Highly Purified Müllerian Inhibiting Substance Inhibits Human Ovarian Cancer *in Vivo*[1]

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ABSTRACT

Purpose: **Müllerian inhibiting substance (MIS) causes Müllerian duct regression in mammalian, avian, and reptilian embryos; MIS also inhibits growth *in vitro* of Müllerian-derived cell lines and primary cells from human ovarian carcinomas. We hypothesize that highly purified MIS delivered parenterally inhibits ovarian cancer *in vivo*.**

Experimental Design: **To test the efficacy of highly purified MIS against ovarian cancer cell lines *in vivo*, we treated immunosuppressed mice with MIS after implanting OVCAR 8 or IGROV 1 human ovarian cancer cells beneath the renal capsules and measured tumor volume over time. Animals were treated with daily injections of 10 μg of purified exogenous recombinant human MIS or by endogenous MIS secreted from cells growing on biodegradable mesh.**

Results: **The average graft size ratio (change in size compared with starting size) of the OVCAR 8 tumor implants was larger in the control animals than in animals treated for 2 weeks ($P < 0.019$) or 3 weeks ($P < 0.001$) with parenteral MIS, or after treating with MIS produced from transfected cells, which impregnated the biodegradable mesh ($P = 0.02$). The average graft size ratio of the IGROV 1 tumors was also larger in the control animals than in those treated with injected MIS ($P = 0.0174$).**

Conclusions: **Highly purified recombinant human MIS, delivered parenterally, or MIS produced endogenously causes inhibition of human ovarian cancer cell lines *in vivo*, providing convincing preclinical evidence to support the use of MIS as a parenteral agent for the treatment of ovarian cancer.**

INTRODUCTION

MIS[3] is a M_r 140,000 disulfide-linked glycoprotein homodimer (1, 2), which induces regression of the Müllerian ducts in the male fetus during prenatal development (3). It is a member of the large transforming growth factor β polypeptide family that functions to regulate tissue growth and differentiation (4, 5). In the absence of MIS, the Müllerian ducts in the female normally develop into the uterus, Fallopian tubes, and upper vagina. Human ovarian epithelial carcinomas, the most common and lethal of the ovarian cancers, are often referred to as Müllerian tumors because they arise from the coelomic epithelium, which invaginates to form the Müllerian duct (6). Because of its role in regression of the Müllerian structures in fetal development, we have proposed that MIS could inhibit tumors of Müllerian origin (7, 8).

There is abundant evidence suggesting a role for MIS in postnatal ovarian function and growth regulation, and in the inhibition of ovarian carcinomas. It is present in females after sexual differentiation, and throughout postnatal and adult life (9, 10). MIS inhibits epidermal growth factor-induced proliferation of and progesterone production by human granulosa/luteal cells *in vitro* (11); it also inhibits oocyte meiosis in the rat (12) and completely ablates the ovary in older transgenic mice producing high levels of MIS (13, 14). Purified MIS inhibits several human ovarian cancer cell lines of Müllerian origin *in vitro* (15–17), and the MIS type II receptor is expressed in these cell lines and in the ascites cells of patients with ovarian cancer (17) providing additional evidence that human ovarian cancer may be a target for MIS as a potential chemotherapeutic agent. To investigate this hypothesis, a number of different preparations were tested *in vivo*.

Tail vein injections of MIS purified from bovine sources caused regression of rat urogenital ridges heterotopically implanted under the renal capsule of immunosuppressed mice (18), demonstrating that MIS traverses the bloodstream and retains biological activity against transplanted fetal Müllerian tissue. This same bovine MIS inhibited the growth of HOC 21, a human ovarian serous cystadenocarcinoma cell line (8). Blood samples from these animals also contained the injected bovine MIS as measured by ELISA. rhMIS delivered via Alzet pumps placed in the peritoneal cavity of nude mice inhibited the growth of a human ocular melanoma cell line implanted beneath the

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[3] The abbreviations used are: MIS, Müllerian inhibiting substance; rhMIS, recombinant human Müllerian inhibiting substance; SCID, severe combined immunodeficient; CHO, Chinese hamster ovary; FPLC, fast performance liquid chromatography; GSR, graft size ratio.

renal capsule (19) and markedly diminished lung metastases of the same tumor after tail vein injections. Also, ocular melanoma cells transfected with the *MIS* gene showed fewer lung metastases after tail vein injections when compared with injected cells transfected with a mutated form of MIS rendering it functionally inactive (20).

The naturally occurring fetal regressor MIS has been shown recently to inhibit the growth of human ovarian cancer cell lines *in vitro* (17, 21). OVCAR 8 is a human ovarian cancer cell line which expresses the MIS type II receptor, and colony growth of this cell line is markedly inhibited *in vitro* both by exogenous treatment with MIS (17) and by MIS secreted after transfection into the target cells (21). Growth inhibition correlates with a block in cell cycle progression and is characterized by apoptosis. These events are mediated by induction of the cyclin dependent kinase inhibitor p16, which results in upregulation of the E2F family of transcription factors that are regulated by members of the pocket protein family (21). IGROV 1 is a human ovarian cancer cell line, which also expresses the MIS type II receptor and responds to MIS *in vitro*. Before proceeding with a clinical trial, it is important to show that growth of a human ovarian cancer can be inhibited *in vivo* by a parenterally delivered MIS preparation of high purity anticipated for use in a clinical trial. Because there are no animal models that produce tumor of the same variety as the human ovarian cancer, we continued to use the model of human ovarian cancer cell lines xenotransplanted into immunosuppressed mice. Biologically active highly purified rhMIS prepared from serum-free (22) or serum-containing medium of MIS transfected cells (23) and delivered parenterally inhibited growth of the OVCAR 8 and IGROV 1 human ovarian cancer cell lines to a degree comparable with the inhibition caused by MIS secreted directly *in vivo* from an implanted polymer-cell graft (15). These experiments contribute to the body of evidence needed to justify undertaking clinical trials of MIS as a chemotherapeutic agent for ovarian cancer.

MATERIALS AND METHODS

Animals. Female athymic nude mice, SCID mice, and RAG 2 deficient mice (Ref. 24; 6 weeks old; 18–20 g) were obtained from the Edwin L. Steele Laboratory, Massachusetts General Hospital, Boston, MA. In some experiments, the mice were additionally immunosuppressed by whole body radiation at a dose of 5 Gy 1 day before tumor implantation (25, 26). All of the experiments conformed to Association for Assessment and Accreditation of Laboratory Animal Care guidelines and were approved by Massachusetts General Hospital Institutional Animal Care and Use Committee.

Cells. OVCAR 8 and IGROV 1 human ovarian cancer cell lines were maintained in DMEM with glutamine, penicillin, streptomycin, and 10% female FCS. Cells were grown at 37°C in a humidified chamber in 5% CO_2 in air. Cells were passed 1:4 when 80% confluent and, because the MIS responsiveness of these cells decreases with high passage number, the number of passages did not exceed 12. Before *in vivo* implantation, OVCAR 8 and IGROV 1 cells were tested for responsiveness to MIS *in vitro* in a colony inhibition assay (17, 21). Nontransfected CHO cells and the CHO B9 cell line transfected with wild-type human genomic MIS (27) were maintained in DMEM with glutamine, penicillin, streptomycin, methotrexate, and 5% MIS-free female FCS. All of these cells were tested for growth in each of the immunosuppressed mouse strains. IGROV 1 cells did not grow in the nude mice but grew well in SCID mice; therefore, this strain was used for additional experiments with IGROV 1 cells. OVCAR 8 cells grew in nude mice and in RAG 2-deficient mice, and CHO cells grew in nude mice; therefore, nude mice were used for OVCAR 8 injection and biodegradable mesh experiments.

Subrenal Capsule Assay. The OVCAR 8 and IGROV 1 tumor cell lines were tested for growth *in vivo* after implantation beneath the renal capsule of immunosuppressed mice following established methods (18, 28, 29). Growth was assessed in irradiated nude mice, nonirradiated nude mice, SCID mice, and RAG 2-deficient mice. To avoid spreading of cells, as occurs by injection in suspension, tumor cells were fixed in fibrin-thrombin clots. Ten million cells were mixed with 300 μg of fibrinogen in PBS (pH 7.4) and 0.16 units of thrombin in double-strength DMEM, and incubated at 37°C for 10–15 min. After incubation, the cell clot was maintained on ice and cut into ~50 fragments (200,000 cells each). After ketamine/xylazine (100/10 mg/kg body weight, i.m.) anesthesia, the left kidney was accessed through a small flank incision and a subcapsular space developed with a #11 scalpel blade; a cell clot of 10 × 10 ocular micrometer units was loaded onto the tip of a 19-gauge needle trocar and introduced beneath the renal capsule. The longest diameter (L1) of the implant and the diameter perpendicular to the longest diameter (W1) were measured using an ocular micrometer on a dissecting microscope. The graft size was then estimated as L1 × W1 × W1. In preliminary experiments dozens of grafts were weighed, and it was determined that they average ~10 μg each at the time of implantation and have a volume of 10 μl. This size is used because larger implants induce tears in the renal capsule. Tumors were remeasured at weekly intervals at the same focal distance and the GSR [(L2 × W2 × W2)/(L1 × W1 × W1)] calculated. The duration of the experiment was chosen as 3 weeks to permit controls to grow at least 3–4-fold so that inhibition could be observed.

Purification of MIS. CHO cells were transfected with wild-type human genomic MIS (27) and a high producing clone, the CHO B9 cell line, was selected. The rhMIS used to test inhibition of OVCAR 8 *in vivo* was secreted from the CHO B9 cells into chemically defined serum-free medium (22) then purified by ammonium sulfate precipitation or lectin affinity chromatography, either of which was followed by anion exchange chromatography (22). Separation by gel electrophoresis revealed a homogenous product (Fig. 1).

MIS secreted from CHO B9 cells into serum-containing medium was used to test inhibition of IGROV 1 growth. This preparation was purified using immunoaffinity chromatography, as described earlier (23), by loading onto a column of Sephadex 4β beads coated with monoclonal antibody (6E11), which recognizes holo MIS (9), and eluting with 20 mM HEPES (pH 3.0).

The resulting MIS fractions were desalted and concentrated into PBS using a Centricon device, the protein quantified by Bradford, and MIS measured by a human MIS-specific ELISA (30). The ELISA does not detect the mouse protein; therefore, all of the MIS measured in the serum was the human protein

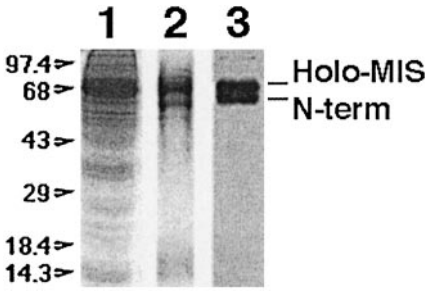

Fig. 1 Purified bioactive rhMIS. Silver-stained SDS PAGE gel (reducing conditions) of ~1 μg of total protein of serum-free medium of CHO cells expressing MIS (*Lane 1*) and of peak of protein purified by serial FPLC (*Lane 2*); corresponding Western analysis of the same blot transferred to a membrane and incubated with a polyclonal antibody raised to holo-MIS (*Lane 3*).

either injected or secreted by the bioengineered tissue. An organ culture assay for regression of the Müllerian ducts (31), and a colony inhibition assay against OVCAR 8 (21) and IGROV 1 (17) was used to test the bioactivity of the purified material.

Injection of Animals with MIS. Selected nude mice injected with MIS were irradiated with 5 Gy whole body irradiation 24 h before OVCAR 8 tumor implantation. On the day of OVCAR 8 tumor implantation, each animal was given 10 μg of purified MIS in 100 μl of PBS by intraperitoneal or i.m. injections, which were alternated to minimize injection trauma in any one site. Animals similarly injected with the same volume of only PBS served as controls. In one experiment, the animals were injected for 2 weeks and the OVCAR 8 tumors harvested 1 week later, and in a second experiment the animals were treated for 3 weeks and the OVCAR 8 tumors harvested the next day. In a third injection experiment, the animals were treated for 3 weeks with daily i.p. injections and IGROV 1 tumors harvested the next day. At the time of tumor harvest, the final tumor size was measured in each animal, and the GSR of MIS treated and untreated animals calculated; immediately after the final tumor measurement, the MIS levels in the serum of the mice was determined by ELISA and the kidney harvested for histological evaluation of tumor growth and invasion.

Preparation and Implantation of MIS-producing Polymer Cell Graft. To compare endogenously produced MIS to exogenously delivered highly purified MIS, we constructed MIS secreting biodegradable polymer cell grafts. The grafts were prepared and implanted into nonirradiated nude mice as described (15). Briefly, biodegradable 1-mm thick polymer sheets of nonwoven fibers of polyglycolic acid, density 70 mg/ml, fiber diameter 14 μm, and average pore size 250 μm (Smith and Nephew, York County, United Kingdom), were sectioned into squares 0.5 cm on each side, an optimal size for implantation in the ovarian pedicle (15). The polymer squares were then sterilized and coated with collagen (Vitrogen 0.0 3 mg/ml in sterile PBS). MIS-transfected CHO B9 or nontransfected wild-type CHO cells ($1–2 \times 10^6$) both suspended in 1 ml of DMEM with 10% female FCS were seeded onto each polymer square and incubated for 1–2 h at 37° in 5% CO_2 to permit absorption of the cells onto the interstices of the polymer. Fresh medium was added and the incubation continued for 3 days. MIS in the medium was assessed serially using an MIS-specific ELISA (9) and an MIS-specific organ culture assay (30, 31).

Three days after seeding with CHO B9 cells the polymer cell graft was implanted into the right ovarian pedicle of nude mice (15, 32). After ketamine-xylazine anesthesia, a 1-cm incision was made in the right flank, and the polymer cell graft was sutured onto the ovarian pedicle with 6–0 prolene. One week later, a fibrin/thrombin clot containing the OVCAR 8 cells was prepared and implanted under the left renal capsule as described above; MIS in the serum of the mice was measured just after tumor implantation using an ELISA specific for human MIS (9). Three weeks after implantation of the OVCAR 8 cell clot, the tumors were measured, the GSR calculated for comparisons between animals implanted with the CHO and CHO B9 polymer, and MIS levels measured by ELISA (9).

Tissue Analysis. The tissue formed from the OVCAR 8 and IGROV 1 cells implanted in fibrin-thrombin clots under the capsule of the left kidney were removed, and the entire kidney fixed in 5% picric acid and 15% formalin in PBS. After tissue processing, 8 μm transverse sections taken at the midpoint of the tumor to beyond the edge of the tumor were stained with H&E for histological analysis to assess viability of the tumors. The CHO B9 biodegradable mesh implants were fixed, sectioned, and stained for routine histological analysis and for immunohistochemistry for MIS (33).

Statistics. GSRs are expressed as mean ± SE. An unpaired *t* test performed by STATVIEW and an ANOVA performed on an EXCEL program were used to determine statistical significance. A $P \leq 0.05$ was considered significant.

RESULTS

Tumor Growth in Immunosuppressed Animals. The OVCAR 8 cells did not grow in SCID mice but formed measurable tumors under the renal capsule of nonirradiated nude, irradiated nude, and RAG 2-deficient mice, reaching a GSR of ≥3–4, 3 weeks after implantation. Histological analysis showed well-formed growths with neovascularization over the surface of the tumor and from the underlying kidney. There was minimal necrosis and inflammatory infiltrate. The CHO cell lines grew well in Swiss nude mice and SCID mice but not in RAG 2-deficient mice. Therefore, nude mice were used for additional studies of OVCAR 8 cells. The IGROV 1 cells formed measurable tumors in the subrenal capsule of SCID mice as confirmed by histological analysis but did not grow well in nude mice. Therefore, SCID mice were used for subsequent IGROV 1 experiments. Because CHO cells did not grow in RAG2 mice it would not be possible to compare results from MIS injections with those of the bioengineered tissue; no additional studies were performed with these animals.

Assays for rhMIS. Purification to homogeneity as determined by gel electrophoresis under reducing conditions was achieved by FPLC of serum-free medium (Ref. 22; Fig. 1). The concentration of protein and purified rhMIS used in the experiment was determined by the Bradford method and by an MIS-specific ELISA, and 10-μg aliquots in 100 μl of PBS were prepared for injection. The purified rhMIS was active in the Müllerian duct regression assay and in a colony inhibition assay against OVCAR 8. When measured 24 h after injections on days

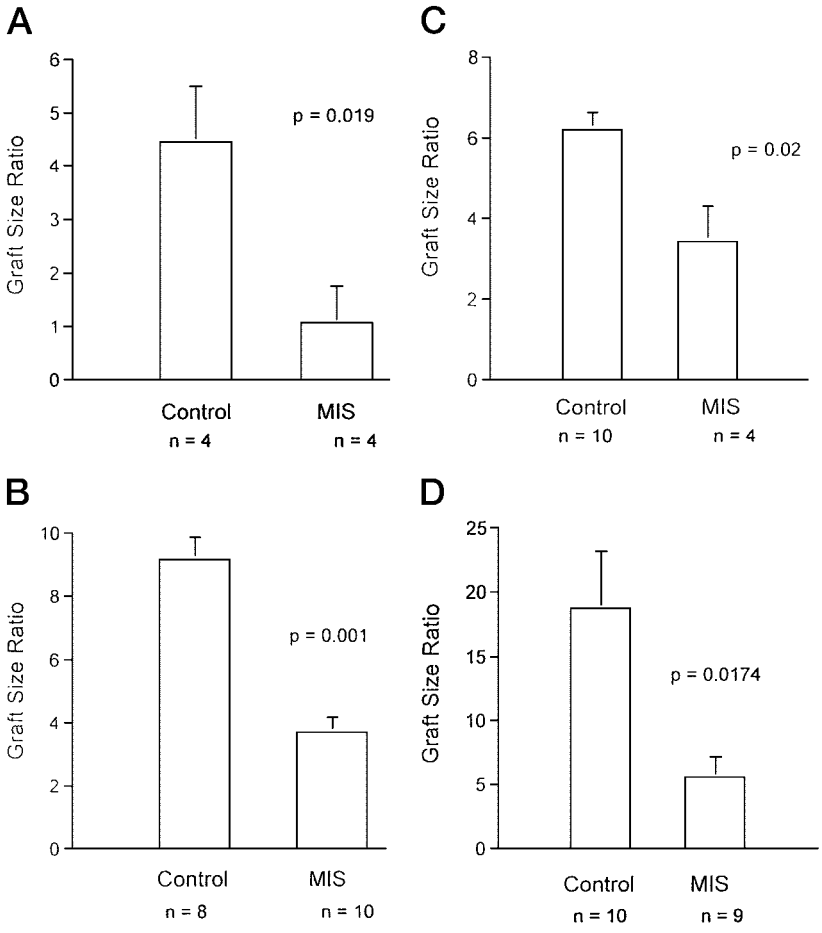

Fig. 2 Highly purified MIS inhibits ovarian cancer cell growth *in vivo*. *A*, the mean (GSR) of OVCAR 8 tumors implanted in MIS-treated nude mice (10 μg/day × 2 weeks; $n = 4$) was significantly smaller than the mean GSR of the same tumors implanted in mice treated with PBS ($n = 4$; $P = 0.019$). *B*, the GSR of the OVCAR 8 tumors implanted in MIS-treated nude mice (10 μg/day × 3 weeks; $n = 10$) was significantly smaller than the GSR of the same tumors treated with PBS ($n = 8$; $P = 0.001$). *C*, the mean GSR of OVCAR 8 tumors implanted in MIS-treated irradiated nude mice ($n = 4$) was significantly smaller than the mean GSR of the same tumors implanted in PBS treated mice ($n = 10$; $P = 0.02$). *D*, the mean GSR of IGROV 1 tumors implanted in MIS-treated SCID mice (10 μg/day × 3 weeks; $n = 9$) was significantly smaller than the mean GSR of the same tumors implanted in PBS treated mice ($n = 10$; $P = 0.017$); *bars*, ±SD.

4 and 5, the FPLC-purified MIS was detectable in the serum of all of the animals at levels of 69 ± 31.2 and 112 ± 37.7 ng/ml (mean ± SE) in nude mice, with similar levels in SCID mice ($n = 5$ for each day). Aliquots of immunoaffinity purified MIS used in the IGROV 1 *in vivo* experiment also showed complete regression of the Mullerian duct (30) and caused inhibition of colony growth (17). Furthermore, immunoaffinity purified MIS also was detectable in all of the treated mice at 24 h after injection on days 4 and 5, at average levels in SCID mice of 24 ± 9.1 and 64 ± 29.8 ng/ml ($n = 5$ for each day), with similar levels in nude mice and at a level of 54.1 ± 8.7 ng/ml on day 18 of the experiment, 24 h after the most recent injection.

Production of MIS *in Vivo* by Polymer Cell Graft. Physiological levels of serum MIS (200–400 ng/ml), as described previously (15), were reached at 10 days after animals were implanted with the CHO B9 polymer cell graft; therefore, the OVCAR 8 cells were implanted under the capsule of the left kidney 1 week after implantation of the MIS producing graft, and tumor size followed for 3 weeks. At the time of tumor harvest the serum MIS levels in the animals implanted with a polymer cell graft averaged 15 μg/ml in each animal as measured by the MIS ELISA (9). However, the rate of rise was not determined. The grafts grew to an average of 1 cm diameter and were limited to the ovarian pedicle without invasion of adjacent organs or peritoneal spread, and continued MIS production by the graft was confirmed by immunohistochemistry as described previously (15).

Inhibition of Tumor Growth by MIS. The OVCAR 8 tumors in the nude mice control population ($n = 4$) reached a GSR of 4.439 ± 1.064 after 3 weeks of growth. The tumors implanted in animals injected with the purified MIS at a dose of 10 μg/day for 2 weeks ($n = 4$) and measured at 3 weeks achieved a mean GSR of 1.065 ± 0.69 after 3 weeks of growth. This difference was statistically significant ($P = 0.019$; Fig. 2*A*). In a separate experiment in which MIS was injected for 3 weeks, the OVCAR 8 tumors implanted in animals injected with PBS ($n = 8$) achieved a mean GSR of 9.16 ± 0.72, whereas animals injected with the purified MIS ($n = 10$) at a dose of 10 μg/day achieved a mean GSR of 3.71 ± 0.471 ($P = 0.001$; Fig. 2*B*). The OVCAR 8 tumors implanted in animals containing the nontransfected CHO polymer ($n = 10$) achieved a mean GSR of 6.22 ± 0.417, whereas tumors implanted in animals containing the bioactive MIS producing CHO B9 polymer ($n = 4$) achieved a mean GSR of 3.43 ± 0.885. This difference was statistically significant ($P = 0.02$; Fig. 2*C*). IGROV 1 tumors implanted in SCID mice injected with 100 μl of PBS ($n = 10$) achieved a GSR of 18.7 ± 4.5, whereas SCID mice treated with MIS ($n = 9$) achieved a GSR of 5.6 ± 1.6 after 3 weeks of growth *in vivo*

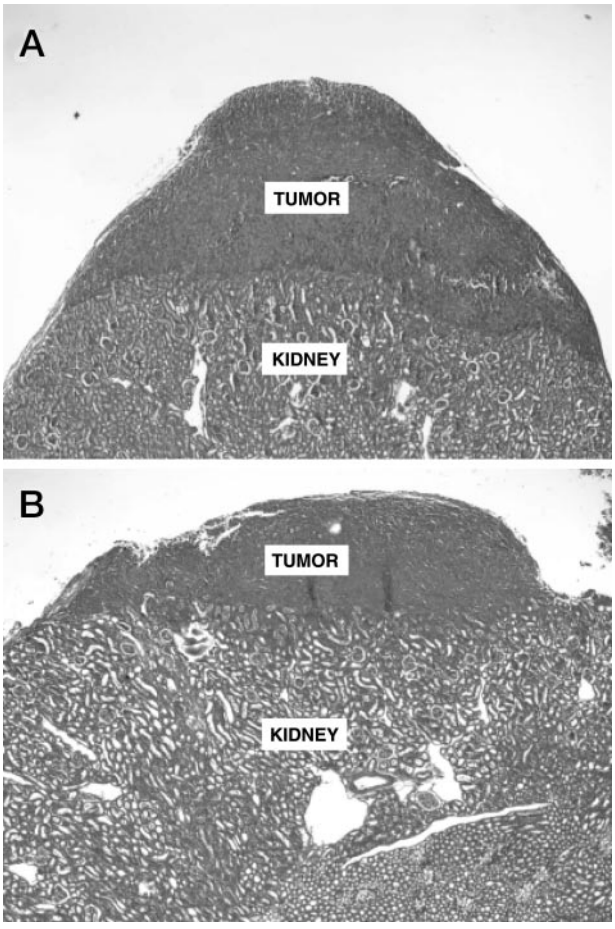

Fig. 3 OVCAR 8 tumors implanted under the renal capsules of nude mice after 3 weeks *in vivo*. *A*, control tumor shows neovascularization and minimal necrosis or inflammation. (magnification, ×40). *B*, MIS-treated tumor (10 μg/day × 3 weeks delivered i.p.) is about one-third the size of the control tumor and has minimal necrosis or inflammation. (magnification, ×40).

($P = 0.0174$; Fig. 2*D*). At the end of all of the experiments, the mice looked healthy with no differences in weight and no apparent infections. Histological analysis after H&E staining confirmed that the size measured represented viable tumor (Fig. 3).

DISCUSSION

Because we carefully defined a molecular pathway (21), which correlated with growth inhibition in the Müllerian-derived human ovarian cancer cell line OVCAR 8 *in vitro* (17), namely up-regulation of the cyclin-dependent kinase inhibitor protein p16 and of the retinoblastoma or "pocket protein" family regulated transcription factor E2F1 mRNA (21), we wished to document inhibition of growth of this tumor *in vivo* using a highly purified preparation of rhMIS, which is suitable for testing in humans. The MIS used for the injections to treat the OVCAR 8 tumors in nude mice was rhMIS expressed in CHO cells, secreted into chemically defined serum-free medium, and concentrated by ammonium sulfate precipitation or lectin affinity followed by anion exchange FPLC (22); its bioactivity was confirmed before injection in the standard organ culture assay for regression of the Müllerian duct (30, 31), and by activity against OVCAR 8 and IGROV 1 *in vitro* in colony inhibition assays. This MIS, which was also detected in the serum of the mice by a sensitive ELISA at selected intervals after parenteral injection, caused inhibition of OVCAR 8 growth. This method of purification provides bioactive MIS in sufficient quantities for preclinical *in vivo* testing described here. MIS produced endogenously from CHO B9 cells grown on a biodegradable polymer scaffold also caused inhibition of the OVCAR 8 human epithelial ovarian cancer cell line, and the degree of growth inhibition achieved with the injected purified material was comparable. We demonstrated that exogenously delivered MIS was also active against another human ovarian cancer cell line, IGROV 1, which responded previously to MIS delivered from implanted biodegradable mesh (15).

Both of these human ovarian cancer cell lines, when tested *in vitro* in colony inhibition assays, were sensitive to MIS, and the inhibition was dose dependent (17). These cells were subjected to no more than 12 passages before use in the *in vivo* assay to avoid the possibility of loss of responsiveness to MIS; moreover, *in vitro* responsiveness to MIS was confirmed in colony inhibition assays just before *in vivo* experimentation. To demonstrate growth inhibition *in vivo*, it was first necessary to document adequate growth of both cell lines in the subrenal capsule assay in a series of immunosuppressed mice, including athymic nudes, both untreated and treated with whole body radiation, SCID mice, and RAG 2-deficient mice; OVCAR 8 cells did not grow beneath the renal capsule of SCID mice. Because CHO cells also grew in nude mice, this strain was used for subsequent studies of OVCAR 8 implanted tumors. IGROV 1 tumors grew in SCID mice, which were used for subsequent studies of injected MIS. Because the growth of xenotransplanted cell lines varies considerably between immunosuppressed mice strains, growth curves must be tested in each strain for each new tumor line before conducting experiments.

Variations in growth of the control tumors between experiments may be because of consistency of the fibrin/thrombin clot. Exact cell numbers implanted in each clot are extrapolated from the total beginning number, because we cannot extract the cells for direct counting without causing lysis. The cell cycle phase of implanted cells was partially controlled from experiment to experiment by synchronizing the cells by plating a set number of cells, growing the cells to near confluency, then dividing 4:1, and repeating this process twice before harvesting the cells at 70–80% confluency at the time of each experiment.

To compare results between experiments the percentage of change from tumor size at implantation was calculated for control and MIS-treated OVCAR 8 animals. At 2 weeks there was 98.5% inhibition of growth ($n = 4$), at 3 weeks 71.3% ($n = 10$), and at 3 weeks exposure to the mesh 62.4% ($n = 4$), giving an overall inhibition of 75.3% for all of the experiments.

The polymer cell graft continuously produces MIS, whereas injection of the purified protein provides bolus dosing; the ideal dosing regimen for MIS has yet to be elucidated and is currently being studied in our laboratory. For example, the rise to supraphysiologic levels of MIS delivered from the biodegradable mesh does not occur until after 2 weeks (15). Hence, longer

periods may be required before implantation of the target tumor beneath the renal capsule. The transfected CHO cells used currently in the polymer cell graft are both transformed and tumorigenic, and, hence, would not be appropriate for clinical use. Human fibroblasts transfected with the human *MIS* gene have been clonally selected for production of rhMIS; those producing MIS at high levels will be grown on polymer scaffolds for implantation in nude mice.

Epithelial ovarian tumors are the most common human ovarian carcinomas, accounting for >90% of all of the human ovarian cancers. The majority of these cancers present as stage III or IV disease, with peritoneal implants at the time of diagnosis. The introduction of platinum-based and Taxol chemotherapy has improved early survival, but unfortunately, because of recurrence of tumors resistant to these drugs, the 5-year mortality rate for advanced disease remains at 70% (34, 35). The subrenal capsule assay can be used to: (*a*) establish the most effective dose schedules for MIS; (*b*) determine its efficacy in combination with other chemotherapeutics such as Taxol or cisplatin; and (*c*) test responsiveness to MIS after the tumors develop drug resistance to standard agents. This assay will also be important to test the efficacy of MIS against: (*a*) target tumors after they have been established for periods of time; and (*b*) other tumors that are potential targets for MIS. For example, we have shown recently that MIS regulates normal breast proliferation and development *in vivo* (36), and can inhibit the growth of human breast cancer cell lines *in vitro* (37); growth of these human breast tumors will be tested in immunosuppressed mice strains using MIS administered either exogenously or endogenously as described above.

We have shown that the highly purified preparations obtained by FPLC can be detected *in vivo* after parenteral delivery by an MIS ELISA. We have also shown that the MIS preparations maintain functional efficacy by exerting a growth inhibitory effect *in vivo* against at least two different xenografted human ovarian cancer cell lines. Given these results, it will be appropriate to initiate clinical trials of MIS against human ovarian cancer in hopes of establishing its role as a promising adjuvant therapy for these highly lethal tumors once more formal pharmacology of this highly purified preparation of MIS has been determined and toxicology studies are performed. MIS may hold particular promise in the treatment of minimal residual disease, which is apparently present and undetectable when patients discontinue standard therapy, or against disease that has documented resistance to Taxol or cisplatin. In fact, these may be its most important applications.

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