Recombinant Human Mullerian Inhibiting Substance Inhibits Human Ocular Melanoma Cell Lines in Vitro and in Vivo

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ABSTRACT

Since Mullerian Inhibiting Substance (MIS) causes regression of the Mullerian duct, the anlagen of the uterus, vagina, and fallopian tube, we expected and have previously observed that purified recombinant human MIS causes regression of gynecological tumors. However, recent experiments indicating that neural crest derivatives might be responsive to MIS prompted study of a group of human ocular melanoma cell lines in 4 in vitro inhibition assays, and a subrenal capsule assay in vivo. Ocular melanoma cell lines that grew well in a respective assay were studied with MIS to determine whether this biological modifier could inhibit growth. Three human ocular melanomas, OM431 (P < 0.01), OM467 (P < 0.02), and OM482 (P < 0.03), were growth-inhibited by highly purified recombinant MIS in soft agarose. A dose-dependent tumor inhibition was noted when OM431 cells were incubated with MIS in a liquid colony inhibition assay (P < 0.05). In addition, OM467 was inhibited (P < 0.05) by MIS in a multicellular tumor spheroid assay. Cell cycle analysis indicated that OM431 cells were inhibited in monolayer by MIS while in G1. At 100-fold lower serum concentrations than required in the media of in vitro assays, MIS delivered via i.p. osmotic pumps inhibited (P < 0.05) in vivo the growth of OM431 implanted beneath the renal capsule of nude and CD-I irradiated mice when compared to mice given implants of pumps containing no MIS. The responsiveness of ocular melanoma to MIS broadens the spectrum of tumors that might be treated with MIS and suggests further investigation of other neural crest tumors.

INTRODUCTION

Ocular melanoma, presumably from conjunctival and/or uveal melanocytes, represents a distinct variety of malignant melanoma that shares a common neural crest embryological origin with cutaneous melanoma derived from skin melanocytes (1), although they differ in many clinical and epidemiological characteristics (2). Although chemotherapeutic agents such as dacarbazine, the nitrosoureas, vinblastine, thiotope, methotrexate, diticene, procarbazine, and cisplatin (3-5) are being used for the treatment of ocular melanoma, the chemosensitivity of these tumors has not been systematically studied in vitro since few cell lines have been established. Since we have been able to grow and maintain a number of human ocular melanoma cell lines (6), we studied the effect of the biological modifier recombinant human MIS2 on such lines in a variety of in vitro and in vivo assays.

MIS, produced by the fetal testis as a Mr 140,000 glycosylated disulfide-linked homodimer (7), is known to cause regression in the male fetus of the Mullerian duct, which in the female fetus forms as an invagination of the coelomic epithelium overlying the genital ridge and develops into the fallopian tubes, uterus, and upper vagina. Although first described by Jost as a "Mullerian inhibitor" more than 40 years ago (8-10), MIS has more recently been implicated in inhibition of oocyte (11, 12) and spermatogonia maturation (13), gonadal differentiation (14), testicular descent (15), fetal lung development (16), and tumor growth (17-20) through a mechanism thought to involve inhibition of autophosphorylation of the EGF receptor (21).

The abbreviations used are: MIS, Mullerian Inhibiting Substance; CHO, Chinese hamster ovary cells; rhMIS, recombinant human MIS; ELISA, enzyme-linked immunosorbent assay; α-MEM, α-minimal essential medium; EGF, epidermal growth factor; FACS, fluorescence-activated cell sorter.

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mm diameter Petri dish that contained confluent, UV-irradiated MRC-5 fibroblasts. The fresh tumor pieces were scratched into the surface of the Petri dish to which was added 10–12 ml of growth medium consisting of Ham's F-12 medium supplemented with 2000 mg/liter glucose, 1 × 10^{-4} M cholaeris toxin, 10 μg/ml of bovine epidermal growth factor, 5% horse serum, and 15% fetal bovine serum. These cultures were maintained at 36.5°C in a humidified chamber perfused with 5% CO_2. As the irradiated feeder layer detached from the surface of the Petri dish, the tumor cells were trypsized and frozen at regular intervals beginning within 8–10 weeks after being placed into culture. After the tumors became established in vitro, they were maintained in a MEM supplemented with only 1 g/liter glucose, 1 g/liter L-glutamine, and 10% fetal bovine serum, which provided better long term growth than the F-12 medium in which they were established. These cells exhibited various Callender (28) morphological criteria, and electron microscopy confirmed the presence of melanin granules consistent with a pigmented cell type. A human diploid or near diploid karyotype was found in each of the different cell lines which were then frozen in Dulbecco's modification of minimal essential media with 10% dimethyl sulfoxide and stored in liquid nitrogen. Before use in these experiments, the cells were thawed; studied for plating efficiency, doubling time, and confluent density (Table 1); tested and found to be negative for adventitious bovine viruses and Mycoplasma; and a portion refrozen. Cells were reared before 20–30 population doublings in α-MEM with ribosides.

**Semisolid Medium (Double Layer) Colony Inhibition Assay.** Cell lines were tested in triplicate for growth in the conventional double layer agarose colony inhibition assay and, for those that grew, the effect of rhMIS on colony growth as described previously (19, 20, 24). The overlay consisted of 0.3% agarose (Sigma Chemical Co., St. Louis, MO) in 10% female calf serum supplemented α-MEM with ribonucleosides and deoxyribonucleosides, the cells to be tested (50,000 cells/ml for OM431 and 25,000 cells/ml for OM467, OM482, and OM464), EGF 10 ng/ml (Sigma), and either rhMIS or vehicle buffer. After incubation for 10–21 days, colony growth was compared as percent survival relative to the control group (number of colonies in the test group × 100/number of colonies in the control group).

**Liquid Medium Colony Inhibition Assay.** The cell lines were tested for their ability to grow and form colonies in liquid media and for the effect of rhMIS on colony growth. Single cell suspensions of OM431 (8250 cells/well) were grown in 24-well culture plates (no. 3047; Falcon, Oxnard, CA) in 0.5-ml media containing 50 ng/ml EGF, as described previously (24). Agents to be tested were added (50 μl/well) to triplicate wells and colonies formed in 5–7 days counted using a computer-based image analyzer (29). After dilution in vehicle buffer, MIS was tested in concentrations of 0.98, 9.8, 25.2, 50.4, 75.6, and 100.8 nM. The results were expressed as percent survival relative to the vehicle buffer control.

**Multicellular Tumor Spheroids Assay.** The cell lines were tested for their ability to grow as spheroids and for the effect of rhMIS on spheroid growth. Multicellular tumor spheroids were produced by the method described by Yuhas et al. (30) and used previously in this laboratory (24). Cells (10^6) of each line on 1% agarose were incubated for 2-5 days when spheroids usually formed; 0.5 ml of 1% agarose was then added to each well of a 24-well culture plate (no. 3047; Falcon). Individual spheroids of similar size (10–250 mm diameter) were transferred by micropipette, one each to each well. The sizes of the spheroids on day 0 were determined by the longest diameter (L), and the diameter perpendicular to the longest one (W), and the volume was expressed as (L × W × W). Six to 10 wells were treated with either rhMIS or vehicle buffer, spheroid volumes were measured at regular intervals, and the size ratio was compared to its initial size. The average size ratio of each group was plotted versus time in a growth curve, and the groups were compared.

**MIS Effect of OM431 on Cell Cycle Kinetics in Monolayer.** Previous investigations by one of us (P. H.) have shown in monolayer culture that, upon restimulation, a G1 arrested human vulvar epithelial cell line (A431) is growth-inhibited by rhMIS. To investigate the effect in OM431 cells, cells were plated on multiple 35-mm tissue culture plates and allowed to grow to confluence. The cells from one plate were analyzed by FACS using a Becton Dickson FACS with a 488-nm argon laser, after fixation in ethyl alcohol, treatment with ribonuclease A, and staining with propidium iodide. The non-analyzed cells were then subcultured to 30% confluency, and a sample of cells (one plate) was analyzed each day by FACS until the population again reached confluency. Cell phase versus time was plotted for this population to establish that the OM431 cell line could be density arrested in G_1. The density arrested OM431 cell line was subdivided to 30% confluency and exposed to either buffer (20 nM N2-hydroxyethyl-piperazine-N2-2-ethanesulfonic acid) or 70 nM rhMIS. At 0 and 24 h, a sample of each group was analyzed by FACS to determine the population's cell phase distribution.

**Subrenal Capsule Assay.** Following the method of Bogden et al. (31), as subsequently modified by Fingert et al. (32), all cell lines were tested for growth in vivo. Cells (10^6) from each line were centrifuged at 1500 rPM for 5 min to form a pellet. Twenty ml of fibrinogen (20 mg/ml; Sigma) dissolved in phosphate-buffered saline (pH 7.4) were added to the pellet, followed by 10 ml of thrombin (20 units/ml; Sigma) dissolved in double-strength Dulbecco's modification of minimal essential medium. This mixture was incubated at 37°C for 15 min. The cell clot thus formed was cut into approximately 50 fragments (1 mm^2 × 10^3 cells) in preparation for implantation.

**Analysis.** Viral and pathogen-free female CD-1 mice (10 weeks old, average weight 35 g; Charles River Breeding Laboratory, Wilmington, MA) were given whole body irradiation of 640 rad by a Mark-1 137Cs irradiator 16–24 h before implantation (33). Female nude mice (nu) (8 weeks old, average weight 24 g; Edwin L. Steele Laboratory, Massachusetts General Hospital, Boston, MA) were also used. All animals were cared for under the guidelines established by the Massachusetts General Hospital and conformed to the instructions to authors. After inducing anesthesia with an i.p. injection of 0.3 ml of 10% pentobarbital (Abbott Laboratory, North Chicago, IL), a subcapsular space was developed in the left kidney with a 19-gauge needle trocar, and a clot introduced. The longest diameter (L) of the implant and the diameter perpendicular to the longest diameter (W) were measured as described previously (24, 33). Initially, all cell lines were implanted for 8 days without MIS in both nude and irradiated CD-1 mice to determine the extent of growth. Histology was reviewed to assure that the implanted tumor was viable and lacked both an inflammatory infiltrate and central necrosis.

**Cell lines that produced a graft size ratio >2, without necrosis or an inflammatory response, were then tested with MIS. Animals containing tumor implants were either treated by rhMIS or vehicle buffer delivered by Alzet pumps placed in the peritoneal cavity at the time of implantation and sacrificed on the eighth day. The Alzet mini-osmotic pumps (no. 2001, fill volume 210 μl; ALZA Corp., Palo Alto, CA), released their contents at a rate of 1 μl/h. Blood samples were obtained on the eighth day from selected nude mice and serum MIS levels were measured by an ELISA for human MIS (25). The longest diameter (L_0) of the tumor, and the diameter perpendicular to the longest diameter (W_0), as measured blindly by 2 independent investigators, were used to calculate the graft size ratio (L_0 × W_0 × W_0)/(L_0 × W_1 × W_1). Kidneys were fixed and tumors with cystic change or lymphocytic infiltrate were not analyzed.

**Statistics.** Liquid medium and semisolid medium colony inhibition, multicellular spheroid, and subrenal capsule assays were expressed as mean ± standard error of the mean (SE) and were analyzed by Student's t test, with P < 0.05 considered statistically significant.
MIS INHIBITS HUMAN OCULAR MELANOMA

RESULTS

Semisolid Medium (Double Layer) Colony Inhibition Assay. Only OM464 failed to grow in soft agarose; the other ocular melanoma cell lines were significantly inhibited by rhMIS. The percentages of survival of the various cell lines, tested in triplicate, were 34% for OM431 (in 30 nM MIS; P < 0.01), 61% for OM467 (in 150 nM MIS; P < 0.02), and 80% for OM482 (in 90 nM MIS; P < 0.03) (Fig. 1).

Dose-dependent Inhibition of Colony Formation by MIS in the Liquid Colony Inhibition Assay. OM431 cells produced colonies whose counts could be automated in the liquid colony inhibition assay. The percentages of survival were 33, 29.5, 56.6, 71, 109.8, and 96.8%, respectively for MIS concentrations of 100.8, 75.6, 50.4, 25.2, 9.8, and 0.98 nM. Significant inhibitions were seen with MIS concentrations of 50.4 (P < 0.05), 75.6 (P < 0.004), and 100.8 (P < 0.006) nM MIS (Fig. 2).

Multicellular Spheroid Assay. OM467 and OM482 grew as spheroids, whereas OM431 and OM464 did not. OM467 was inhibited with the average size ratios of the spheroids in the control group (n = 6) being 3.85 ± 0.43 (SE), 4.85 ± 0.64, and 5.52 ± 0.78 at days 5, 8, and 11, respectively, whereas in the MIS (220 nM) group (n = 6) they were 2.17 ± 0.26, 2.79 ± 0.33, and 3.91 ± 0.59. This difference was significant (P < 0.02). The average size ratio of OM482 spheroids in the control group (n = 10) were 2.18 ± 0.17 and 8.22 ± 0.81, respectively, at days 3 and 6, whereas in the MIS (330 nM) group (n = 10), they were 1.93 ± 0.14 and 9.0 ± 0.67 (Fig. 3).

Cell Cycle Kinetics in Monolayer Growth. When OM431 was density-arrested and then passed to 30% confluency, FACS analysis of the population done at 0 and 24 h shows that rhMIS (10 µg/ml or 70 nM by ELISA)-treated cells were delayed from exiting G1, whereas buffer-treated cells progressed to S phase (Table 2).

Subrenal Capsule Assay. Each cell line was tested in 5 CD-1 mice for growth without MIS over 8 days. The graft size ratio was 3.68 ± 0.56 for OM431, 1.97 ± 0.67 for OM482, and 1.34 ± 0.45 for OM467. OM464 had graft size ratio of <1. In nude mice, OM467 and OM464 had graft size ratios of one or less, whereas OM431 had a ratio of 3.08 ± 0.27. Histologically, OM482 showed spreading growth under the renal capsule with significant invasion into the renal parenchyma that precluded accurate quantitation of tumor growth. Since we had previously seen inhibition of growth in the CD-1 irradiated mice (24), the OM431 cell line was then tested against MIS in nude mice. In 2 assays using nude mice, with a total of 11 controls and 9 MIS-treated mice, the graft size ratios of the OM431 tumors were significantly greater, 3.02 ± 0.17 and 3.14 ± 0.40, respectively, for the controls, versus 1.68 ± 0.09 (P < 0.001) and 1.69 ± 0.43 (P < 0.005) for the MIS-treated group. In the first nude mouse assay, the MIS-treated group received 44.7 µg rhMIS over 8 days whereas the animals in the second assay received 130 µg rhMIS. The average MIS serum levels of the rhMIS-treated mice on the 8th day of the nude mouse assays were 570 and 749 pm, respectively. The measured controls had MIS levels of less than 10 pm (Fig. 4). The OM482 cell line was also tested against rhMIS (51 µg) using 5 controls and 5 treated mice. As noted above, growth could not be quantitated, but limited tissue invasion was noted in the rhMIS-treated group.

DISCUSSION

In the male fetus, MIS causes the regression of the tissues that form the anlagen of the fallopian tubes, uterus, cervix, and upper vagina. We hypothesized earlier that MIS might suppress the growth of malignant tumors derived from Mullerian duct structures (17), and using MIS purified from bovine testes demonstrated an inhibitory effect on endometrial and ovarian carcinoma cell lines both in vitro and in vivo (18, 19). This antitumor effect was also seen in a large series of primary human Mullerian-derived tumors in vitro using highly purified bovine MIS (20). Since cloning the human MIS gene and expressing and amplifying it in CHO cells (22), human MIS has become available for expanded testing. This rhMIS from the conditioned media of the amplified CHO cell line was then shown to inhibit endometrial, cervical, ovarian, and vulvar tumor cell lines and indicated that an ocular melanoma cell line was similarly sensitive (24). Here we examined the effects of highly purified rhMIS on a number of ocular melanoma cell lines.
In the reliable and reproducible double-layer inhibition assay, despite its rather lengthy incubation time (10–21 days), 3 of 4 cell lines (OM482, OM431, and OM467) grew well in soft agarose, and their colony growth was significantly inhibited by MIS at concentrations of 30–150 nM. The liquid colony inhibition assay as developed by Chin et al. (24) was used to study the effect of MIS on the OM431 cell line since OM467 and OM482 failed to grow discrete colonies in this assay and did not lend themselves to automated counting. When colony growth can be effectively achieved, as with OM431, this assay is rapid (5–7 days) and uses relatively little MIS; colony counting can be automated (29) and doses can be repeated and their schedules modified. OM431 showed a clear dose response with significant inhibition of colony formation in vitro with rhMIS concentrations at 50 nM and above. The percent survival of OM431 correlated well between the 2 assays. The multicellular spheroid assay was used to recapitulate tumor microregions with cell-cell interactions and nutrient-affected growth patterns (34, 35). OM467 and OM482 formed satisfactory spheroids, whereas OM431 did not, and OM467 was significantly inhibited while the growth of OM482 was unaffected by MIS. The assay also permitted selection of optimal dose and repeated dose treatment schedules. The availability of multiple in vitro assays permits selection of the optimal conditions in which to test each tumor.

Investigations in progress6 show in monolayer culture that, upon release from density arrest, a human vulvar epithelial cell line (A431) is growth-inhibited by rhMIS. OM431 cells were grown to confluence and as with A431, this maneuver was found by FACS analysis to have arrested a majority (>80%) of cells in G1. This density-arrested population was then subdivided to 30% confluency; when then exposed to rhMIS, OM431 cells are delayed in cell cycle progression as evidenced by the high percentage remaining in G1 at 24 h, inferring that OM431 is responsive to MIS in the G1 phase of the cell cycle.

The subrenal capsule assay was used to test the effect of MIS in vivo in nude mice. Of the 4 cell lines investigated, OM431 and OM482 grew to a sufficient graft size ratio. After peritoneal implantation, MIS was delivered via a constant infusion Alzet pump for 8 days. Though designed for rats, the mice tolerated the larger, longer delivery pumps, placed at the time of tumor insertion, without difficulty. After 8 days, the growth of OM431 was significantly inhibited by rhMIS. It is encouraging that the in vivo inhibitory effect in this subrenal assay was achieved at pM concentrations of serum MIS, whereas in vitro inhibition required 100-fold higher concentrations. These differences may reflect a failure of the in vitro milieu to provide the conditions required 100-fold higher concentrations. This observation will stimulate future study of MIS against metastasizing tumors.

When density arrested, 84.4% of cells were in G1, as determined by FACS analysis. Upon passage to 30% confluency, cells treated with buffer alone progressed in the cell cycle, whereas cells exposed to rhMIS (10 μg/ml) were delayed in G1 (86% versus 56.2%).

<table>
<thead>
<tr>
<th>Density arrest</th>
<th>Buffer, 24 h</th>
<th>MIS, 24 h</th>
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<tbody>
<tr>
<td>G1 (%)</td>
<td>84.4</td>
<td>94.0</td>
</tr>
<tr>
<td>S (%)</td>
<td>7.9</td>
<td>8.1</td>
</tr>
<tr>
<td>G2/M (%)</td>
<td>7.7</td>
<td>7.9</td>
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P. L. Hudson et al., manuscript in preparation.
Fig. 4. OM431 cells were implanted beneath the murine renal capsule (35) to investigate the effect of rhMIS on cell growth in vivo. In 2 assays (A and B) using 11 control and 9 MIS-treated nude mice, the graft size ratios of the OM431 tumors were significantly greater, 3.02 ± 0.17 and 3.14 ± 0.40, respectively, for the controls, versus 1.68 ± 0.09 (P < 0.001) and 1.69 ± 0.43 (P < 0.005) for the MIS-treated group. In the first assay, the MIS-treated group received 44.7 μg MIS over 8 days while in the second assay, the animals received 130 μg of another preparation of MIS. The average MIS serum levels of the MIS-treated mice on the eighth day of the nude mouse assays were 0.57 ± 0.75 nm, respectively. The measured controls had MIS levels of less than 0.01 nm.

REFERENCES


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