Recombinant Human Mullerian Inhibiting Substance Inhibits Long-term Growth of MIS Type II Receptor–Directed Transgenic Mouse Ovarian Cancers In vivo

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Abstract

Purpose: Mullerian inhibiting substance (MIS) is a glycoprotein hormone that causes Mullerian duct regression in male embryos. In short-term experiments, recombinant human MIS (rhMIS) inhibits xenotransplanted human ovarian cancer cell lines that are thought to be of Mullerian origin. Because this highly lethal cancer has a high recurrence rate after conventional chemotherapy, new treatments are warranted. We examined whether rhMIS as a novel, nontoxic, naturally occurring growth inhibitor can be an effective anticancer drug in long-term studies in vivo against allograft tumors that recapitulate human ovarian carcinoma.

Experimental Design: Mouse ovarian carcinoma (MOVCAR) cell lines expressing the early region of the SV40 virus, including the large and small T-antigen genes under transcriptional control of a portion of the murine MIS receptor type II (MISRII) gene promoter, were derived from TgMISRIIR-TAg transgenic mice. rhMIS was tested against MOVCAR cells in growth inhibition assays in vitro, and in vivo in 6-week-old female nude mice. Tumor growth in animals was measured at weekly intervals for up to 20 weeks.

Results: MOVCAR cells and tumors express MISRII by Western blot, immunohistochemical, and Northern blot analyses. rhMIS significantly inhibited MOVCAR cell growth in vitro and in vivo in three separate long-term allotransplantation experiments.

Conclusions: Because rhMIS is an effective anticancer agent in vitro and in long-term in vivo preclinical experiments against MISRII-positive tumors, we predict that rhMIS can be used safely and effectively to treat human ovarian malignancies.

Mullerian inhibiting substance (MIS; refs. 1–3) is a 140 kDa glycoprotein disulfide linked homodimer with ~15% carbohydrate. It causes regression of Mullerian ducts, the precursors to the Fallopian tubes, uterus, cervix, upper third of the vagina, and the ovarian lining. In humans, the MIS gene is located on chromosome 19 (4) and its expression is sexually dimorphic. In males, MIS is high in fetal Sertoli cells and these levels continue until puberty, whereupon expression decreases to basal levels. In females, MIS is only produced postnatally in granulosa cells from prepuberty through menopause at levels similar to adult males. The detection of MIS after birth (5–7) suggests postnatal roles for MIS.

MIS biological activity requires binding to a heterodimer of type I (8–11) and II single transmembrane spanning serine threonine kinase receptors, leading to cross-phosphorylation of kinase domain of each receptor initiating intracellular signaling cascades that lead to altered gene transcription (12–16), including proteins that regulate cell cycle (17). MIS receptor type II (MISRII) is expressed in the urogenital ridge and then in mesenchymal cells surrounding the Mullerian duct epithelium,4 as well as in the fetal and adult gonads (18–20) and the ovarian coelomic epithelium. Several type I MIS receptors have been identified, including Alk3 (21), Alk2 (8, 11, 22) that predominate in rodents, and Alk6 (9). How these receptor(s) interact in human tissues is not clear but all are expressed in several human cell lines.5

The 5-year mortality rate of ovarian cancer is over 70% (23). Although most patients initially respond to chemotherapy, relapse (24) is common due to drug resistance. Thus, ovarian cancer becomes a chronic disease of the peritoneum for which novel therapies are needed. The hypothesis that MIS could be used to treat ovarian cancer (25) is based on...
the fact that embryonic Mullerian duct histology (26–29) is recapitulated in the common ovarian adenocarcinomas (30). That human ovarian cancer may respond to human MIS is strongly suggested by a series of in vitro (17, 31–34) and in vivo studies using xenotransplanted human ovarian cancer cell lines (31–33, 35–37) with recombinant human MIS (rhMIS).

Given these encouraging findings and the need to use MIS in long-term preclinical trials not permitted by xenotransplantation, we tested rhMIS against rapidly proliferating MISRII-expressing cell lines taken from ascites in a transgenic ovarian cancer mouse model, which closely mimics the human disease (38).

The model we chose was developed by using a portion of the 5’ upstream regulatory sequence of the mouse MISRII gene to drive the expression of oncogenic SV40 T antigen, which inactivates p53 and the pocket protein, Rb (38). The TgMISRII-TAg transgenic mice form bilateral ovarian carcinomas that are accompanied by tumor invasion of peritoneal organs and ascites (38). The tumors faithfully recapitulate the phenotype of the most common human ovarian cancer, serous cystadenocarcinoma. Ascites cells were used to establish cell lines [mouse ovarian carcinoma (MOVCAR)], which form colonies in soft agar and produce tumors in immunodeficient mice. Several MOVCAR cell lines were used in a series of in vivo experiments as reproducible models for extended preclinical trials to show its therapeutic potential of MIS before testing in humans.

Materials and Methods

Cell lines and cultures. The human ovarian cancer cell line OVCAR8 transfected with the rat MISRII (39) served as a positive control for MISRII expression (17). COS7 cells served as a negative control for expression of MISRII but as a positive control for T antigen (40). The mouse ovarian cancer cell lines, MOVCARs, were developed at the Fox Chase Cancer Center (Philadelphia, PA; ref. 38). MOVCAR cell lines were maintained in 4% female fetal bovine serum (MIS-free) at the Fox Chase Cancer Center (Philadelphia, PA; ref. 38). MOVCAR control for expression of MISRII but as a positive control for T antigen OVCAR8 transfected with the rat MISRII (39) served as a positive control.

MOVCAR cell lines were used in a series of in vivo experiments as reproducible models for extended preclinical trials to show its therapeutic potential of MIS before testing in humans.

MISRII detection by Northern blot analysis. Total RNA was isolated using Trizol (Invitrogen Life Technologies) and quantified by absorbance at 260 nm. Samples (10 μg) were denatured with DMSO and glyoxal at 65°C, separated in a 1.5% agarose gel, and electroblotted to an Immobilon-P membrane (Millipore, Rockford, IL). The blots were washed thrice with 0.1% SDS and exposed to radiographic film with intensifying screens at −70°C.

Purification of rhMIS. rhMIS was purified from serum-free conditioned medium of Chinese hamster ovary cells transfected with the human MIS gene (41, 42). Briefly, rhMIS was concentrated by wheat germ lectin affinity chromatography and purified by fast protein liquid chromatography anion-exchange chromatography (42).

Inhibition of MOVCAR cells by rhMIS in vitro. rhMIS inhibition of cell proliferation was measured in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (43). MOVCAR cells were plated in 96-well plates at 1,000 cells per well. Twenty-four hours after plating, cells were treated with either rhMIS or vehicle buffer. At day 5 or 7 of treatment, cells were quantified for each group by recording the absorbance at 550 nm (43) and the degree of inhibition calculated.

Growth of MOVCAR cells in vivo. To study rhMIS effects in vivo, the time to appearance and changes in tumor volume were measured after injection of MOVCAR7 cells. Six-week-old female Swiss nude mice were injected with 6 × 10⁶ cells into the s.c. tissue above the quadriceps of the left hind limb or in the dorsal fat pad between the scapulae using a 27-gauge needle with the bevel turned downward and the exit site clamped with forceps as the needle was removed to prevent wide dispersion of the tumor cells. The skin incision exposing the fat pad was closed with stainless-steel clips. All experiments were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee (A3596-01).

The mice were randomly divided into two treatment groups, five to eight in each group. One week after tumor injection, i.p. treatment with rhMIS (10 or 20 μg/animal/d) or the same volume of buffer was initiated for 5 days/wk. Tumor volume (length × width²) was measured with calipers at weekly intervals. Animals that developed hematomas at the beginning of the study were excluded. When tumors reached the maximum size allowed by the animal use institutional review board, animals were sacrificed and tumors were harvested. Tumor tissue was fixed for histology, immunohistochemistry, and immunofluorescence, or frozen for mRNA or protein analyses.

MISRII antibody. Rabbit polyclonal antibodies were raised against the highly conserved MISRII-specific kinase domain (amino acids 176-400) expressed in Escherichia coli. The antibody designated 153P was ligand affinity purified and recognizes the receptor kinase domain and full-length protein produced by either SP9 cells or E. coli at 40 and 60 kDa, respectively, and endogenous and transfected receptor in lysates of OVCAR 8 by Western blot analysis (17). The antibody also recognizes a 65 kDa protein in MIS-responsive human cervical carcinoma cell lines CaSki and C33A (31), and in the endometrial carcinoma cell line AN3CA (44).

Western blot analyses. Proteins from tumor tissues and MOVCAR7, COS, and OVCAR8 cells were harvested in Cell Signaling (Danvers, MA) × SDS sample buffer [187.5 mmol/L Tris-HCl (pH 6.8), 6% w/v SDS, 30% glycerol, and 150 mmol/L DTT] with added protease inhibitors (Complete Mini Protease Inhibitors; Roche Diagnostics, Indianapolis, IN). SDS-PAGE (40 μg protein per lane) was done under reducing conditions (NuPAGE LDS, sample buffer and reducing agent; Invitrogen, Carlsbad, CA) at 70°C for 10 minutes and electroblotted to an Immobilon-P membrane (Millipore, Danvers, MA). Membranes were blocked in TBST [TBS and Tween 20: 25 mmol/L Tris (pH 7.4)/136 mmol/L NaCl/5 mmol/L KCl/0.1% Tween 20] containing 5% milk for 1 hour at room temperature. The SV40 TAg antibody pAb101 from Santa Cruz Biotechnology (Santa Cruz, CA) and MISRII antibodies (12 μg/mL) were incubated in 5% milk/TBST overnight at 4°C. The blots were washed three times with TBST and then incubated with the horseradish peroxidase–conjugated secondary antibodies (1:100, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Bound antibodies were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Immunohistochemical and immunofluorescence recognition of MISRII and T antigen. Mouse tumor tissues and rat embryonic urogenital ridges were fixed in 4% paraformaldehyde + 5% picric acid and embedded in paraffin. Eight-micrometer sections were deparaffinized, heated with citrate buffer at 85°C for 10 minutes in a microwave oven, allowed to cool to room temperature, and quenched with 1.5% H₂O₂. The slides were washed with TBS, blocked with 10% normal goat serum, and incubated with primary antibodies at dilutions of 1:100 of anti SV40 T-Ag (pAb101) and 1:50 of 153P, respectively, overnight at 4°C. After washing three times, slides were incubated with secondary antibody for 1 hour with amplification using avidin biotin
solution (Vectorlabs, Burlingame, CA) and color was developed with diaminobenzidine substrate.

For immunofluorescence, tissues were fixed overnight in 4% paraformaldehyde at 4°C, washed thrice with 10% sucrose, embedded in OCT, and preserved in liquid nitrogen before sectioning. Epitope retrieval was done in 0.1 mol/L sodium citrate with microwave heating at 85°C for 60 seconds. Sections were washed with TBS, blocked using 5% normal donkey serum (Jackson ImmunoResearch) for 1 hour, and incubated with 153P primary antibody at dilution of 1:100 overnight at 4°C. Sections were then incubated with Cy2 or FITC-conjugated secondary antibodies for 1 hour. Nuclei were stained using propidium iodide (1:1,000) for microscopy using a Nikon Eclipse E400 microscope. Photographs were then obtained using the SPOT camera.

**Statistical analysis.** Student’s *t* test were used for the statistical analyses of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. For *in vivo* tumor growth inhibition studies, serial measurements of tumor size were compared between rhMIS- and PBS-treated animals at weekly intervals and analyzed by repeated-measures ANOVA. Where a significant difference (*P* < 0.05) was found, the interaction implied that the difference between rhMIS and control changed over time. Two-tailed *t* tests and one-tailed *t* tests for unequal variances were then used for each time point.

### Results

**Detection of MISRII in MOVCAR cells.** Before evaluating MOVCAR cell responsiveness to rhMIS, we studied whether they expressed MISRII. Northern blot analyses detected the 2 kb MISRII mRNA in MOVCAR7 and MOVCAR8 cells, and in the positive control testis but not in cerebellum (Fig. 1).

**rhMIS inhibits proliferation of MOVCAR cells in vitro and in vivo.** To test rhMIS inhibition of proliferation of MOVCAR7, cells were treated with increasing concentrations of rhMIS from 7 to 70 nmol/L. The IC₅₀ was 35 nmol/L for MOVCAR8 (Fig. 2A) and 21 nmol/L for MOVCAR7 (not shown). rhMIS inhibited cell proliferation in six replicate experiments (*P* < 0.000001; Fig. 2B).

In one *in vivo* study, 10 animals were injected with 6 × 10⁶ MOVCAR7 cells into the dorsal fat pad. After 1 week, they were randomly assigned to control (*n* = 5) or rhMIS groups (*n* = 5) and treated with buffer or 20 µg rhMIS, 5 d/wk, for the remainder of the experiment. Repeated-measures ANOVA showed significant rhMIS inhibition (*P* < 0.05). Tumors became apparent at 54 days with a statistically significant difference in volumes between control and rhMIS-treated groups (*P* = 0.05); thereafter, *P* < 0.01 at 62 days, *P* < 0.025 at 72 days, *P* = 0.03 at 78 days, and *P* < 0.03 at 82 days, by two-tailed *t* test (Fig. 3A). These results were confirmed with a second group of 13 animals. Seven animals in the PBS group and six in the rhMIS group were studied for 75 days after tumor injection. rhMIS and PBS groups were again compared using ANOVA (*P* < 0.05) over the entire experiment. Tumors became apparent at 33 days with a statistically significant difference apparent at 36 days (*P* = 0.05); thereafter, *P* = 0.01 at 40 days, *P* = 0.02 at day 47, *P* = 0.05 at day 56, *P* = 0.004 at day 61, *P* = 0.01 at day 68, and *P* = 0.06 at day 75 by two-tailed *t* test (Figs. 3B and 4A) and *P* = 0.04 by one-tailed *t* test.

In another experiment, 10 animals were injected with 4.8 × 10⁶ MOVCAR7 cells into the left leg and randomly separated into vehicle- and MIS-treated groups. The two groups were significantly different when compared by ANOVA, with a *P* < 0.05 over the course of the experiment. There was a statistically significant difference in tumor volume (1,577 mm³ versus 398 mm³ after 82 days of treatment). Two-tailed *t* tests showed a statistically significant difference in tumor volume between control and rhMIS groups, *P* < 0.001 at 35 days, *P* < 0.001 at 47 days, *P* < 0.002 at 57 days, *P* < 0.0005 at 62 days, *P* < 0.009 at 70 days, and *P* < 0.001 at 82 days after injections commenced (Figs. 3C and 4A).

**Fig. 1.** Northern blot analysis of MISRII mRNA expression in MOVCAR cells. The migration of the λ/HindIII size marker is indicated on the left. MISRII is present in MOVCAR7 and MOVCAR8 and testis but not in the cerebellum.

**Fig. 2.** rhMIS inhibits MOVCAR cell lines in *in vitro* A, rhMIS dose-dependent inhibition of MOVCAR8 in an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The IC₅₀ was 5 µg/mL (35 nmol/L). Points, percentage of growth; bars, SD. B, rhMIS at 70 nmol/L inhibits MOVCAR7 proliferation. When means of six different experiments were compared, significant inhibition was observed (*P* < 0.000001). OD, absorbance.
tumors is evident after as few as 35 days of treatment but persisted for up to 80 days when control animals had to be sacrificed because of tumor size (Figs. 3C and 4A).

In several cases, we continued to treat the rhMIS groups. However, after an additional 5 weeks of treatment, dorsal fat pad tumors in three of five animals began to grow whereas two remained small. Two of four animals remained small in the group injected in the leg after another 3 weeks, one of five animals with leg tumors remained responsive to rhMIS. Therefore, a 30% sustained response to rhMIS was observed. The total length of treatment of these animals was close to 20% of their 2-year life span.

By fluorescence immunohistochemistry, 153P specifically recognizes MISRII in granulosa cells of developing follicles and in the surface coelomic epithelium of the mouse ovary (Fig. 5A), cells known to express MISRII. 153P also recognizes the mesenchyme around the embryonic (E15) Mullerian duct (Fig. 5B) as a positive control. MOVCAR-derived tumors expressed both TAg and MISRII by immunohistochemistry (Fig. 6A) and T antigen was detected by Western blot analysis in extracts of tumors that formed in the dorsum of three PBS-treated mice and in two MIS-treated mice, and in the positive control COS cells (Fig. 6B). Human endometrial (AN3CA) and ovarian (OVCAR 8) cell lines were negative controls for TAg. Western blot analysis detected MISRII in tumor extracts and the MOCAR 7 cells used for the fat pad injections but not in the negative control COS cells (Fig. 6C).

Discussion

In 1970, the coelomic epithelium was proposed as the source of cystadenocarcinoma (30). The fact that human ovarian cancer cell lines (17), a human ovarian surface cell line (45), and the surface epithelium of the adult mouse ovary (Fig. 5A) express MISRII provides compelling evidence that the ovarian surface epithelium is a target for MIS and that MIS may serve as a therapeutic for these ovarian cancers.

Transgenic TgMISRII-TAg mice developed large ovarian cancers that closely recapitulate human ovarian cystadenocarcinomas; cells shed into the peritoneal cavity (38) constituted the MOVCAR cell lines used in the present studies. After demonstrating that rhMIS inhibited growth of the MOVCAR cell lines in vitro, efficacy was tested in vivo.
MOVCAR cells show sustained growth in nude mice without the signs of immune rejection seen with xenotransplants (36, 37), allowing treatment of the allotransplanted tumors with rhMIS for up to 20 weeks, which represents almost a quarter of the life span of a mouse. Significant inhibition of tumor growth was observed without obvious toxicity. Thus, this allografted tumor model can serve as a preclinical evaluation of rhMIS efficacy and for full toxicology studies when GMP preparations of rhMIS are available.

It is now important to assess the incidence of MISRII and MIS inhibition of growth in ovarian cancer cells, which can be easily harvested in humans (34) as a marker to select patients for MIS therapy. Other markers of functional response being investigated include p16 (17); the pocket proteins p107, p130, and Rb; and the E2Fs (31, 44), which are associated with cell cycle arrest and apoptosis. Up-regulation of activated Smads in the MIS pathway, as seen in the urogenital ridge (8), is another possibility. The use of response markers together with MISRII may increase the percentage of MIS-responsive patients.

MIS treatment may be expanded to endometrial (30, 36, 41, 44) and cervical carcinomas (31, 37) in which MIS caused growth inhibition associated with G1 arrest and apoptosis. Human breast and prostate and their tumor cells express MISRII (12–14, 46). In addition, in vivo response to MIS was seen in our laboratory (47) in human breast cancer cell lines transplanted into severe combined immunodeficient mice and in T-antigen transgenic animals engineered to developed breast cancer (48).

The allotransplant model can be used to test MIS at various doses and in combination with other therapeutic agents. The present experiments will provide a template or model for these combination studies, as GMP preparation of rhMIS becomes available. Such in vitro and in vivo investigations will help to establish the optimal strategies by which MIS should be deployed as a therapeutic for human disease.

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References


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