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Minimal Antiproliferative Effect of Recombinant Mullerian Inhibiting Substance on Gynecological Tumor Cell Lines and Tumor Explants

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ABSTRACT

Mullerian Inhibiting Substance (MIS) is a testicular hormone that promotes involution of the Mullerian duct during embryogenesis. The Mullerian duct gives rise to adult female reproductive ducts including the fallopian tubes, uterus, and upper vagina. Thus, testicular MIS ensures the regression of female sex organ primordia. Partially purified bovine MIS was reported to inhibit proliferation of tumor cells derived from human gynecological cancers. These observations suggest that MIS might be an effective anticancer agent for some human tumors. Recombinant human MIS (rHu-MIS) has recently become available. To assess the antiproliferative activity of rHu-MIS, we examined its effects on 11 ovarian, six endometrial, and two nongynecological human tumor cell lines. rHu-MIS had no effect on proliferation of these cell lines in five independent assays. Forty-three primary human tumor explants were also examined in human tumor colony forming assays, gel-supported primary culture assays, and subrenal capsule assays. rHu-MIS significantly inhibited the growth of five of these tumors including four ovarian and one small cell lung cancer explant. The four ovarian cancer responses include three of 13 (23%) explants tested in human tumor colony-forming assays and one of eight (12.5%) explants tested in gel-supported primary culture assays. We conclude that rHu-MIS may have antiproliferative activity against some human ovarian cancers.

INTRODUCTION

MIS[2] is a glycoprotein hormone produced by the testes during mammalian embryogenesis (1). MIS promotes the regression of Mullerian duct tissues that would otherwise develop into the adult uterus, fallopian tubes, and upper vagina. Testicular production of MIS thus ensures that the female reproductive duct primordia are eliminated during male sexual differentiation. MIS bioactivity can be measured *in vitro* by assaying for regression of fetal rat Mullerian duct tissue (2). Using this assay, Donahoe (3) and Josso (4) have succeeded in purifying biologically active MIS from bovine testes. MIS isolated from bovine testes is a glycoprotein with an approximate molecular weight of 140,000 (4, 5). This M_r 140,000 species is composed of two identical disulfide-linked polypeptides with molecular weights of approximately 72,000. MIS cDNA clones have been isolated from bovine testicular cDNA libraries (6, 7). Analysis of these cDNAs indicated that the amino acid sequence of MIS is significantly homologous to another growth regulatory molecule: TGF-β (6, 8). A full-length genomic clone of human MIS has also been isolated. Using this genomic clone, rHu-MIS has been expressed in mammalian cells (6). rHu-MIS is also produced as a M_r 140,000 glycoprotein that exhibits full biological efficacy in the fetal rat Mullerian duct regression assay.

In addition to its developmental role in promoting the regression of Mullerian duct tissue, MIS may possess other biological activities including inhibition of ovarian meiosis (9), promotion of testicular descent (10), and inhibition of tumor cell growth in gynecological malignancies (11). The antitumor activity of MIS is particularly intriguing because of its therapeutic implications. MIS has been reported to inhibit the growth of human gynecological tumor cell lines and primary human tumor explants *in vitro* and *in vivo* (12–14). However, Josso and coworkers reported that bovine MIS did not inhibit the growth of a human endometrial carcinoma cell line (15). All of the antiproliferative studies reported to date have employed bovine testicular MIS. Therefore, it was unclear what effects highly purified rHu-MIS would have on tumor cell growth.

To assess the antiproliferative activity of rHu-MIS on human tumor cells we tested 19 cell lines in five different cell growth assays including growth in monolayer cultures, colony inhibition in semisolid media, and growth in nude mice. We also examined the effect of rHu-MIS on the growth of 43 independent primary human tumor explants in HTCFAs (16), three-dimensional GSPCAs (17), and SRC assays (18).

MATERIALS AND METHODS

Cell Culture. Human ovarian carcinoma cell lines CAOV-3, CAOV-4, NIH/OVCAR-3, PA-1, and SKOV-3 and human endometrial carcinoma cell lines AN3CA, HEC-1A, HEC-1B, KLE, and RL95-2, as well as the human colon carcinoma cell line SW-48, were obtained from the American Type Culture Collection (Rockville, MD). Human ovarian carcinoma cell lines SKOV-4, SKOV-6, SW626, A-7, A-10, and Schict-1 were obtained from the Human Tumor Cell Line Bank, Memorial Sloan-Kettering Cancer Center (New York, NY). The human endometrial carcinoma cell line, Ishikawa 3-H-4 (19), was a gift from Dr. Masato Nishida, University of Tsukuba, Japan. The human epidermoid carcinoma cell line, A431, was supplied by Dr. Patricia K. Donahoe, Massachusetts General Hospital, Boston, MA. The MIS-producing cell lines were obtained by transfecting CHO cells deficient in DHRF with the human MIS gene under the control of the SV40 early promoter, along with the DHFR cDNA. CHO cell line 311-A9B7 was selected in 30 nM methotrexate and produces 0.1 mg rHu-MIS/l/day, while CHO cell line L2-58 produces approximately 0.5 mg rHu-MIS/l/day. Both cell lines were grown in a minimal essential medium lacking ribonucleosides and deoxyribonucleosides (GIBCO, No. 410-2000), containing 10% dialyzed fetal bovine serum. Cell line 311-A9B7 was used for producing purified rHu-MIS used in this study, while cell line L2-58 was used for the cocultivation and dual tumor assays described below. All cell lines were maintained in growth media containing DMEM (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and L-glutamine (2 mM). Fetal calf serum lots were screened to ensure that they did not promote rat Mullerian duct regression *in vitro*.

MIS Protein. The rHu-MIS producing cell line 311-A9B7 was grown

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[2] The abbreviations used are: MIS, Mullerian Inhibiting Substance; cDNA, complementary DNA; TGF-α, transforming growth factor-α; rHu-MIS, recombinant human MIS; HTCFA, human tumor colony forming assay; GSPCA, gel-supported primary culture assay; SRC, subrenal capsule; CHO, Chinese hamster ovary cells; DHRF, dihydrofolate reductase; DMEM, Dulbecco's modified essential medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MTT, 3-[3,4-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; TCA, trichloroacetic acid; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

in 10-liter cell factories in α-MEM containing 10% dialyzed fetal bovine serum, streptomycin (1 mM), gentamicin (50 μg/ml), L-glutamine (4 mM), pyruvate (1 mM), and methotrexate (30 nM). After the cells reached confluence, they were washed three times with the above medium without serum or methotrexate (3 liters), and then grown in the serum free medium (10 liters). Conditioned medium was collected every 4 days, filtered and stored at 4°C until further processing. After four or five collections from one 10-liter cell factory, the cell population was regenerated by adding medium with 10% dialyzed fetal bovine serum and then the collection cycle was repeated.

Two hundred liters of conditioned medium was diluted with 200 liters of 40 mM Tris-HCl, pH 7.7 and loaded on a 2 liter Fast Q column (Pharmacia). The protein was eluted with a 250 mM NaCl, 20 mM Tris-HCl, pH 7.7 step. Fractions containing rHu-MIS were identified by Western blotting and concentrated to 100 ml by ultrafiltration. The sample was diluted to a conductivity equivalent to 100 mM salt, reconcentrated to 100 ml by ultrafiltration, and passed over a 100-ml DEAE-cellulose column. rHu-MIS was further purified from the DEAE flow-through by adsorption to a 10-ml lentil lectin column followed by elution with 10% *N*-acetyl glucosamine, 25 mM Tris, pH 7.7 and 300 mM NaCl. rHu-MIS (60% pure based on gel analysis) was frozen and stored at −70°C until further purification using immunoaffinity chromatography with a mouse monoclonal antibody raised against rHu-MIS. rHu-MIS was eluted from the immunoaffinity column with 2 M NaSCN, 150 mM NaCl and 15 mM sodium phosphate, pH 6.3 (4). The chaotrope was removed and replaced with MIS storage buffer (10% glucose, 300 mM NaCl, 10 mM HEPES, pH 7.5) in a P6DG desalting column (BioRad). The final preparations were aliquoted and stored at −70°C. Prior to use, rHu-MIS was filter sterilized by passage through a 0.22 μm millex-GV_4 filter unit (Millipore, Bedford, MA). After filtration, the rHu-MIS protein was characterized by isoelectric focusing, reducing and nonreducing SDS-PAGE, amino acid composition analysis and NH_2-terminal amino acid sequencing. These analyses indicated that rHu-MIS was >95% pure and was consistent with the predicted amino acid composition and NH_2-terminal sequence of human MIS (6). A final yield of 6 mg of rHu-MIS was obtained from 200 liters of conditioned medium. Protein concentration was determined by amino acid composition analysis. Biological activity of the purified rHu-MIS was demonstrated *in vitro* using the rat Mullerian duct regression assay as modified by Donahoe *et al.* (20). Briefly, urogenital ridge tissue was obtained from 14-day-old female rat embryos and cultured on agar coated grids. These cultures were exposed to various concentrations of rHu-MIS for 3 days at 37°C. Thereafter the tissue explant was fixed, sectioned, and stained. Mullerian duct regression was graded on a scale from I to V with grade I indicating minimal activity.

MTT Assay of Cell Proliferation. For each cell line a standard curve of A_{570} values was generated from MTT (21) assays using various cell densities in 96 well plates (Costar, Cambridge, MA). From this curve a cell density was chosen which gave A_{570} values within the linear range. MIS was added to cell suspensions at final concentrations of 10, 1, 0.1, and 0.01 μg/ml. Vehicle buffer was included in all assays as the negative control. 50 μl of each sample was added to triplicate wells of 96-well plates and incubated at 37°C in 5% CO_2 for 48, 72, or 96 h. 10 μl of 5 mg/ml MTT (Sigma, St. Louis, MO) was added and the plates were incubated at 37°C for 4 h. The medium was aspirated and 0.1 ml of isopropanol was added to each well to solubilize the purple formazan crystals formed in viable cells. The A_{570} was measured using an EL-310 (Bio-Tek) plate reader. Percentage decrease in cell viability was determined by comparing the average A_{570} from triplicate wells of MIS-treated cells to the negative control.

[^{3}H]Thymidine Incorporation Assay of Cell Proliferation. Cells were seeded at various densities into 24-well plates and a standard curve of [^{3}H]thymidine incorporation was generated. For each cell line a seeding density was chosen which produced maximum [^{3}H]thymidine incorporation in the linear range of the curve comparing seeding densities with total cell proliferation after 24, 48, 72, and 96 h in culture. Cell suspensions containing rHu-MIS at 1, 0.1, and 0.01 μg/ml, or vehicle buffer only, were seeded in triplicate wells into 24-well plates (Costar) and incubated at 37°C, 5% CO_2 for 36, 60, or 84 h. One μCi of [^{3}H]-thymidine (Amersham, Arlington Heights, IL) was added to each well and the plates were incubated for 15 h. One ml of lysis buffer (10 mM NaCl, 10 mM EDTA, and 1% SDS) was added directly to each well. The cell lysate was mixed with an equal volume of 10% TCA and kept on ice for 10 min. TCA-insoluble material was trapped on a 25 mm, 0.45 μm HA filter (Millipore, Bedford, MA) and [^{3}H]thymidine incorporation was measured in a liquid scintillation counter. Percentage of inhibition was determined by comparing average cpm from triplicate wells of rHu-MIS treated cells to the negative control.

Anchorage-independent Growth Inhibition in Semisolid Media. One-milliliter underlayers of 0.6% low-gelling-temperature agarose (Sigma) in growth media were poured into six-well plates (35-mm diameter/well, Costar) and solidified at 4°C. One ml overlayers containing 0.3% low-gelling-temperature agarose, 5×10^6 cells, and 10 μg/ml, 1 μg/ml, or 0.1 μg/ml, of rHu-MIS or vehicle buffer as the negative control, were poured in triplicate plates. The overlayers solidified at room temperature and were incubated at 37°C in 5% CO_2 until colonies were macroscopically visible. Colonies greater than 50 cells per colony were counted from a 2 mm x 5 mm area of the plates (equivalent to $>5 \times 10^3$ cells). Percent inhibition of colony formation was determined by comparing the average number of colonies from triplicate plates of rHu-MIS treated cells to the negative control plates.

Cocultivation Growth Inhibition. 35-mm millicell cups (0.45-μm pore size, HJA filter, Millipore) were prewetted in DMEM and placed in empty wells of six-well tissue culture plates. Four milliliters of L2-58 or CHO cell suspensions (1.6×10^6 cells/ml) in DMEM was placed into the cup. Four milliliters of growth media was added to the well surrounding the cup and the plates were incubated for 24 h. Ovarian carcinoma and endometrial carcinoma cells were seeded into six-well tissue culture plates at 5×10^4 cells/well in 4 ml of DMEM and incubated at 37°C in 5% CO_2 for 24 h. Cups with CHO cells or L2-58 cells were transferred to the wells containing ovarian carcinoma or endometrial carcinoma cells and the two cell lines were cocultivated for 5 days. Culture medium from inside and outside the cups was collected separately and passed through 0.45-μm Uniflo filters (Schleicher and Schuell, Keene, NH) prior to assay for rHu-MIS by quantitative ELISA. The cups were discarded and the ovarian carcinoma and endometrial carcinoma cells were rinsed with PBS, trypsinized, and centrifuged at $250 \times g$. The cells were resuspended in 150 μl of PBS and 150 μl of 0.4% trypan blue stain (GIBCO). Viable cells were counted by visual inspection using light microscopy. Percentage inhibition of growth was determined by comparing viable cell counts from duplicate wells containing cups with CHO cells to wells containing cups with L2-58 cells.

Dual Tumor Inhibition. Nude mice (Harlan Sprague-Dawley Inc., Indianapolis, IN) were injected s.c. in the left flank with 1×10^7 L2-58 cells producing rHu-MIS or CHO cells in 0.5 ml of DMEM. One week later, 5×10^6 ovarian carcinoma or endometrial carcinoma cells in 0.5 ml DMEM were injected s.c. into the right flank of the animals previously injected with L2-58 or CHO cells. Separate groups of animals received only the ovarian carcinoma or endometrial carcinoma cells in their right flanks. Twenty-eight days later the mice were sacrificed, blood samples were taken, and the tumors measured. Serum rHu-MIS levels were measured by ELISA. Tumor volume was calculated from caliper readings in three dimensions. Percentage inhibition of ovarian or endometrial tumor growth was determined by comparing tumor volumes from animals bearing L2-58 tumors with animals bearing CHO tumors.

Sandwich ELISA for rHu-MIS. A sandwich ELISA was used to quantitate rHu-MIS in conditioned medium from cell culture experiments or serum from nude mouse studies. Plates (96 well) were coated with 100 μl/well of 12.5 μg/ml monoclonal anti-MIS antibody (Biogen) in 0.05 M $Na_2CO_3/NaHCO_3$, pH 9.6. The monoclonal antibody was raised against rHu-MIS. The plates were incubated at room temperature for 2 h, washed with PBS, incubated with blocking buffer (1% bovine serum albumin in PBS) at room temperature for one hour and washed with PBS. One hundred microliters/well of sample diluted in 0.67% BSA in PBS (with 1% normal mouse serum when appropriate) was added. The plates were incubated overnight at 4°C, washed with PBS, and 100 μl/well of rabbit polyclonal anti-MIS (Biogen, diluted 1:500) was added. The polyclonal antibody was raised against the M_r 140,000

band eluted from nonreduced polyacrylamide gels following electrophoresis of rHu-MIS. The plates were incubated at room temperature for 1 h, washed with PBS, and incubated with alkaline phosphatase-conjugated, donkey anti-rabbit IgG (The Jackson Immunoresearch Labs, Inc., Avondale, PA, diluted 1:500) at 37°C for 1 h. The wells were washed with PBS, incubated with 1 mg/ml *p*-nitrophenyl phosphate (Sigma) in diethanolamine buffer (Kirkegaard and Perry Labs, Inc., Gaithersburg, MD) at room temperature for 30 min. The A_{405} was measured using an EL310 plate reader and the rHu-MIS concentration determined by comparison to a standard curve generated using purified rHu-MIS. This assay yields a linear curve for rHu-MIS concentrations as low as 20 pg/ml (see Fig. 3). Net optical density represents the O.D. of the standard or test sample minus the O.D. of a blank (culture medium or normal serum) sample.

Human Tumor Colony Forming Assay. Patients' tumors were collected in the operating room under sterile conditions, cut into 5-mm fragments and placed in transport media (McCoy's 5A + 10% calf serum). Malignant effusions were collected with 10 units of preservative-free heparin per cc of malignant effusion. Single cell suspensions were prepared by mechanical means. rHu-MIS was provided in aliquots ready for use and was diluted in the culture media. Four concentrations were utilized including 0.01, 0.1, 1.0, and 10 μg/ml. Cells were exposed to rHu-MIS for the entire culture period of 14 days. Culture of primary tumors was performed using a capillary cloning system (22). Six capillary tubes were utilized for each data point. To assure that an adequate single cell suspension had been used, the nonspecific toxin orthosodium vanadate was used as a positive control for inhibiting tumor colony formation (acceptable positive control = <30% survival of tumor colony-forming units with this agent). Vehicle buffer was included as a negative control with each tumor explant assay.

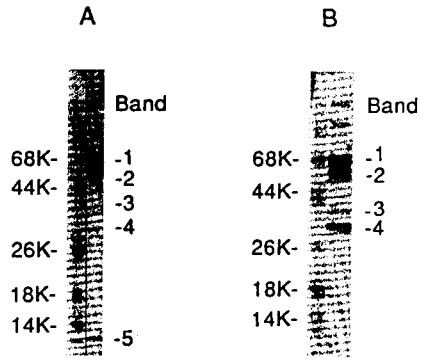
Three-Dimensional Gel Supported Primary Culture Assay. Human tumor tissue was grown on collagen gel rafts as previously described (23). Briefly, 1 mm^3 of tumor tissue was placed on a collagen gel raft in Eagle's minimal essential medium plus 10% fetal calf serum and antibiotics. Five to 7 days later, the medium was changed to MEM with rHu-MIS at 10 or 1 μg/ml. The medium was changed twice daily for 3 days after which [^{3}H]thymidine was included. Twice daily changes of medium continued for 4 more days. At each medium change fresh rHu-MIS was added at the concentrations indicated. A cocktail of cisplatin (15 μg/ml), doxorubicin (290 ng/ml), and melphalan (10 μg/ml) was used as the positive control for tumor cell killing. Vehicle buffer was used as the negative control. Autoradiography of the cultured tumor sections was used to count [^{3}H]thymidine localized to tumor cell nuclei.

Subrenal Capsule Assay. Fresh human tumor explants of ovarian, uterine, or fallopian tube origin were assayed for growth inhibition in SRC assays as previously described (24). Briefly, a 1-mm^3 piece of tumor was implanted under the renal capsule of normal immunocompetent BDF_1 mice. The mice were then treated with twice daily i.p. injections of rHu-MIS at 2 μg per injection. On Day 6 the mice were sacrificed and the size of the tumor implants were measured by ocular micrometer readings. Cisplatin injections (40 μg/mouse/day) were used as a positive control for inhibition of tumor cell growth. Separate groups of mice receiving vehicle buffer or phosphate buffered saline were used as negative controls.

RESULTS

Before the antiproliferative effects of rHu-MIS could be assessed two sets of preliminary experiments had to be completed. First, biologically active rHu-MIS protein had to be isolated and analyzed. Second, the growth properties of a panel of human gynecologic tumor cell lines had to be characterized *in vitro* and *in vivo*.

Characterization of rHu-MIS Protein. rHu-MIS was produced in 311-2A9B7 cells from a genomic clone of the human MIS gene. These cells secrete rHu-MIS into their culture medium. rHu-MIS was isolated from 311-2A9B7 cell conditioned medium by ion exchange, lectin affinity, and immunoaffinity chromatography (25). Fig. 1 shows a Coomassie-stained SDS-polyacrylamide gel and a Western blot of rHu-MIS. The rHu-MIS and breakdown products of rHu-MIS comprised >95% of the protein visualized in these gels. Amino terminal amino acid sequence analysis confirmed that the three major species, bands 1, 2, and 5, are derived from human MIS. Bands 2 and 5, are proteolytic fragments of rHu-MIS generated by cleavage at a site near the carboxy-terminus. Processing of rHu-MIS in this manner is analogous to the processing of TGF-β (26). The additional bands, 3 and 4, detected in Western blots are generated by a second minor cleavage.



N-TERMINAL SEQUENCE ANALYSIS:

Band 1- L R A E E P A V G T
Band 2- L R A E E P A V G T
Band 5- S A G A T A A D G P

Fig. 1. Photographs of SDS-polyacrylamide gel electrophoresis analyses of rHu-MIS run under reducing conditions. *A*, Coomassie staining of polyacrylamide gel used to visualize proteins; *B*, Western blot of polyacrylamide gel using an anti-human MIS polyclonal antibody. *Left lane*, prestained molecular weight markers; *right lane*, 6.0 μg of protein from the final step of rHu-MIS purification. Amino terminal sequence analysis was performed using material eluted from individual gel slices containing the indicated bands.

The rHu-MIS was quantitated by ELISA using an anti-human MIS monoclonal antibody (see "Materials and Methods"). The biological activity of the purified rHu-MIS was assessed in fetal rat Mullerian duct regression assays (see "Materials and Methods"). Biological activity was assessed immediately after isolation of rHu-MIS and at periodic intervals up to 5 months following storage at −70°C. rHu-MIS isolated and stored under these conditions retained full biological potency (Grade IV regression of Mullerian ducts using 6.25 μg/ml rHu-MIS).

Characterization of Human Cell Lines. Eleven human ovarian carcinoma cell lines and six human endometrial carcinoma cell lines were maintained in monolayer cultures. In addition, one human colon carcinoma cell line (SW-48) and one human epidermoid carcinoma cell line (A-431) were examined. Table 1 lists the cell doubling times and plating densities used for all 19 cell lines. The plating densities reflect the concentration of cells required to produce linear growth rates between 24 and 96 h following seeding into 96-well culture plates. Anchorage-independent growth in soft agarose and the ability of each cell line to form s.c. tumors in nude mice was also tested. Ten cell lines formed colonies in soft agarose while 12 cell lines produced s.c. tumors in nude mice.

Cell Proliferation Assays *in Vitro*. The effect of rHu-MIS on the growth of human tumor cell lines *in vitro* was measured in four independent assays. [^{3}H]Thymidine incorporation into newly synthesized DNA and reduction of the tetrazolium salt

Table 1 *Growth properties of human tumor cell lines*

	Approximate doubling time (h)	Plating density for 96-well trays	Soft agar colonies per 10^3 cells	Tumor growth in Nude mice	
				Sub Q	IP
Ovarian carcinoma cell lines					
CAOV-3	64	5×10^3	0	0	0
CAOV-4	165	2.5×10^4	0	0	0
NIH-OVCAR-3	21	1.2×10^4	20.9	+	0
PA-1	11	3×10^3	30	+	+
SKOV-3	17	7×10^3	0	+	+
SKOV-4	17	3×10^3	0	+	+
SKOV-6	21	3×10^3	0	+	+
SW626	25	1.2×10^4	0	0	0
A-7	19	6×10^3	0	0	+
A-10	17	6×10^3	0	0	+
Schict-1	24	6×10^3	20	+	+
Endometrial carcinoma cell lines					
AN3CA	23	7×10^3	142	+	+
HEC-1A	17	7×10^3	72.6	+	+
HEC-1B	13	6×10^3	74.5	+	+
KLE	14	5×10^3	0	0	0
RL95-2	116	5×10^4	54.8	+	+
Ishikawa	84	4×10^4	11.1	0	0
Other human carcinoma cell lines					
A431	36	5×10^4	46	+	NT[a]
SW48	60	5×10^4	135	+	NT

[a] NT, not tested.

Table 2 *Growth inhibition of human tumor cell lines in monolayer cultures*

Cells were plated in the presence of rHu-MIS, cell culture medium, or cisplatin (1.5 μg/ml) for 48, 72, or 96 h. Only the 72-h data is shown here. Similar results were obtained after 48 and 96 h of incubation. Data is presented as percentage inhibition compared to control cultures that did not receive drugs. Each result represents the mean values obtained from three independent experiments. [^{3}H] Thymidine incorporation values for each cell line ranged between 10^4 and 10^5 cpm per assay.

	[^{3}H]Thymidine incorporation rHu-MIS (μg/ml)			MTT assay				
				rHu-MIS μg/ml				
Cell line	1.0	0.1	0.01	10.0	1.0	0.1	Medium	Cisplatin
A431	0	7	0	0	0	0	0	99
SW48	0	0	0	NT[a]	NT	NT	NT	NT
A7	7	0	0	0	0	1	0	95
A10	0	6	0	0	2	0	4	90
CAOV3	0	0	0	4	0	7	0	90
CAOV4	0	0	0	0	0	10	0	100
SKOV3	0	0	0	0	0	0	3	94
SKOV4	0	0	0	0	0	0	10	98
SKOV6	0	3	0	9	0	2	0	86
NIH-OVCAR-3	0	0	0	7	0	5	0	100
PA-1	2	0	0	0	0	NT	0	99
Schict-1	0	0	7	11	0	11	0	85
SW626	3	5	9	2	0	0	0	87
AN3CA	1	0	4	0	5	3	0	50
HEC1A	0	0	0	0	0	0	0	84
HEC1B	0	0	8	0	0	NT	5	94
KLE	10	8	2	0	0	0	4	92
RL95-2	0	0	0	0	0	0	0	100
Ishikawa	0	5	0	4	0	NT	0	100

[a] NT, not tested.

MTT by mitochondrial enzymes (27) are biochemical parameters that reflect mammalian cell proliferation. Both of these parameters were measured in all nineteen cell lines at 48, 72, and 96 h following exposure to rHu-MIS. Concentrations of rHu-MIS up to 10 μg/ml were tested (Table 2). None of the cell lines exhibited significant growth inhibition in response to rHu-MIS in either of these assays. By contrast, cisplatin treatment (1.5 μg/ml) dramatically reduced cell growth (84–100%) in 18 of 19 cell lines. Both the [^{3}H]thymidine incorporation assays and the MTT cell proliferation assays were performed in monolayer cultures. To assess the capacity of rHu-MIS to inhibit anchorage-independent tumor cell growth, we exposed 10 cell lines to rHu-MIS and plated these cells in soft agarose. Only one cell line exhibited statistically significant growth inhibition in this assay. Schict-1 cells produced 13.4% fewer colonies in the presence of 10 μg/ml of rHu-MIS than in the presence of vehicle buffer (Table 3).

The [^{3}H]thymidine incorporation, MTT, and soft agarose colony inhibition assays relied on a single treatment of rHu-MIS. This form of therapy may not provide sufficient exposure time to rHu-MIS for inhibition of the growth of human tumor cell lines. Therefore, a cocultivation assay was designed to allow continuous exposure of the tumor cell lines to newly synthesized rHu-MIS. L2-58 cells were grown in plastic cups on nitrocellulose membranes that allow the diffusion of rHu-MIS but do not permit L2-58 cell migration out of the cup. The cups containing L2-58 cells were then placed in larger tissue culture wells containing the individual human tumor cell lines (see Fig. 2). rHu-MIS was quantitated by ELISA (Fig. 3) in the culture fluid within the L2-58 cell containing cups and in medium from the surrounding tissue culture well after 48 h of cocultivation. In each case rHu-MIS concentrations were approximately equal

Table 3 *Growth inhibition of human tumor cell lines in semisolid media and in nude mice*

	Geometric mean (GM) and % inhibition of tumor cell colonies		
Tumor cell lines	Buffer, GM (±SEM)	1 μg/ml MIS (% inhibition)	10 μg/ml MIS (% inhibition)
NIH-OVCAR-3	166.5 ± 5.5	<0	<0
PA-1	302.0 ± 3.5	<0	<0
Schict-1	870.0 ± 5.8	9.9	13.4[a]
AN3CA	306.8 ± 15.9	0.7	9.6
HEC1A	212.2 ± 31.4	<0	3.6
HEC1B	100.0 ± 1.0	<0	<0
KLE	246.9 ± 30.2	<0	6.0
RL95-2	120.5 ± 0.5	<0	<0
Ishikawa	124.6 ± 10.0	1.3	7.0
A431	343.2 ± 34.9	5.3	1.0

	Geomtric mean and % inhibition of tumor volume (cc)		
Tumor cell line	Human tumor cell line only (±SEM)	Human tumor cell line with L2-58 cells (±SEM) %	inhibition
SKOV3	0.40 ± 0.05	0.50 ± 0.11	<0
SKOV4	0.17 ± 0.04	0.21 ± 0.04	<0
SKOV6	0.23 ± 0.01	0.20 ± 0.01	11.7
AN3CA	8.60 ± 1.70	8.99 ± 0.68	<0
HEC1A	0.16 ± 0.06	0.21 ± 0.06	<0
HEC1B	0.33 ± 0.04	0.37 ± 0.04	<0
RL95-2	0.05 ± 0.13	0.43 ± 0.18	14.3
A431	5.03 ± 0.67	4.48 ± 0.96	10.9
SW48	2.37 ± 0.52	2.09 ± 0.14	11.9

[a] $P < 0.05$ by one-sided Dunnett's procedure (35). All other cell lines and drug doses failed to produce statistically significant differences in colony numbers or tumor volumes.

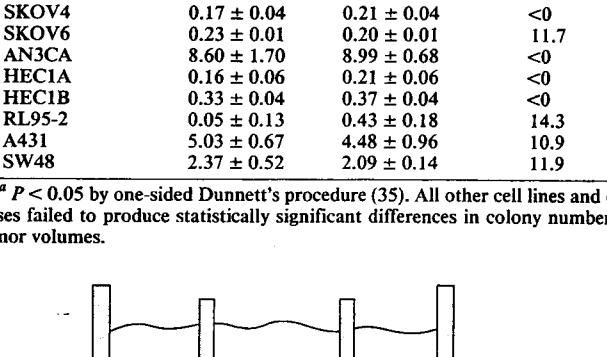


Fig. 2. Schematic diagram of cell culture chambers used in cocultivation experiments. Note, L2-58 cells producing rHu-MIS are physically separated from the human tumor cell line under investigation in the outer culture well.

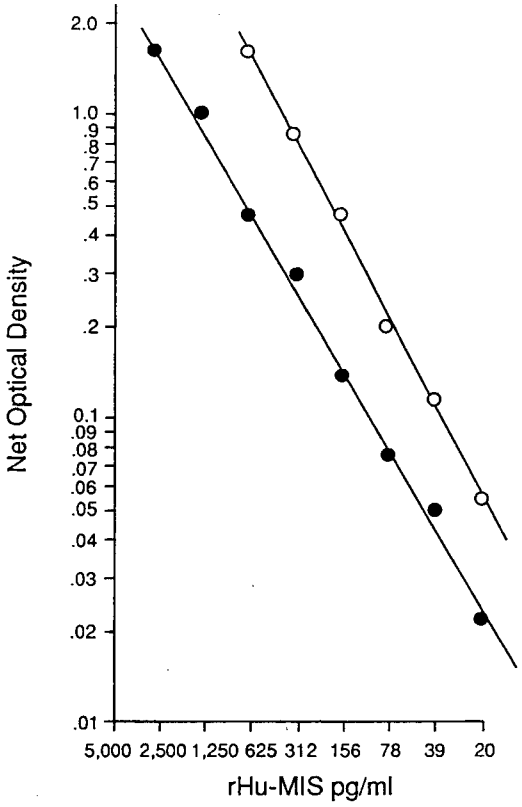


Fig. 3. Graphic representation of sandwich ELISA data used to quantitate rHu-MIS. ●, standardization experiments performed in mouse serum; ○, standardization experiments performed in cell culture medium.

Table 4 *Cocultivation of human tumor cell lines with cells producing rHu-MIS*

Human tumor cell lines	Cocultivation cell line: Parental CHO cells	Cocultivation cell line: CHO/L2-58 cells
A431	17.0[a]	0[b]
A7	—[c]	
A10	—[c]	
CAOV3	7.6	3
CAOV4	8.5	5
SKOV3	4.3	0
SKOV4	3.0	0
SKOV6	3.9	0
NIH/OVCAR-3	2.8	7
PA-1	3.5	0
Schict-1	—[c]	
SW626	3.4	0
AN3CA	18.0	0
HEC1A	15.0	0
HEC1B	17.0	0
KLE	3.8	0
RL95-2	8.6	0
Ishikawa	7.0	10

[a] Viable cell counts × 10^5. Each result represents the mean values from three independent experiments.
[b] Percentage inhibition of viable tumor cell numbers after 5 days.
[c] These cell lines were not viable after 48 h of cocultivation with the parental CHO cells.

inside and outside the cups ranging from 0.32 to 1.4 (median 0.75) μg/ml. After 5 days of cocultivation the number of viable tumor cells was determined. Despite the continuous exposure to freshly produced rHu-MIS, the growth of the tumor cell lines was not inhibited compared to control cultures cocultivated with CHO cells (Table 4).

Cell Proliferation Assays *in Vivo*. Although rHu-MIS did not affect the growth of tumor cell lines *in vitro*, it was possible that other factors not present in cell culture might be needed to enhance the antiproliferative effect of MIS. Therefore, a series of *in vivo* experiments were performed to assess the effect of continuous exposure to rHu-MIS on tumor cell lines grown in nude mice. L2-58 cells were used to form s.c. tumors in nude mice. Serum samples were taken from these animals to document the presence of circulating rHu-MIS. ELISA titers of rHu-MIS ranged from 0.4 to 100.0 (median 9.5) μg/ml of serum in mice bearing tumors >0.5 g. Eight human tumor cell lines were also inoculated s.c into the contralateral flanks of these animals. After 3 weeks of dual tumor growth, the mice were sacrificed and their tumors were examined. Each of the human tumor cell lines grew equally well in the presence or absence of L2-58 tumors that secreted rHu-MIS (Table 3).

Primary Tumor Explants *in Vitro*. Two independent assays were used to assess the effect of rHu-MIS on the growth of human primary tumor explants *in vitro*. Human tumor colony forming assays in soft agar and three-dimensional gel supported primary culture assays were performed on 84 tumor samples taken from 83 cancer patients. Twenty-four of 74 HTCFAs were judged adequate for evaluation as evidenced by adequate tumor colony formation and an antiproliferative response to the positive control agent, orthosodium vanadate. A ≥50% reduction in tumor colonies in the HTCFA is defined as a positive response (28). rHu-MIS at 10.0 μg/ml caused a >50% reduction in tumor colonies in three ovarian tumor cell explants. Ten other ovarian tumor explants were exposed to rHu-MIS, but none achieved the 50% level of growth inhibition. One of three small cell lung cancer explants exhibited >50% growth inhibition. Three breast, one colon, and four non-small cell lung cancer tumor explants were also examined but failed to exhibit significant growth inhibition (Table 5).

All 10 tumor explants tested in the three-dimensional GSPCA were judged adequate as evidenced by uptake of [^{3}H]-thymidine into the tumor cell nuclei and preservation of the histological architecture within the explant tissue. In this assay an 80% reduction in cell growth correlates with a positive response to therapy in patients.[3] One ovarian tumor explant exhibited 80% growth inhibition at 10 μg/ml of rHu-MIS. Seven other ovarian cancer, one carcinoma of the cervix, and one uterine cancer explant did not exhibit significant growth inhibition in response to rHu-MIS (Table 5). By contrast, all 10 tumor cell explants were suppressed >98% on exposure to a cocktail of cisplatin, doxorubicin, and melphalan.

Primary Tumor Explants *in Vivo*. To assess the effects of rHu-MIS on primary human tumor explants *in vivo*, 10 gynecological (six ovarian, three uterine, and one fallopian tube) tumor explants were placed beneath the renal capsule of BDF_1 mice. These mice received 20 μg of rHu-MIS by i.p. injections over a 5-day period. Tumor explant size was measured at the time of implantation and on Day 6. Nine of these explants were judged adequate for evaluation as evidenced by growth of the control samples (>−0.5 ocular micrometer readings) in the presence of vehicle buffer or saline. rHu-MIS did not induce statistically significant growth inhibition in any of these nine tumor explants (Table 6).

DISCUSSION

rHu-MIS did not inhibit the growth of any of the human tumor cell lines examined under the culture conditions of this study. Nineteen independent cell lines were tested in four *in vitro* assays designed to measure both biochemical and biological parameters of cell growth. None of these assays revealed a

[3] R. M. Hoffman, unpublished observations.

Table 5 *Growth inhibition of primary human tumor explants in HTCFAs and GSPCAs*

Tumor type	HTCFA no. responses[a]/no. evaluable (μg/ml) 0.01	0.1	1.0	10.0
Breast	0/3	0/3	0/3	0/3
Colon	0/1	0/1	0/1	0/1
Lung (non small-cell)	0/4	0/4	0/4	0/4
Lung (small cell)	0/3	1/3 (35%)[b]	0/3	1/3 (33%)[b]
Ovary	1/13 (36%)	2/13 (21, 36%)	1/13 (33%)	3/13 (21, 23, 40%)
Total	1/24	3/24	1/24	4/24
Tumor type	**GSPCA no. response[c]/no. evaluable (μg/ml) 0.01**	**0.1**	**1.0**	**10.0**
Ovary	0/8	0/8	0/8	1/8 (82%)[d]
Uterus	0/1	0/1	0/1	0/1
Cervix	0/1	0/1	0/1	0/1
Total	0/10	0/10	0/10	1/10

[a] Response is defined as ≤50% survival of colony forming units.
[b] The same tumor sample was responsive at 0.1 and 10.0 μg/ml but failed to respond at 1.0 μg/ml of rHu-MIS. Numbers in parentheses, percentage of surviving colony forming units.
[c] Response is defined as >80% reduction in [^{3}H]thymidine incorporation into nucleus. In all 10 cases the positive control treatment with cisplatin, doxorubicin, and melphalan produced 99% inhibition in [^{3}H]thymidine incorporation.
[d] Number in parenthesis, percentage inhibition in [^{3}H]thymidine incorporation.

consistent inhibition of cell proliferation in the presence of rHu-MIS. In those assays where rHu-MIS was associated with minimal growth inhibition, comparisons with other growth assays using the same cell line failed to substantiate an inhibitory response to rHu-MIS. For example, rHu-MIS treatment of KLE cells was associated with 10% inhibition in the [^{3}H]-thymidine incorporation assay, but treatment of the same cell line with the same or higher doses of rHu-MIS in the MTT assay had no effect on cell growth (Table 2). Since little is known about the mechanism of MIS antitumor activity, it was possible that rHu-MIS required postsynthetic modifications for full activity. These modifications might only occur *in vivo* through the action of blood or tissue factors. Alternatively, MIS antitumor activity might require the cooperation of nontransformed effector cells (*e.g.*, macrophages or natural killer cells) to inhibit tumor cell growth. Therefore, we tested the effect of rHu-MIS on eight tumor cell lines grown as s.c. tumors in nude mice. Again rHu-MIS had no effect on the growth rates of the human gynecologic tumor cell lines tested *in vivo*. Our current results differ from the previously published reports of MIS antiproliferative activity against human tumor cell lines. However, it should be noted that the present studies were carried out using highly purified (>95%) recombinant human MIS. The earlier studies of the antiproliferative effects of MIS were, of necessity, performed with MIS purified from bovine testes. The antiproliferative activity in these preparations may have been due to contaminants copurified with bovine MIS.

Human tumor cell lines are not always accurate predictors of the clinical utility of antitumor agents (29). Therefore, we also examined the antiproliferative effects of rHu-MIS on primary human tumor explants grown *in vitro* and *in vivo*. Forty-three independent human tumors were evaluated in one of three assays: 24 in human tumor colony forming assays, 10 in gel supported primary culture assays, and nine in subrenal capsule assays in mice. In contrast to the lack of inhibition by rHu-MIS in tumor cell lines, 11.6% (five of 43) of the primary tumor explants demonstrated significant growth inhibition. Most of these positive responses were recorded in the HTCFA where four of 24 (16.6%) tumor explants exhibited >50% reduction in tumor colony formation on exposure to rHu-MIS. These positive responses included three of 13 (23%) ovarian cancer explants and one of 11 nongynecological tumor explants. However, only one of 10 tumors examined in the GSPCA exhibited meaningful growth inhibition, and none of the tumors tested in the subrenal capsule assays *in vivo* exhibited significant growth inhibition. The one positive response in the GSPCA also occurred with an ovarian cancer explant. Therefore, the total response rate for ovarian cancer explants examined in HTCFAs and GSPCAs was four of 21 (19%). If the five ovarian cancer explants tested in the SRC assays *in vivo* are included, the overall response rate for ovarian cancer was four of 26 (15.4%). While the response rates reported here are not dramatic, it should be noted that false-positive antitumor effects are unusual (<3%) in these types of assays (30). Therefore, the limited responses obtained with rHu-MIS *versus* primary tumor explants probably represent genuine antiproliferative activity. Similar antiproliferative effects have also been observed with recombinant TGF-β treatment of human breast cancer explants.[4]

Table 6 *Growth inhibition of primary human tumor explants in subrenal capsule assays in mice*

Human tumor explants	Implant size (OMU[a]) ± SEM rHu-MIS	Cisplatin	Control[b]
Ovary	2.3 ± 2.8	1.0 ± 1.9	2.2 ± 1.8
Ovary	1.8 ± 1.1	1.5 ± 1.0	2.6 ± 0.9
Ovary	4.0 ± 2.4	1.6 ± 2.1	2.5 ± 1.5
Ovary	1.3 ± 1.9	1.0 ± 1.0	1.8 ± 1.3
Ovary	2.1 ± 1.8	3.6 ± 4.1	1.0 ± 3.3
Ovary	−1.3 ± 2.5	−2.8 ± 2.2	−1.1 ± 1.6
Fallopian tube	−0.7 ± 2.9	−2.6 ± 2.3	0.1 ± 1.8
Uterus	1.3 ± 1.9	−4.8 ± 1.4	1.0 ± 2.4
Uterus	0.3 ± 1.8	−0.1 ± 1.1	0.0 ± 1.1
Uterus	−2.3 ± 1.4	−1.2 ± 1.1	−0.2 ± 2.9

[a] OMU, ocular micrometer units.
[b] Control explants were treated with either vehicle buffer or PBS.

The maximum dose of rHu-MIS used in these studies was 10 μg/ml. All five of the primary tumor explants that responded in rHu-MIS did so at this maximum dose. Three of these explants also responded to one or more lower doses of rHu-MIS. It is unclear if higher doses (>10 μg/ml) of rHu-MIS would have produced growth inhibition in a larger percentage of primary tumor explants. Ten μg/ml of rHu-MIS is approximately 71 nM. Most protein hormones that affect cell proliferation exert their biological effects at concentrations below this level. For example, platelet-derived growth factor affects cell proliferation at concentrations below 1 nM (31) and TGF-β,

[4] D. D. Von Hoff, unpublished observations.

which bears amino acid homology with MIS, can inhibit tumor cell growth at concentrations <50 pM (32). It should also be noted that samples of rHu-MIS from the same lot used in our cell proliferation assays reproducably caused Grade IV regression of Mullerian ducts at 6.25 μg/ml. Therefore, the dose range of rHu-MIS (0.01–10.0 μg/ml) explored in our studies should have been adequate to assess the antiproliferative effects of this hormone.

The potential clinical utility of rHu-MIS remains to be established. The studies reported here suggest that the antiproliferative effect of rHu-MIS on human gynecological tumor cells is limited to ovarian cancers. However, more ovarian tumors were sampled than any other type of cancer. Additional tumor explants from a variety of human cancers must be examined before the precise spectrum of rHu-MIS' antitumor activity can be defined. Nonetheless, an overall response rate of 15.4–23% in ovarian tumors is intriguing. These rHu-MIS responsive tumors may define a subset of ovarian cancers. Histological examination of the ovarian tumors accessed in this study did not reveal any characteristics that distinguished responsive explants from nonresponders. However, the MIS receptor status of these tumors was not determined. Estrogen receptor status is clearly useful in defining subsets of breast cancer patients who are likely to respond to hormonal therapy (33, 34). Similarly, MIS receptor status on ovarian cancer cells may have predictive value in identifying ovarian tumors that will respond to rHu-MIS. If MIS receptor positive tumor cells are generally growth inhibited by rHu-MIS, then it may be possible to develop rHu-MIS as an antitumor agent for this subset of cancer patients.

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