Human Müllerian Inhibiting Substance Inhibits Tumor Growth in Vitro and in Vivo

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ABSTRACT

Müllerian inhibiting substance (MIS) causes regression of the müllerian duct in the male fetus. Bovine MIS has been reported to inhibit the growth of some gynecological tumors. Recombinant human MIS (rhMIS) produced in transfected Chinese hamster ovary cells has been highly purified by immunoaffinity chromatography. The introduction of a salt wash prior to elution of MIS from the affinity column removes a growth-stimulating factor(s) derived from Chinese hamster ovary cells. This immunopurified rhMIS caused significant inhibition (34–59% survival) of A431 (a vulvar epidermoid carcinoma), HT-3 (a cervical carcinoma), HEC-1-A (an endometrial adenocarcinoma), NIH:OVCAR-3 (an ovarian adenocarcinoma), and OM431 (an ocular melanoma) human cell lines in colony inhibition assays. Two cell lines, Hep 3B (a hepatocellular carcinoma) and RT4 (a bladder transitional cell papilloma), were unresponsive to immunopurified rhMIS. Using in vivo subrenal capsule assay in irradiated CD-1 mice, the growth of A431 and OM431 cells was inhibited by immunopurified rhMIS. We conclude that rhMIS inhibits the growth of certain tumor cell lines in vitro and in vivo.

INTRODUCTION

The müllerian duct in the female fetus develops into the fallopian tubes, uterus, and upper vagina. MIS is produced by the fetal testis as a 140-kDa glycosylated disulfide-linked homodimer and causes regression of the müllerian duct in the male fetus. The protein can be enzymatically cleaved in vitro into two fragments, composed of dimers with subunits of M, 57,000 and 12,500. Since first described by Jost as a fetal regressor of the müllerian ducts more than 40 years ago, MIS has also been shown to play a role in inhibition of oocyte meiosis (1), testicular descent (2), inhibition of fetal lung development (3), inhibition of autophosphorylation of the EGF receptor (4–5), and inhibition of tumor growth (6–9). The antitumor effect of MIS was demonstrated in vitro using the rat müllerian duct regression organ culture assay (11). MIS concentrations shown to play a role in inhibition of oocyte meiosis (1), testicular descent (2), inhibition of fetal lung development (3), inhibition of autophosphorylation of the EGF receptor (4–5), and inhibition of tumor growth (6–9). The antitumor effect of MIS was demonstrated in vitro using the rat müllerian duct regression organ culture assay (11). MIS concentrations

MIS and the dihydrofolate reductase genes (10). The transfected CHO cells were amplified in methotrexate and grown at 37°C in α-minimal essential medium without ribonucleosides and deoxyribonucleosides, supplemented with 10% bovine MIS-free female FCS. Two different MIS purification protocols were used to provide either partially pure DG-MIS (1–10%) or homogeneous (90–95%) IAP-MIS. The first used serial anion exchange and dye affinity chromatography (12), and the second used immunoaffinity chromatography and an anti-human MIS monoclonal antibody (13). Previous immunoaffinity protocols resulted in rhMIS preparations that contained low levels of several other proteins which counteract MIS activity in some in vitro systems. These proteins can be eluted from the immunoaffinity columns with salt-containing buffers, prior to elution of highly purified rhMIS.

The biological activity of MIS was detected in vitro using the rat müllerian duct regression organ culture assay (14). MIS concentrations were estimated using an ELISA for MIS (13), and protein concentrations were measured by the method of Bradford (15).

Preparation of Monoclonal Antibody for MIS Absorption. Monoclonal antibody was produced by immunizing female A/J mice (The Jackson Laboratory, Bar Harbor, ME) with immunoaffinity-purified rhMIS by methods previously described for bovine MIS (13, 16). Spleen cells producing anti-MIS antibodies were harvested, and hybridomas were produced as described by Kohler (17). A monoclonal line (6E11) was selected and amplified in Dulbecco’s modified essential medium supplemented with 15% FCS. The antibody was precipitated from 6E11 conditioned medium with 50% (NH4)2SO4 and further purified by protein A-Sepharose CL-4B (Sigma, St. Louis, MO) chromatography.

Cell Lines. A431, a cell line derived from a human vulvar epidermoid carcinoma; HT-3 from a human lymph node metastasis of a cervical carcinoma; NIH:OVCAR-3 from a human ovarian adenocarcinoma; HEC-1-A from a human endometrial adenocarcinoma; RT4 from a human bladder transitional cell papilloma; and Hep 3B from a human hepatocellular carcinoma were obtained from the American Type Culture Collection. OM431, from a human ocular melanoma, was obtained from Dr. James Epstein of Massachusetts General Hospital.

The A431, HT-3, NIH:OVCAR-3, OM431, and Hep 3B cells were maintained with α-MEM+ supplemented with 10% female FCS and 2 mM L-glutamine. HEC-1-A and RT4 were maintained with McCoy’s 5A medium supplemented with 10% female FCS. Cells were passed 1:2 every 2–3 days, and experiments were performed at 70–80% confluency. Flourescence-activated cell sorting analysis of this population in A431 cells shows a consistent phase distribution, with approximately 70% of the cells in S phase. This proliferating cell population was then centrifuged at 1500 rpm for 5 min and resuspended with 10% female FCS-supplemented medium. Cells were counted using a hemocytometer.

Semisolid Medium (Double-Layer) Colony Inhibition Assay. The effects of IAP-MIS and the IAP-salt were tested using A431, HT-3, HEC-1-A, NIH:OVCAR-3, OM431, and Hep 3B cells in the conventional double-layer agarose colony inhibition assay (18, 19). The underlayer of the 35-mm culture dishes contained 1 ml of 0.6% agarose (Sigma) in 10% female FCS-supplemented α-MEM+. The overlay consisted of 0.3% agarose in 10% female FCS-supplemented α-MEM+.

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3 The abbreviations used are: MIS, müllerian inhibiting substance; CHO, Chinese hamster ovary; rhMIS, recombinant human MIS; FCS, fetal calf serum; DG-MIS, serial anion exchange and dye affinity-purified MIS; IAP-MIS, immunoaffinity-purified MIS; IAP-salt, salt-eluted fraction from immunoaffinity column; ELISA, enzyme-linked immunosorbent assay; α-MEM+, α-minimal essential medium with ribonucleosides and deoxyribonucleosides; EGF, epidermal growth factor.
the cells to be tested (50,000 cells/ml for A431, HT-3, and OM431; 25,000 cells/ml for HEC-1-A, NIH:OVCAR-3, and HEP 3B), 10 ng/ml EGF (Sigma), and one of the following: IAP-MIS (final concentration, 30 nM); IAP-salt (final protein concentration, 19.3 μg/ml); or vehicle buffer as a negative control. The dishes were incubated in humid air with 5% CO2 at 37°C for 10–21 days. Colonies with a diameter greater than 30 μm were counted with an inverted microscope (Nikon). The results are expressed as percentage survival relative to a control group.

No. of colonies in the test group x 100
No. of colonies in the control group

Liquid Medium Colony Inhibition Assay. Single-cell suspensions were placed and grown in 24-well culture plates (no. 3047; Falcon, Oxnard, CA). After cell attachment, only those with good single-cell dispersion without clumping were used for further study. Agents to be tested were added in a volume less than 1/10 of the total volume in a well and were allowed to incubate in triplicate. The cells were incubated in humid air with 5% CO2 at 37°C. Colonies that formed in 5–7 days were stained with Giemsa solution, and those with more than 30 cells were counted with an inverted microscope or by a computer-based image analyzer. The assay was used to compare the response of different cell lines to various preparations of rhMIS and to establish a dose-response curve to rhMIS in A431 cells.

Antibody Absorption of MIS. MIS monoclonal antibody (6E11) and normal rabbit IgG (Lot 108; Dako Corporation, Denmark) were diluted into serum-free α-MEM+ before use. Previous experiments showed that normal rabbit IgG does not bind to MIS. Therefore, 17.4 μg of 6E11 were added to 5.6 μg of DG-MIS. Normal rabbit IgG was diluted with culture medium and added to MIS in the same 1:3 ratio to determine nonspecific absorption. Appropriate controls were run in parallel to check for any nonspecific inhibition. An equivalent amount of protein purified from conditioned medium of untransfected wild-type CHO cells served as a negative control when mixed 1:3 with antibody as above. The preparations were mixed at 4°C for 2 h. Protein A-Sepharose CL-4B (Sigma), after being washed in serum-free medium, was added and incubated at 4°C for another 2 h. The mixtures were centrifuged, and the supernatants were tested in the liquid medium colony inhibition assay using A431 cells (25,000 cells/ml; EGF, 50 ng/ml). The percentage survival of each group was calculated by comparing the number of colonies in each group to the wild-type negative control.

Multicellular Tumor Spheroid Assay. Multicellular tumor spheroids of HT-3 and HEP 3B cells were produced by the method described by Yuhas et al. (21). In brief, 106 cells of either HT-3 or HEP 3B in 10 ml of 10% female FCS-supplemented α-MEM+ were plated on the top of 1% agarose in a 10-cm culture dish and incubated in humid air with 5% CO2 at 37°C. Spheroids usually formed in 2–5 days. Agarose (0.5 ml of 1%) was added to each well of a 24-well culture plate (Falcon) to form a bottom layer before use. Individual spheroids of similar size (approximately 250 μm in diameter) were selected using a dissecting microscope and transferred by a micropipet into the desired space with a segment of 5-0 nylon suture (approximately 1 mm long), which was used both to calibrate ocular micrometer measurements and to localize the tumor. Twenty-four mice were implanted with A431 cell clots and 12 mice with OM431 cell clots. The longest diameter (L) of the implant, the one perpendicular to the longest one (W1), and the length of the suture were measured with the ocular micrometer of a dissecting microscope. The animals were treated by either IAP-MIS or vehicle buffer delivered by the Alzet miniosmotic pump placed in the peritoneal cavity. Blood samples at 6, 24, 48, 120 (fifth day), and 192 h (eighth day) were obtained from selected animals by orbital bleeding, and serum MIS levels were measured by ELISA. The animals were sacrificed on the eighth day. The longest diameter (L0) of the tumor, the one perpendicular to the longest one (W0), and the length of the suture were measured blindly by two independent investigators. After calibration of the measurements, the graft size ratio was represented by

\[
\frac{L_0 \times W_2 \times W_2}{L_1 \times W_1} \times \frac{W_1}{W_0}
\]

Histological sections of the kidneys were also obtained and examined. Tumors with cystic change were excluded.

Statistical Analysis. The results of the liquid medium and semisolid medium colony inhibition assays were analyzed by the χ2 test with Yates correction, while the multicellular spheroid and the subrenal capsule assays were tested by Student’s t test (P < 0.05 was considered statistically significant).

RESULTS

Semisolid Medium (Double-Layer) Colony Inhibition Assay. The percentage survival of the various cell lines after incubation with 30 nM of IAP-MIS was 45% for A431, 47% for HT-3, 54% for HEC-1-A, 59% for NIH:OVCAR-3, 34% for OM431, and 114% for HEP 3B. When compared to controls, the survival after treatment with MIS in all cell lines except HEP 3B was significantly inhibited by IAP-MIS (P < 0.05). The growth of HEP 3B was not inhibited by MIS. The percentage survival after incubation with IAP-salt was 172% for A431, 93% for HT-3, 92% for HEC-1-A, 120% for OM431, 105% for NIH:OVCAR-3, and 173% for HEP 3B. The stimulatory effect was significant for A431, OM431, and HEP 3B cells (P < 0.05) (Fig. 1).

Effect of IAP-MIS and IAP-salt on Colony Formation, by the Liquid Medium Colony Inhibition Assay. The percentage survival of the various cell lines after incubation with 30 nM of IAP-MIS was 45% for A431, 36.8% for OM431, 100.5% for HT-3, and 115.8% for RT4 when treated with 30 nM IAP-MIS. The percentage survival was 169.0% for A431, 226.7% for OM431, 101.6% for HT-3, and 96.5% for RT4 when treated with IAP-salt (final protein concentration, 19.3 μg/ml).

MÜLLER INHIBITING SUBSTANCE ANTITUMOR EFFECT

Semisolid Medium Colony Inhibition Assay

**Fig. 1.** Semisolid medium (double-layer) colony inhibition assay. Left, effect of IAP-MIS. Colony formation of A431, HT-3, HEC-1-A, NIH:OVCAR-3, and OM431 cells was significantly inhibited by IAP-MIS (30 nM). Right, effect of the salt fraction preeluted from the immunoaffinity column (IAP-salt). Stimulation of A431, OM431, and Hep 3B colony formation was seen when treated with the salt fraction. *, P < 0.05 when compared with control.

Liquid Medium Colony Inhibition Assay

**Fig. 2.** Liquid medium colony inhibition assay. Left, effect of IAP-MIS. The colony formation of A431 and OM431 cells was significantly inhibited by IAP-MIS (30 nM). Right, effect of the salt fraction preeluted from the immunoaffinity column (IAP-salt). Stimulation of A431, OM431, and Hep 3B colony formation was seen when treated with the salt fraction. *, P < 0.05 when compared with control.

Dose-dependent Inhibition of A431 Colony Formation by MIS. The percentage survival was 103.4, 81.4, 61.3, and 26.5%, respectively, for DG-MIS concentrations of 0.9, 1.8, 3.5, and 7.0 nM. Significant inhibitions were seen with DG-MIS concentrations of 3.5 and 7.0 nM (P < 0.05). The percentage survival was 109.7, 71.1, 56.6, and 33.3%, respectively, for IAP-MIS concentrations of 12, 24, 48, and 96 nM. Significant inhibitions were seen at IAP-MIS concentrations of 24, 48, and 96 nM (Fig. 3). DG-MIS thus was 10–14 times more potent than IAP-MIS.

Antibody Absorption of MIS. The MIS levels, measured by ELISA for MIS, were 9.0 nM for the positive control (MIS alone), 8.8 nM after normal IgG absorption (nonspecific), and 0.62 nM after the monoclonal antibody absorption (specific). All the wild-type negative controls had an MIS level equal to 0. The percentage survival was 42.9% for MIS alone, 38.0% for normal IgG (nonspecific absorption), and 74.2% for the monoclonal antibody (specific absorption). There was no significant nonspecific absorption of MIS activity (P > 0.05 compared with negative control). The percentage survival after the monoclonal antibody absorption was significantly higher than that of positive control (MIS alone) and normal IgG (Fig. 4).

The Salt-eluted Fraction (IAP-salt) Inhibits IAP-MIS. The percentage survival of A431 cells was 51.3% for IAP-MIS alone inhibitory effect of IAP-MIS and the stimulatory effect of IAP-salt were significant for A431 and OM431 colony formation (P < 0.05) (Fig. 2).

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percentage survival of the combination was significantly higher than that of MIS alone. *, P < 0.05 when compared with IAP-MIS alone, although the MIS combination of these two preparations reduces MIS-inhibitory effect. The per
cells/ml; EGF. 50 ng/ml) in the liquid colony inhibition assay. IAP-MIS was
(50 HM), 116.9% for IAP-salt alone, and 81.6% for the combi
and upper vagina. The müllerian duct is formed by an invagi
DISCUSSION
In the male fetus, MIS causes the regression of the müllerian duct, which is the anlagen of the fallopian tubes, uterus, cervix, and upper vagina. The müllerian duct is formed by an invagination of the coelomic epithelium overlying the genital ridge. The serosal surface of the normal ovary, the tissue of origin of the majority of primary ovarian tumors, is also derived from this same coelomic epithelium. We hypothesized that MIS might suppress the growth of malignant tumors derived from müllerian duct and hence coelomic epithelial structures (6).

Using MIS partially purified from bovine testes we demonstrated an inhibitory effect on endometrial and ovarian carcinoma cell lines both in vitro and in vivo (7, 8). This antitumor effect was also seen in a large series of primary human coelomic epithelium/müllerian duct-derived tumors in vitro using highly purified bovine MIS (9). The human MIS gene has since been cloned, expressed, and amplified in CHO cells (10) so that sufficient human MIS has become available for testing. Thus, rhMIS from the conditioned media of the amplified CHO cell line, purified by either DG-MIS or IAP-MIS, was examined against various cell lines in a number of growth assays.

The reliable and reproducible double-layer colony inhibition assay was used, but the lengthy incubation time (10–21 days) and the relatively large quantity of sample required for assessment proved to be disadvantageous. To overcome some of these difficulties, a more rapid (5–7-day) liquid medium inhibition assay using less test material was developed. While some cell lines failed to grow colonies in the liquid medium colony inhibition assay, when so formed their counting can be automated. OM431 and A431 required EGF for colony formation. This EGF-dependent colony formation is paradoxical in view of the fact that EGF is known to inhibit the A431 cell in monolayer (20). Overall, the results of the liquid and semisolid assays were quite similar, with the exception of the HT-3 (cervical carcinoma) cell line, which was inhibited by highly purified MIS in the semisolid assay but not in the liquid assay. The multicellular spheroid assay was used to recapitulate tumor microregions with cell-cell interactions and nutrient-affected growth patterns (25, 26). HT-3 cells formed satisfactory spheroids that were inhibited by MIS. No MIS effect was noted on the spheroid growth of Hep 3B (hepatocellular carcinoma). The availability of all three in vitro assays permits selection of optimal conditions for each tumor.

Not all cell lines respond to MIS. Initial studies included only cell lines and tumors derived from tissue of coelomic epithelium/müllerian duct origin, in which the majority were inhibited by MIS (6–9). As we expanded our investigations to evaluate tumor cell lines unrelated to müllerian-derived tissue, we found that a vulvar epithelial and an ocular melanoma tumor

**DISCUSSION**

The average size ratios of HT-3 spheroids in the control group (n = 6) were 1.15 ± 0.04, 1.44 ± 0.02, and 1.96 ± 0.11 at days 3, 6, and 9, respectively, while in the MIS group (n = 6) they were 0.98 ± 0.04, 0.94 ± 0.04, and 1.08 ± 0.06 (Fig. 6A). The average size ratios of Hep 3B spheroids in the control group (n = 6) were 2.56 ± 0.05, 5.64 ± 0.53, and 13.07 ± 1.09, respectively, at days 3, 6, and 9, while in the MIS group (n = 6), they were 2.36 ± 0.25, 5.77 ± 0.54, and 14.30 ± 0.54. The growth of Hep 3B spheroids was faster and uninhibited by MIS (Fig. 6B).

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cell line were also inhibited by MIS, thereby suggesting that other diverse tumor lines may have sensitivity to MIS.

DG-MIS, although less purified, consistently showed inhibition of cell growth in vitro. The specificity of the MIS effect was demonstrated by blocking the inhibitory effect with an MIS monoclonal antibody (13). Recent findings suggest that MIS is processed by at least one requisite posttranslational proteolytic cleavage and that both the amino- and carboxy-terminal fragments are required for full biological activity (27, 28). The monoclonal antibody to holo-MIS thus may not recognize all MIS moieties present in the preparations, which could account for the incomplete inhibition of the response.

Wallen et al. (11) reported only a minimal antiproliferative activity when an immunopurified MIS preparation was tested against a variety of established cell lines. We observed the same poor response with IAP-MIS before the salt elution step was added prior to elution with 1 M acetic acid. In addition, the salt fraction eluted from the immunoaffinity column actually showed a growth-stimulating effect in some cell lines. Electrophoresis of this salt fraction shows several bands in the region of M, 14,000–43,000, which are absent in the fraction subsequently eluted by 1 M acetic acid (Fig. 5B). When the concentrated culture medium of untransfected wild-type CHO cells was subjected to the same purification process, the salt fraction, which also showed a stimulatory effect on A431 cells (Figs. 1 and 2), revealed the presence of the same bands at M, 14,000–43,000. These protein products of the untransfected CHO cells are being studied as putative growth-stimulating factors that might serve to mask the MIS effect. This hypothesis was supported when the salt-eluted fraction added to the acetic acid-eluted fraction altered the MIS inhibition (Fig. 5A). Transforming growth factors and other polypeptides are known to be produced as autocrine growth factors by certain malignant cells (29–32).

When the dose responses to highly purified IAP-MIS and DG-MIS were compared, it was noted that DG-MIS was 10-fold more potent than the more highly purified IAP-MIS. It is possible that purification of the cleaner preparation may have removed the proteases required to cleave and activate MIS. Furthermore, irreversible aggregation may occur; to reduce these effects, EDTA and a nontoxic concentration of Nonidet P-40 (0.001%) were added to the eluted IAP-MIS. In addition, the relatively harsh immunoaffinity column elution protocols may denature a binding protein essential for the antiproliferative effect of MIS. Such binding proteins have been shown to be essential for the activity of other biological systems (32).

The subrenal capsule assay was used to test the effect of MIS in vivo. By delivering MIS via a constant i.p. infusion Alzet pump inserted at the time of tumor implantation, the growth of a vulvar epidermoid carcinoma cell line, A431, and an ocular melanoma line, OM431, were inhibited in vivo. It is essential in evaluating this assay that histology be documented for each different tumor and that the experiment be completed before central necrosis occurs. Thus the assay was terminated on day 8, since longer duration led to variable cystic change. Such changes can vary the graft size ratio enough to make comparisons unreliable due to imbibition of fluid and cystic changes. By this careful analysis, the postimplantation characteristics of each tumor cell line can be established, and artifacts can be avoided. The in vivo effect in this subrenal assay is achieved at physiological or picomolar concentrations, as was also observed in vivo in suppression of lung surfactant in late embryo rats (3). In vitro studies, on the other hand, require 50- to 500-fold higher levels, which may reflect failure of activation.
Although we do not yet know the maximum tolerable dose of systemic MIS, no obvious toxicity to animals was observed. Much must be done to understand the mechanism of action of MIS, but the in vivo environment appears to create the conditions appropriate for MIS to exert its maximal antiproliferative effects. The results of these experiments encourage continued evaluation of this biological modifier as an effective chemotherapeutic agent.

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