Human Ovarian Cancer, Cell Lines, and Primary Ascites Cells Express the Human Mullerian Inhibiting Substance (MIS) Type II Receptor, Bind, and Are Responsive to MIS

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ABSTRACT

Six human ovarian cancer cell lines and samples of ascites cells isolated from 27 patients with stage III or IV ovarian papillary serous cystadenocarcinoma were studied individually to test whether recombinant human Mullerian inhibiting substance (rhMIS) acts via its receptor. To do these experiments, we scaled up production of rhMIS and labeled it successfully with biotin for binding studies, cloned the human MIS type II receptor for mRNA detection, and raised antibodies to an extracellular domain peptide for protein detection. These probes were first tested on the human ovarian cancer cell lines and then applied to primary ovarian ascites cells. rhMIS inhibited colony growth of five of six cell lines that expressed the human MIS type II receptor mRNA by Northern analysis while not inhibiting receptor-negative COS cells. Flow cytometry performed on MIS-sensitive ovarian cancer cell lines demonstrated specific and saturable binding of rhMIS (Kd = 10.2 nM). Ascites cells from 15 of 27 or 56% of patients tested bound biotinylated MIS (MIS-biotin) and, of the 11 that grew in soft agarose, 9 of 11 or 82% showed statistically significant inhibition of colony formation. Of the 15 patients who bound biotinylated MIS, mRNA was available for analysis from 9, and 8 of 9 expressed MIS type II receptor mRNA by reverse transcription-PCR, showing a statistically significant correlation, compared with binding, by χ² analysis (P = 0.025). Solid ovarian cancers were positive for the MIS type II receptor protein by immunohistochemical staining, which colocalized with staining for antibody to CA-125 (OC-125). Thus, the detection of the MIS type II receptor by flow cytometry may be a useful predictor of therapeutic response to MIS and may be a modality to rapidly choose patients with late-stage ovarian cancer for treatment with MIS.

INTRODUCTION

The most common human ovarian cancers are derived from coelomic epithelium; the histology of these tumors resembles the fetal Mullerian structures that form in the embryo by invagination of the coelomic epithelium and that regress in the male embryo under the influence of MIS3 (1, 2). MIS is produced in fetal and postnatal testes at high levels that persist until puberty, after which, the concentration of circulating MIS drops to low levels, where it persists throughout adult life (3–6). In contrast, MIS is undetectable in the fetal and early postnatal ovary or in serum but becomes detectable in the developing antral follicles of the ovary and in the serum of the adolescent and adult female (3, 7–9). The concentration of MIS persists at low levels until menopause, after which it drops to undetectable levels (3, 6). The function of MIS in the adult gonads of either sex has not yet been fully elucidated, but evidence exists for inhibition of meiosis of rat oocytes (10), inhibition of growth and progesterone production by human granulosa cells (11), and suppression of growth and of steroidogenesis in Leydig cells (12–14). Because in the male fetus the Mullerian ducts, the anlagen for the uterus, Fallopian tubes, and vagina, regress under the influence of MIS, we examined whether the common human Mullerian-derived ovarian cancers are responsive to MIS through a receptor-mediated process. Recent cloning of the MIS type II receptor (15–18) has made such an analysis possible.

MIS signals through a heteromeric receptor, one partner of which is the MIS type II receptor, which was found to share homology with the TGF-β and activin type II receptors (15–18) as a conserved single transmembrane serine/threonine kinase.

3 The abbreviations used are: MIS, Mullerian inhibiting substance; rhMIS, recombinant human MIS; TGF, transforming growth factor; RT-PCR, reverse transcription-PCR.
As with other members of the TGF-β family of receptors, the MIS type II receptor is assumed to form a complex with a type I receptor that, as a class, are homologous to type II receptors in the kinase domain (19). In this complex, the type II receptor renders specificity by selective ligand binding, and phosphorylation of the type I receptors by the type II receptors initiates downstream signaling. In situ hybridization shows that MIS type II receptor mRNA is developmentally expressed in the urogenital ridges of both sexes in the epithelium and mesenchyme of the Mullerian ducts, on the coelomic epithelium, and in the tubules of embryonic gonads (15, 16, 18). Northern analysis of a variety of tissues shows high expression of the MIS type II receptor in the gonads, urogenital ridge, and uterus (18). Inactivation of MIS (20) or its type II receptor (21) by homologous recombination results in males with retained Mullerian ducts, Leydig cell hyperplasia, and Leydig cell tumors. In female transgenic mice, overexpression of MIS results in regression of the Mullerian ducts in the embryo and complete ablation of the ovaries in the adult (22), suggesting that the ovaries could be a target for MIS action and express MIS receptors. To investigate this possibility, we examined ovarian cancer cell lines and primary human ovarian cancer ascites cells using tools and probes developed in this laboratory. We scaled up production of rhMIS, successfully labeled it with biotin for binding studies, cloned the human MIS type II receptor for mRNA detection, and raised antibodies to the type II receptor for protein detection. After careful validation, we found that the cell lines and primary ascites cells that functionally responded to MIS by inhibition of growth express the MIS type II receptor and bind MIS. These results suggest that human ovarian cancers may be therapeutic targets for MIS.

MATERIALS AND METHODS

Cell Culture in Monolayer and Suspension. The Mullerian-derived human ovarian carcinoma cell lines OVCAR3, OVCAR8, OV1063, IGROV-1, and SKOV3 and the negative control COS-1 (SV40-transformed, non-Mullerian-derived African Green Monkey kidney) cells were obtained from American Type Cell Culture. OVCAR5 was a kind gift from Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA). All cells were grown in 10% MIS-free female FCS (Aries Oncology Service, Richardson, TX) supplemented with penicillin and streptomycin (100 U/ml) and 1-glutamine in 5% CO₂ at 37°C. The medium used varied with the cell line as follows: OVCAR3, OVCAR5, and OV1063 were grown in RPMI; COS, OVCAR8, and IGROV-1 used DMEM; and SKOV3 cells were maintained in McCoy’s 5A. Only OVCAR5 cells could also be adapted for growth in suspension in roller bottles.

Study Subjects. Ascites from 27 patients, who ranged in age from 21 to 91 years (mean, 61 years) with stage III or IV ovarian cystadenocarcinoma, were studied, and all but four were postmenopausal. The study was approved by the Subcommittee on Human Studies of Massachusetts General Hospital, and consent was obtained from each patient in our Gynecological Oncology Service.

Purification of Bioactive rhMIS and a Bioinactive Mutant (L9). Wild-type rhMIS or the mutant MIS (L9) was purified by immunoaffinity chromatography from MIS gene-transfected Chinese hamster ovary cells as described (23–25). The protein was quantified by the Bradford assay (26), and its bioactivity was measured in the MIS-specific fetal rat organ culture bioassay (27). L9 is not biologically active (28) and is the negative protein control for active ligand. The Massachusetts General Hospital Institutional Animal Care and Use Committee approved all animal studies.

Nonadherent Colony Growth of Ascites Cells and Ovarian Cancer Cell Lines in Semisolid Media. All of the human ovarian cancer cell lines, the negative control COS cells, and ovarian cancer ascites cells were cultured in agar as a semisolid support in 30-mm-grid dishes in quadruplicate using a method described previously (29, 30) in which OVCAR3 (31) had been shown to be responsive and SKOV3 to be unresponsive to MIS (32) to test whether MIS can inhibit their growth. The number of cells plated in these studies varied from 10,000 to 50,000 for the cell lines to 125,000 to 250,000 for the ascites samples. Increasing doses of MIS from 3.5 to 107 nM, the inactive preparation (L9) at 71 nM, or vehicle control were added to the cells. Seven to 14 days after plating, colonies measuring >4 μm in diameter and only those in the mid-cruciate pattern of 81 squares (to prevent edge effects) were counted.

Adherent Colony Growth of Ovarian Cancer Cell Lines Transfected with MIS. OVCAR8 and IGROV-1 were also grown as colonies on a solid plastic support, after stable cotransfection with a plasmid conferring drug resistance (hygromycin), and either vector DNA or an MIS construct cloned into pcDNA 3.1(+) (Invitrogen, Carlsbad, CA) vectors. Transfected cells were grown in hygromycin for 2 weeks and stained with crystal violet to measure colony growth and MIS secreted into the media measured by a specific ELISA (3). The MIS construct used in this assay was the readily cleavable MIS-RR bioactive isoform generated in this laboratory (33). A mutant MIS that lacks the leader sequence, and thus cannot be secreted, was used as a negative control.

Construction and Screening of a Human Fetal Testis Library for the MIS Type II Receptor. A human fetal testis cdNA library was prepared in lambda UniZap II (Stratagene, La Jolla, CA). Total RNA was extracted from the testes of fetuses, 17–22 weeks of age, by homogenization as described (34), from which mRNA was purified using the PolyAtract system (Promega Corp., Madison, WI). cdNA sized from 1 to 5 kb was ligated into UniZap XR vector arms and packaged with Gigapack Gold generating >5 × 10⁶ independent clones, of which 1.5 × 10⁵ were amplified.

Screening was done by hybridization (34) with a radiolabeled fragment generated by PCR using conserved type II receptor-specific primers (17) at 42°C in 50% formamide with 2.4 × 10⁶ clones on replicate nitrocellulose filters. Two overlapping human type II receptor cdNAs in pBluescript were excised from lambda phase clones, combined, sequenced (35), and deposited in GenBank with accession number AF172932.

Analysis of Primary Ascites Cells from Ovarian Cancer Patients. Ascites was obtained from 27 patients undergoing laparotomy or ultrasound-guided paracentesis. The fluid was placed on ice and centrifuged to isolate the cellular component that was resuspended in media (CMRL-1066). Hypotonic lysis and sedimentation were used to remove erythrocytes. Cells were counted using a Coulter counter and aliquoted for analysis of
MIS binding, detection of the type II receptor mRNA by RT-PCR, and assessment of colony growth in soft agarose with or without MIS. Whenever sufficient cells (7 \times 10^6) were available, flow cytometric analysis of ascites included staining for epithelial tumor and/or mesothelial cell populations (cytokeratin; Becton Dickinson, San Jose, CA) or CA-125 (using OC-125 antibody) and for hematogenous cells (using anti-CD3 or CD45 antibodies). In each MIS binding study, the hematogenous population served as an internal negative control, whereas cytokeratin identified the epithelial population, which should contain the tumor cells and was present in all of the 27 patients samples tested. If fresh ascites was available within 4 h of collection, an aliquot of cells (10^9) was placed in guanidinium thiocyanate for RT-PCR using primer sets that spanned the extracellular domain of the MIS type II receptor. If the collection provided additional cells (7 \times 10^9), cells were plated in soft agarose in quadruplicate at 250,000 cells/plate to examine colony growth in the presence or absence of rhMIS.

**PCR Amplification of MIS Type II Receptor from Ovarian Cancer Cell Lines and Patient Ascites Cells.** Total cellular RNA was isolated using guanidine hydrochloride/SDS or guanidinium thiocyanate from human ovarian cancer cell lines OV1063, OVCAR3, and OVCAR5, patient ascites cells, the monkey kidney cell line COS-1, and from testis of a 15-year-old child. cDNA synthesis was carried out at 42°C using random hexamers, deoxynucleotide triphosphates, RNasin, and SuperScript II reverse transcriptase (Life Technologies, Inc., Long Island, NY). PCR amplification of the entire MIS type II receptor in ovarian cancer cell lines was carried out for 30 cycles out using type II receptor specific primers (sense, 5'-CTTCCAGAAGATGCTAGGGTCT-3' at the beginning of exon 1; and antisense, 5'-CTGGATAATTTACACAGGAGA-3' at the end of exon 11) and the PCR products analyzed on a 1% agarose gels. To determine whether the PCR fragment was identical to the MIS type II receptor DNA, RNA was analyzed by a modified Southern analysis (34) using a radiolabeled 32P-random primed cDNA probe specific for the human type II receptor. The blot was prehybridized for 4 h at 42°C with a solution containing 50% formamide, 1 M NaCl, 0.5% SDS, 50 mM Tris (pH 7.5), and 100 μg/ml salmon sperm DNA. PCR to detect exons 2 through 6 of MIS type II receptor (17) in ascites cells was performed using MIS type II receptor-specific primers labeled 1 and 4 as follows: 1 is at the 5' end of exon 2 (5'-TTTTGAGGCCTGGAGTGG-3'); and 4 is at the 3' end of exon 6 (5'-CTGGTG-TGCTTGGAGCCTGTCCG-3'). Shorter fragments encompassing exons 2, 3, and 4 were detected by combining primer 1 with primer 3 at the 3' end of exon 4 (5'-AGGAGCAGCAGGACTTTG-3'). Exons 4, 5, and 6 could be observed by combining primer 2 at the beginning of the exon (5'-CTCTCCATATCGATGGAGCCTGT-3') with primer 4 for the PCR reactions. All of these primers and those used for human cytokeratin 8 (forward, CTGCCATCTCTGTGGTGTTGTTGTTGTT; and backward, ACCGCCATAGCAGCAGCTTT) and cytokeratin 18 (forward, GCCATGGGGGGGAGGCTTG; and backward, GGCCTTTTACTCCTCTTGCTG) were synthesized by the Massachusetts General Hospital Molecular Biology Core Laboratory.

**Northern Analysis of Ovarian Cancer Cell Lines Using a Human MIS Type II Receptor Probe.** Total RNA was isolated from all of the ovarian cancer cell lines and COS cells using RNA-Stat 60 according to the instructions provided by the manufacturer (Tel Test, Friendswood, TX). Poly(A)+ RNA selection was done using FastTrack 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA), according to the protocol provided, and 3–4 μg of poly(A)+ RNA were separated on a 1% agarose gel and transferred onto Nytran membrane. The blot was probed with radiolabeled human MIS type II receptor cDNA.

**Preparation of an MIS Type II Receptor Extracellular Domain Antibody.** Two antibodies were developed against a keyhole limpet hemocyanin-conjugated synthetic type II receptor peptide of the rat receptor (from amino acids 111–127, C-G-T-D-F-C-N-A-N-Y-S-H-L-P-P-S-G), which differs from the human type II receptor by only one amino acid (P to S). Bold S denotes which of the two in the sequence differs between rat and human receptors. This unique sequence was selected from the extracellular region of the type II receptor near the transmembrane domain because of its favorable antigenicity profile (MacVector 5.0; Oxford Molecular, Campbell, CA). One antibody was raised in chickens (Covance, Denver, PA), and the IgY fraction was purified with Eggstract (Promega). Another antibody was raised in a rabbit and purified with protein A-Sepharose. Specificity of the antibodies to their cognate peptide was documented by dot blot analysis and immunohistochemical staining (1:100 dilution of primary antibody) of the 15½-day male and female embryonic urogenital ridge (36, 37) and to liver, stomach, pancreas, lung, and small intestine as negative control tissues using methods described earlier. Staining was also tested in four different primary human ovarian tumors. The secondary donkey antichicken or antirabbit antibody conjugated to horseradish peroxidase was purchased from Vector Labs (Burlingame, CA).

**Western Analysis and Flow Cytometry to Detect MIS Type II Receptor in Transfected Cells and Cancer Cell Lines.** To validate the specificity of the chick MIS type II receptor antibody, the MIS type II receptor was overexpressed in COS cells after transfection with a pCMV-6 fusion construct containing the wild-type MIS type II receptor (pCMV-6-MIS-II). To prepare lysates, cells were washed once in cold PBS, scraped off the plate, and centrifuged at 400 × g for 5 min at 4°C. The pellet was resuspended in 50 μl of RIPA [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% deoxycholate, and 0.1% SDS] lysis buffer, and protein concentration was determined by Bradford analysis (26). One hundred μg of protein were reduced with β-mercaptoethanol, separated on 10% SDS polyacrylamide gels, and transferred to nitrocellulose. The blots were blocked with 10% w/v Carnation instant milk, incubated with 1:500 to 1:1000 dilutions of chicken or rabbit anti-MIS type II receptor antibody or preimmune serum for 2 h, and washed prior to the addition of a 1:1000 to 1:3000 dilution of the species-specific reporter antibody conjugated to horseradish peroxidase. Reactive protein bands were detected using ECL. In addition, an expression construct (pCMV-8/MISrII) was made with a Flag tag (“DYKDDDDK”) at the NH2 terminus of the rat MIS type II receptor. This epitope was recognized by a 1:2000 dilution of the mouse monoclonal antibody M2 (Anti-Flag M2; Eastman Kodak, St. Louis, MO). Endogenous MIS type II receptor pro-
tein in OVCAR3, OVCAR5, OVCAR8, and IGROV-1 cells was detected by Western analyses in essentially the same manner as the overexpressed cloned protein, except that Immobilon membranes were used and blocking was done with 30% milk. Untransfected COS cells were negative controls. Parallel blots were incubated with anti-receptor antibody preincubated with a 2-fold excess of the peptide antigen overnight. For detection of expressed receptor by flow cytometry, transfected cells were incubated with either the chicken MIS type II receptor antibody or the mouse IgG anti-Flag antibody, and the complexes were detected with either an antimouse or antichick FITC IgG conjugate (both detected at 520 nm).

**Biotinylation of rhMIS.** rhMIS was conjugated with biotin using a 40-fold molar excess NHS-LC biotin (Pierce) in PBS on ice for 30 min at neutral pH. Unreacted biotin was quenched with 100 mM Tris, and MIS-biotin was separated from unconjugated MIS using an avidin affinity column (Ultralink; Pierce). Elution fractions were analyzed for total protein (26) prior to bioassay and Western analyses, and the degree of biotinylation of MIS was measured as described earlier (38). No difference in biological activity of MIS was observed when results of 33 assays with MIS-biotin were compared with 133 assays of unconjugated protein at doses of 1, 2, 3, 4, and 5 µg/ml. Avidin affinity chromatography, therefore, essentially eliminated the possibility that bioactive, nonbiotinylated rhMIS was contaminating the MIS-biotin preparations. The labeled MIS was then used to detect binding, first to MIS-sensitive cell lines, and subsequently to ascites cells harvested from patients with advanced ovarian cancer.

**rhMIS Binding Assay on Ovarian Cancer Cell Lines and Ascites Cells.** OVCAR5 cells were cell cycle synchronized by four consecutive passages and after reaching 80–90% confluence were grown in suspension culture and were harvested by centrifugation at 3- to 5-day intervals. The cell pellet was resuspended in binding buffer (0.5% BSA, 150 mM NaCl, 10 mM MgCl₂, 10 mM KCl, and 20 mM HEPES, pH 7.4) at 10⁶ cells/ml. OVCAR8, IGROV-1, SKOV3, and COS cells grown in monolayer were harvested by incubation with trypsin-EDTA, centrifuged, and resuspended in binding buffer at 10⁶ cells/ml. MIS-biotin was added to cells, with or without excess unconjugated MIS, and incubated for 2 h at 4°C to avoid receptor internalization. Multiple replicates for 12 serial dilutions of MIS-biotin were used in each experiment. COS cells, which do not express MIS type II receptor, were used as a negative control. The samples were washed and resuspended in PBS, and 7 µg of avidin phycoerythrin and 10 µg of anti-cytokeratin-IgG FITC or OC-125 and mouse-anti-human-IgG-FITC were added for 30 min in the dark, on ice. The samples were washed, resuspended in PBS, and analyzed by flow cytometry with a Becton Dickinson FACScan flow cytometer (488 nm for excitation, and detection at 585 nm ± 20). Propidium iodine-positive (dead cells) were assessed in a separate aliquot and excluded from these analyses. The LYSIS software (Becton Dickinson) generated a frequency of distribution histogram of cell number versus fluorescence/cell. The arithmetic mean fluorescence/cell was plotted against concentration of MIS-biotin added to generate binding curves. The concentration of MIS-Biotin needed to reach 50% saturation of binding sites is the dissociation constant of the MIS-receptor complex. Nonspecific binding of rhMIS biotin is that remaining in the presence of 750 nM unbiotinylated rhMIS. Nonspecific binding is subtracted from total binding to estimate specific binding levels.

MIS-biotin binding assays on ascites cells were done in the same manner as the cell lines, except that erythrocytes were hypotonically lysed prior to the assays; binding incubations were for 12 h at 4°C, and up to six serial dilutions of MIS-biotin were used.

**Statistical Considerations.** The sensitivity of MIS flow cytometry MIS binding assays on ascites cells, defined as the ability to detect the receptor when the message is also detected by PCR, was determined by comparing the results of the two techniques using a 2 × 2 contingency table. Specificity of binding, or the ability to detect the absence of receptor, was determined in the same manner. If PCR is used as the predictor of MIS type II receptor expression, the predictive value for the MIS binding in detecting the receptor mRNA was calculated by determining the total number of patients in which both binding and mRNA were detected (n = 8), divided by total number of positive PCR results (n = 10). Correlation between the tests, binding, RT-PCR, and colony inhibition assays was tested by χ² analysis. Because the replicate experiments could be done on the cell lines, Student’s t test was used to determine statistical significance.

**RESULTS**

**Determination of MIS Responsiveness.** Before evaluating the ovarian cancer ascites cells, a series of ovarian cancer cell lines were studied to determine whether they respond to MIS and could serve as controls for future experiments. To assess responsiveness of the Mullerian-derived ovarian cancer cell lines to MIS, colony growth of the human ovarian cancer cell lines OVCAR3, OVCAR5, OVCAR8, OV1063, IGROV-1, and SKOV3 was compared with colony growth of the non-Mullerian COS cells. Cells were plated in soft agar to produce 200–600 nonadherent colonies in 7–14 days in the control untreated cultures. Ten to 15 µg/ml (71–107 nM) of MIS produced maximal inhibition that was not observed with 71 nM of the inactive MIS mutant, L9. Greater than 50% inhibition was achieved in >81% of colony inhibition experiments (n = 33; Table 1). These results compare favorably with results observed
previously in human Mullerian and ocular melanoma cell lines, which were inhibited at similar concentrations of MIS in vitro (28, 31, 37, 39). Neither COS nor SKOV3 cells responded to MIS in this antiproliferative assay, which in the case of SKOV3 had been observed previously (31, 32). When response to 15 μg/ml of MIS was compared between cell lines, OVCAR5 and OVCAR8 were the most sensitive to MIS, which caused nearly complete inhibition of growth (Fig. 1A). Furthermore, inhibition of OVCAR8 growth was dose dependent over a range of 0.5 to 15 μg/ml (3.5–107 nM), which was not observed with vehicle control (not shown). The expression of a wild-type MIS cDNA construct in transfected OVCAR8 cells results in the secretion of rhMIS, as detected by ELISA (3) at 48 h, and growth of fewer colonies when compared with OVCAR8 cells transfected with either an empty vector (pcDNA) or an MIS leaderless mutant cDNA construct (pcDNA-K1) that fails to secrete MIS (not detected by ELISA; Fig. 1B). The number of adherent colonies was inhibited by 89.7% ± 8% (n = 3). The number of adherent colonies of similarly transfected IGROV-1 cells was inhibited by 50% (data not shown).

Because MIS inhibited the growth of established ovarian cancer cell lines, we tested it against the primary ascites cells of patient’s known to have ovarian cancer as the cause of their ascites. Only ascites preparations that were positive for cytokeratin, and thus could contain tumor cells, were analyzed. MIS was found to mediate inhibition of growth of ovarian cancer ascites cells in stem cell assays. Of the cells of patients that bound MIS-biotin (15 of 27 or 56%) and exhibited sufficient colony formation in the untreated controls (11 of 15), 9 of 11 (82%) showed a statistically significant functional response to MIS (Table 2). Of the 12 that did not bind MIS, 9 had no growth or insignificant inhibition; the remaining 3 were inhibited in colony inhibition assays.

Detection of the MIS Type II Receptor Message. The cell lines were then studied for the presence of MIS type II receptor cDNA, which was isolated using a 5′ probe. cDNA was synthesized from total cellular RNA isolated from the ovarian cancer cell lines OVCAR3, OVCAR5, and OV1063. cDNA amplified by PCR using MIS type II receptor-specific primers, designed from the extreme 5′ and 3′ end of the coding region of the receptor cDNA, produced a single 1.7-kb fragment, the expected size for the full-length MIS type II receptor (Fig. 2A). A modified Southern blot analysis of the PCR-amplified products showed that a radiolabeled DNA probe, specific for human MIS type II receptor, hybridized to the 1.7-kb band (Fig. 2B). Northern analysis of poly(A)1 RNA isolated from OVCAR3, OVCAR5, OV1063, OVCAR8, and SKOV3 demonstrated the expression of a 2-kb MIS type II receptor mRNA in all five cell lines (Fig. 2C). In a separate experiment, poly(A)1 RNA from the sixth cell line, IGROV-1, hybridized to the MIS type II receptor mRNA band at 2 kb, which at the same time was undetectable in COS cells, which failed to respond to MIS in antiproliferative assays (not shown). SKOV3 cells, which failed to respond to MIS, expressed the
Patient characteristics and experimental results

Ascites fluid was collected from 27 patients with stage III or IV ovarian cancer. All 27 cases contained cells that bound cytokeratin. Assays were prioritized, depending upon the number of cells obtained. Binding of MIS-biotin was always to the cytokeratin-positive epithelial tumor population; cells from 15 of 27 patients (56%) exhibited MIS-biotin binding (+). RT-PCR was performed only if cells could be studied immediately; hence, the number of patients available for this analysis is smaller. Of the 15 patients who exhibited binding, 9 were analyzed for exons 2–6, which include the extracellular domain of the MIS type II receptor mRNA, and 8 of 9 expressed the message (+). The two patients that did not bind MIS but were positive by RT-PCR may reflect the sensitivity of PCR-based assays. The ascites cells of 11 of the patients who bound MIS were used in antiproliferation assays. Inhibition of colony growth was seen in 82% of these patients.

<table>
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<th>Patient no. (not sequential)</th>
<th>Binding of MIS-biotin to ascites cells (+/−)</th>
<th>MIS type II receptor message in ascites cells (+/−)</th>
<th>Colony inhibition of ascites cells</th>
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<tr>
<td>1</td>
<td>+</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>65% &lt;0.05</td>
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<td>4</td>
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<td>+</td>
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aN, insufficient cells remained for additional study, or in the case of RT-PCR analysis, that the sample was received >4 h after collection.

b Cells did not form colonies in soft agar and, therefore, could not be evaluated.

c, statistical tests not performed because cells did not grow.

d NS, not significant.

Native MIS type II receptor gene (Fig. 2C). cDNA was synthesized from total cellular RNA isolated from the ovarian cancer cell line OVCAR8, sequenced, and found to be full-length and identical to the clones isolated by Imbeaud et al. (17).

Extending this analysis for message to the primary ovarian cancer ascites cells, mRNA was available for analysis from the mixed population of ascites cells from 9 of the 15 patients whose cells bound ligand. Total RNA was extracted, and RT-PCR was performed using oligonucleotide primers unique to the extracellular domain of the MIS type II receptor. These primers detected mRNA fragments of appropriate sizes for exons 2–4 (454 bp), 4–6 (431 bp), or 2–6 (885 bp) from the ascites cells of eight of nine (89%) of the patients whose cells bound MIS-biotin. mRNA was present in two of the nonbinders, which may reflect the increased sensitivity of the PCR-based assays. These cDNA fragments, shown in a representative experiment using cDNA from patient no. 3, were of a size similar to those detected from human fetal testis cDNA from which the MIS type II receptor was cloned (Fig. 2D). A sufficient number of cells were available from 27 patients to perform MIS binding assays, and ascites cells from 15 of the 27 (56%) patients bound rhMIS (Table 2). RT-PCR detection of type II MIS receptor transcripts, however, could be completed in only 15 cases: 9 from MIS binders, and 6 from MIS nonbinders. MIS type II receptor transcripts were detected in eight of the nine binders, whereas only two of the six nonbinders expressed measurable receptor. The positive predictive value of the MIS binding to detect the presence of MIS type II receptor in cells in the ascites fluid is 89% (eight of nine). Using ² analysis, there was a statistically significant correlation between MIS binding and expression of the MIS type II receptor mRNA (P = 0.025). Alternatively, there is 67% agreement among the receptor-negative cases (four of six tested), i.e., no binding correlates with no detection of receptor mRNA.

Detection of MIS Type II Receptor Protein Using the Anti-MIS Type II Receptor Antibodies. Chicken and rabbit MIS type II receptor antibodies were developed to the peptide sequence from the extracellular juxtamembrane domain of the rat type II receptor (15, 18) to confirm the presence of the MIS type II receptor protein on the surface of the cells that respond...
Fig. 2 Expression of the MIS type II receptor mRNA in ovarian cancer cells. A, detection of PCR amplified full length (1.7 kb) MIS type II receptor from OVCAR3, OVCAR5, and OV1063 cDNAs. Left, size markers. B, to determine whether the fragments generated by PCR were identical to the MIS type II receptor, DNA was analyzed by a modified Southern analysis using a radiolabeled $^{32}$P-random primed cDNA probe specific for human MIS type II receptor. Left, size markers. C, Northern analysis of MIS type II receptor mRNA expression in ovarian cancer cell lines. Three μg of poly(A)$^+$ RNA from OVCAR3, OVCAR5, OV1063, OVCAR8, and SKOV-3 ovarian cancer cell lines were analyzed by Northern blot using a radiolabeled probe against the human MIS type II receptor. The migration of the 2-kb molecular weight marker and 18S RNA are indicated. D, MIS type II receptor in human ovarian cancer ascites cells cDNA. PCR-amplified DNA fragments of the MIS type II receptor were detected in this representative sample (patient no. 3) at the expected size for exons 2–4 (454 bp; primers 1 and 3), exons 4–6 (431 bp; primers 2 and 4), and exons 2–6 (885 bp; primers 1 and 4). cDNA from human fetal testes was used as a positive control, and RNA or water were negative controls. Primers for cytokeratins 8 and 18 were used to confirm the presence of epithelial/mesothelial cDNA in the preparations. One-kb ladders were included in the first and last lanes to compensate for slight differences in electrophoretic mobility across the gel.
to MIS and express the receptor mRNA. This peptide sequence is not present in the type I (19) or type II receptors of other members of the TGF-β family (40, 41) and was identified by its cognate antibody by dot blot analysis (not shown). To validate the antibody, we showed that the chick antireceptor antibody recognizes, by flow cytometric analysis, the receptor on the surface of COS cells transfected with the receptor gene but does not bind to nontransfected COS cells. The transfected receptor gene was also Flag tagged at its NH₂ terminus. The fact that these cells also were identified with an NH₂ terminal Flag antibody indicates that the complete extracellular domain of the protein was expressed. Western analysis of transfected COS cell extracts also showed a M₆, 65,000 protein, the expected size for the MIS type II receptor (not shown). A similar M₆, 65,000 band, presumed to be the endogenously expressed MIS type II receptor, was seen in the Western analyses of protein lysates of OVCAR3 using the chicken antibody (not shown). Adsorption of the chicken antibody with the original peptide antigen prevents receptor detection by Western analysis that was observed in lysates of IGROV-1 (Fig. 3A) or OVCAR5 (Fig. 3B). A similar M₆, 65,000 band, not present in preimmune serum, was detected in OVCAR8 using the rabbit antibody (Fig. 3C).

MIS type II receptor protein expression was also detected in frozen sections from patients’ tumor samples using the chicken anti-MIS type II receptor antibody. When the type II receptor antibody was used for immunohistochemical analysis of the 15½-day postcoitum male rat urogenital ridge (Fig. 4A), MIS type II receptor staining was observed in the mesenchyme of the Mullerian ducts, where the message for the type II receptor was localized previously by in situ hybridization (18). No specific signal was observed in the same 15½-day rat embryo when preimmune IgY or the chick antibody preadsorbed to its antigen was used (Fig. 4B). When tested in solid tumors from four patients with ovarian carcinoma, in each case the MIS type II receptor antibody stained the papillary epithelial tumor cells (representative section, Fig. 4C), which was not seen when tested with preimmune serum (Fig. 4D). These same cells also stained with OC-125, the antibody against CA-125 (Fig. 4E). A similar pattern of staining was observed when the rabbit antibody was used. This antibody did not recognize receptor in small intestine, liver, stomach, pancreas, or lung (not shown).

Analysis of MIS-Biotin Binding by Flow Cytometry. When the cell lines OVCAR5, OVCAR8, and IGROV-1, which displayed sensitivity to rhMIS in antiproliferative assays, were studied by flow cytometry for binding of MIS-biotin, cells from each cell line were found to bind MIS in a concentration-dependent manner, as measured by mean fluorescence/cell. Binding of MIS-biotin to these human ovarian cancer cell lines was competed by excess unlabeled MIS (see Fig. 5A for specific binding to OVCAR5). COS cells that did not express the MIS receptor, and SKOV3 cells which are not inhibited by MIS in antiproliferative assays, did not bind MIS. The concentration of MIS-biotin required to reach half saturation was 10.2 nM, a measure of the dissociation constant of the MIS-receptor complex. Total binding of MIS-biotin to OVCAR5 cells approached saturation at a concentration of 75 nM and was specifically competed with a 10-fold molar excess of unconjugated MIS (Fig. 5B). Similar results were achieved with OVCAR8 and IGROV-1 (data not shown). These values are comparable with the physiological concentration range of human serum MIS (3–6).

Binding studies performed on human ascites cells using flow cytometry demonstrated that the patient’s cellular ascites were heterogeneous with respect to cell type, consisting of epithelial or mesothelial cells and hematopoietic cells. However, it was only the epithelial/mesothelial subpopulation detected by expression of cytokeratin or CA-125 that bound MIS-biotin in a concentration-dependent manner, as measured by mean fluorescence/cell. Total binding of MIS-biotin to the ascites cells mirrored the binding and the saturation (75 nM) observed previously in the five human ovarian cancer cell lines. The CD45-positive hematopoietic cells do not bind MIS (Fig. 5C). Binding of MIS-biotin could be competed with excess unlabeled MIS.
and, importantly, results were available within 24 h. Furthermore, binding predicted the expression of the MIS receptor with 80% sensitivity and 80% specificity and a functional response to MIS with 75% sensitivity and 70% specificity.

The results in aggregate show that ovarian cancer cell lines that express MIS type II receptor mRNA or protein respond to MIS in functional assays. Similarly, primary ovarian cancer ascites cells that express MIS type II receptor mRNA or protein also respond to MIS in functional assays.

DISCUSSION

Despite encouraging early response rates to cytotoxic therapies such as cisplatin, paclitaxel (Taxol), and topotecan, the recurrence rate for ovarian cancer is high, and the 5-year mortality for stages III and IV disease remains at 70%. The search for other therapeutic agents has led to the investigation of biological modifiers that might augment the armamentarium of presently available therapeutics and help control, with less toxicity, the proliferation of these tumors. We have hypothesized that MIS could serve as such a biological modifier because it causes apoptosis and regression of the Mullerian ducts in the embryo and because ovarian carcinomas recapitulate the histology of the fetal Mullerian ducts (42). In addition, the ovary appears to express the MIS type II receptor (15, 18) and to be a target for MIS action (7–11, 43), particularly in MIS-overexpressing transgenic mice (22). Having expressed and labeled rhMIS, cloned the human MIS type II receptor, and raised antibodies to the MIS type II receptor, we tested this hypothesis.

Expression of the MIS type II receptor was demonstrated, at the transcriptional and translational level, in a series of human ovarian cancer cell lines that are inhibited by MIS in both semisolid and adherent colony antiproliferative assays. In addition, using biotin-labeled MIS that retains biological activity, we have demonstrated binding that is specific, saturable, and of high affinity in the cell lines that were most responsive to MIS. These experimental findings using cell lines derived from human ovarian cancers that originate from the coelomic epithelium provide the first evidence that the type II receptor is present in cell lines that are growth inhibited by MIS, suggesting that the MIS effect occurs as a result of a specific receptor-mediated process rather than from random toxicity. SKOV3, the ovarian cancer cell line that was not inhibited by MIS also failed to bind MIS. Despite expressing the native MIS type II receptor as determined by Northern analysis, this cell line, however, is known to lack p16 (44), which may be important in MIS downstream signaling. Thus, OVCAR5 or OVCAR8 cell lines, which should contain all other MIS receptor components, could be used as positive controls for each assay involving patient’s ascites cells. OVCAR5 would be an ideal positive control for patient ascites binding assays because it can be grown in suspension culture. Furthermore, the OVCAR5 and OVCAR8 cell lines should be very useful in identifying the MIS type I receptor from the seven known type I receptors (19, 45–47) and to decipher downstream signal pathway components and the genes regulated by MIS in ovarian cancer cells.

To test whether an MIS response could be receptor mediated in ovarian cancer patients, we examined primary ascites

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Footnote:


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Fig. 4 Immunohistochemical analysis of protein expression. The chick type II receptor antibody localizes to the mesenchyme around the regressing Mullerian duct (M) in this male embryo (A). The result using preimmune serum is shown in B for comparison. The same anti-type II receptor antibody recognizes the epithelial component of an ovarian serous cystadenocarcinoma (C). Antibody to CA-125 colocalizes with the antibody to the MIS type II receptor (E). Preimmune serum shows no staining (D). Sections were viewed at x250, and all antibodies (1 mg/ml) were used at 1:100 dilution.
cells isolated from 27 patients with stage III or IV ovarian cancer undergoing staging laparotomy or palliative paracentesis. We attempted to analyze all 27 patients cells for: (a) binding by flow cytometry; (b) detection of the presence of the type II receptor by RT-PCR; and (c) assessment of colony growth in soft agarose with or without MIS. However, because the number of cells in each patient’s ascites varied substantially, it was not possible to perform each study for all patients. For example, if the patient had received prior chemotherapy or underwent previous palliative paracentesis, the ascites might be hypocellular. Hence, analyses were prioritized according to cell number and the time of ascites acquisition. It is important to note that if cells could not be isolated within 4 h of surgery to limit RNA degradation, RT-PCR was not performed for that patient. If cell numbers were limited, only MIS binding was assessed. Given these limitations, 13 of 27 (48%) of the patients cells were studied by all three tests.

Ascites cells from 15 of 27 (56%) of the patients with late-stage ovarian cancer bound MIS-biotin. This number may be an underestimation of the actual incidence of receptor-positive tumors, because patients forming voluminous ascites secondary to obstructed lymphatics who require repeated palliative paracenteses may have ascites deplete of tumor cells. The non-tumor, epithelial/mesothelial cells from these patients might be CA-125 and cytokeratin positive but would be nonbinders and nongrowers in stem cell assays and hence could not be evaluated for MIS response in growth inhibition assays. In the future, we would recommend performing simultaneous cytology to evaluate the presence of tumor cells and to differentiate them from the non-tumor epithelial population. Twelve patients did not bind

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Fig. 5  Specific binding of biotinylated MIS to ovarian cancer cells. Histograms of OVCAR5 cells show a shift in the mean surface fluorescence/cell with 50 nM MIS-biotin binding OVCAR5 cells that is competed by 10-fold excess unlabeled MIS (A). Increasing concentrations of MIS-biotin incubated with OVCAR5 cells result in increasing shifts in mean fluorescence/cell. The composite figure for total and specific binding from three experiments normalized against background fluorescence is shown in B. The dissociation constant, $K_d$, of 10.2 nM was calculated at half saturation of MIS specific binding. Bars, SE. Increasing concentrations of MIS-biotin were incubated with human ovarian cancer ascites cells isolated from a patient with cystadenocarcinoma (C). The cytokeratin-positive population bound MIS in a dose-dependent manner, as characterized by a shift in surface fluorescence, whereas the CD45-positive hematopoietic cells did not shift above background. Graded molar excess of unconjugated MIS progressively inhibited binding to cells coincubated with 75 nM MIS-biotin ($n = 1$ for each molar excess; D).
MIS. Six of the 12 patients whose cells did not bind MIS, however, provided enough cells for RNA analysis; of these, 4 had no evidence of type II receptor mRNA. One of the remaining two patients’ cells failed to grow in colony inhibition assay, but of the four that grew, three showed statistically significant inhibition, probably reflecting the technical difficulty of the binding assay. Thus, the colony inhibition assay is a reasonable functional test to predict that all components are present in a patient’s cells to permit a functional MIS response. The length of time (7–10 days) required to obtain sufficient colonies in the soft agarose assays for comparison to MIS-treated cultures makes it necessary to use other determinants of response such as the MIS receptor mRNA or protein, together with binding of MIS, in predicting sensitivity to MIS. In future, the use of all three modalities, but in a prioritized paradigm, can give a comprehensive prediction of possible MIS response.

The two patients whose cells bound MIS but did not respond to MIS in antiproliferation assays could reflect mutations in the cytosolic domain of the MIS type II receptor, mutations in the MIS type I receptor, or defects in the molecules downstream of the receptor complex involved in the signal transduction pathway. Once the candidate genes in this pathway are defined, we envision systematic screening of all ovarian cancer patients for sequence abnormalities of all known pathway genes as a fingerprint of each patient’s tumor. Despite these potential logistical drawbacks, we can safely say that ovarian cancer cell lines that express the MIS type II receptor by various criteria (Figs. 1 and 3) respond to MIS (Fig. 1 and Table 1), and that patients whose ascites cells express the receptor (Figs. 4 and 5, C and D) also respond to MIS (Table 2). The frequency of MIS receptor expression on the ascites cells of ovarian cancer patients is similar to the proportion of breast cancer patients expressing estrogen receptor, a marker used to predict patients who will respond to antiestrogen therapy (48). The data presented in the present pool of patients confirm our hypothesis that papillary serous-cystadenocarcinoma ovarian cancers are targets for MIS therapy. Over half of the cases contain the MIS type II receptor and bind MIS. Furthermore, MIS can block proliferation of these cancer cells in a receptor-dependent manner in vitro, suggesting the MIS receptors detected by MIS binding and RT-PCR are functional. The number of in vitro evaluations that can be performed on the clinical cases depends upon the quantity of tumors cells recovered in ascites. Because cell number varies widely from case to case, the tests performed on the clinical samples must be prioritized. The highest priority is given to MIS binding assays because they require the fewest number of cells and the least amount of time to complete. Also, MIS binding predicted the presence of MIS type II receptor mRNA. With rhMIS, the MIS type II receptor, and antibodies to the receptor and ligand, along with methods of production and purification of rhMIS, these carefully developed tools are now in hand to proceed to clinical trials that will test rhMIS as an adjuvant antineoplastic agent for such patients.

We envision that flow cytometry with labeled MIS and/or MIS type II receptor probes would be used to select such patients with recurrent ovarian cancer as candidates for an expeditious Phase I clinical trial for MIS toxicity. Such a well-constructed trial would not preclude early resumption of conventional chemotherapy. Colony inhibition, mRNA, and protein detection would be used for selection of patients for later efficacy trials.

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Human Ovarian Cancer, Cell Lines, and Primary Ascites Cells Express the Human Mullerian Inhibiting Substance (MIS) Type II Receptor, Bind, and Are Responsive to MIS

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