECD fusion proteins, the isolation of scFv molecules reactive with this novel target and their use in the construction of bivalent scFv:Fc molecules. Two of the clones exhibit highly specific binding to MISIIR, both the recombinant ECD and the native molecule expressed on the surface of human ovarian cancer cells. These first fully human anti-MISIIR antibodies may prove useful for the detection and treatment of ovarian cancer.

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