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 mono-layer 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-1 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 decarbazine, the nitrosoureas, vinblastine, thiotepa, 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 MIS1 on 4 such lines in a variety of in vitro and in vivo assays.

MIS, produced by the fetal testis as a M, 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). Both the bovine and human MIS genes have been cloned and the human MIS protein expressed in CHO cells (22). We tested purified natural bovine MIS against a variety of primary and established gynecological tumors and observed regression (17-20). rhMIS, however, initially showed only marginal antiproliferative activity (23), but after modifying purification protocols to remove antagonistic copurifying proteins, we observed a consistent inhibitory effect of highly purified rhMIS against established human endometrial, cervical, and ovarian tumor cell lines of Mullerian duct/coelomic epithelium origin, whereas human bladder and hepatoma cell lines showed no response (24). Recent immunohistochemical observations suggestive of binding of MIS to embryonic neural crest derivatives and of response to MIS of an ocular melanoma cell line originally studied as a negative control (24), prompted the present study of a group of human ocular melanoma cell lines for their response to this highly purified preparation of rhMIS.

MATERIALS AND METHODS

Production, Purification, and Assays of rhMIS. After cloning MIS genomic DNA, dihydrofolate reductase-deficient CHO cells co-transformed with a linear construct of both the human MIS and the dihydrofolate reductase genes on a pAdD26 promoter (22) were amplified in methotrexate and grown at 36.5°C in the a-modification of Eagle's medium without ribonucleosides and deoxyribonucleosides, supplemented with 10% bovine MIS-free female fetal calf serum. Immunoaffinity chromatography using an anti-human MIS monoclonal antibody (25) was used to purify the rhMIS (90-95% pure). Previous immunoaffinity protocols resulted in rhMIS preparations that contained low levels of several other coeluted proteins that were found to counteract MIS activity in these same in vitro assays (24). These proteins are now pre-eluted from the immunoaffinity columns with salt-containing buffers, before acid elution of highly purified rhMIS. The biological activity of MIS was detected in vitro using the rat Mullerian duct regression organ culture assay (26). MIS concentrations were estimated using an ELISA for MIS (25), and protein concentrations were measured by the method of Bradford (27), and further characterized by gel electrophoresis (25) and Western analysis (25) using antibodies to holo MIS and to peptides from the carboxy and amino terminal fragments.2

Cell Lines. The ocular melanoma cell lines were established in vitro from primary tumor specimens obtained at enucleation (6). freshly excised tumor was rinsed aseptically in balanced salt solution and minced into 1-mm3 sections. Three sections were placed into a 100-

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 cholaer 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% CO2. As the irradiated feeder layer detached from the surface of the Petri dish, the tumor cells were trypsinized 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 the α-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 cell lines 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 retired before 20–30 population doublings in α-MEM with ribosides.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Plating efficiency</th>
<th>Doubling time (h)</th>
<th>Confluent density (cells/cm² × 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM431</td>
<td>35%</td>
<td>24</td>
<td>2.31</td>
</tr>
<tr>
<td>OM464</td>
<td>N/A</td>
<td>&gt;72</td>
<td>0.68</td>
</tr>
<tr>
<td>OM467</td>
<td>18%</td>
<td>38</td>
<td>1.06</td>
</tr>
<tr>
<td>OM482</td>
<td>37%</td>
<td>22</td>
<td>1.96</td>
</tr>
</tbody>
</table>

**MIS inhibits human ocular melanoma**

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 G0. The density arrested OM431 cell line was subdivided to 30% confluency and exposed to either buffer (20 nm N-2-hydroxyethyl-piperazine-N'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 mm²–2 × 10^6 cells) in preparation for implantation.

**Table 1 Classification and characteristics of the human ocular melanoma cell lines used in this study**

**Mixed morphology of OM464, -467, and -482, is primarily spindle.**

<table>
<thead>
<tr>
<th>Human ocular melanomas</th>
<th>Cell line</th>
<th>Calender morphology</th>
<th>Plating efficiency</th>
<th>Doubling time (h)</th>
<th>Confluent density (cells/cm² × 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM431</td>
<td>Epithelioid</td>
<td>35%</td>
<td>24</td>
<td>2.31</td>
<td></td>
</tr>
<tr>
<td>OM464</td>
<td>Mixed</td>
<td>N/A</td>
<td>&gt;72</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>OM467</td>
<td>Mixed</td>
<td>18%</td>
<td>38</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>OM482</td>
<td>Mixed</td>
<td>37%</td>
<td>22</td>
<td>1.96</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. Cell lines were tested in triplicate for their ability to grow and respond to rhMIS in a double layer agarose colony inhibition assay. The percent survival (number of colonies in the test group x 100/number of colonies in the control group) of the various cell lines was 34% for OM431 (in 30 nM MIS; P < 0.01), 61% for OM4467 (in 150 nM MIS; P < 0.02), and 80% for OM482 (in 90 nM MIS; P < 0.03).

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 mM 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 will permit 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 progress 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 confluency and as with A431, this maneuver was found by FACS analysis to have arrested a majority (>80%) of cells in G0. 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 G0 at 24 h, inferring that OM431 is responsive to MIS in the G0 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. These differences may reflect a failure of the in vitro milieu to provide the conditions apparently present in vivo for MIS cleavage (36) and activation (37), which may be essential for MIS to exert antiproliferative effects.

The results of these experiments encourage continued evaluation of this biological modifier as an effective chemotherapeutic agent against ocular melanoma. The logical extension of this work will be to investigate the effect of MIS on cutaneous melanoma and other tumors of neural crest origin; neuroblastomas and ganglioneuromas are worthy targets. Normal neural tissue also will be studied as potential target tissues. Future studies with larger supplies of MIS will also examine the in vitro and in vivo effects of combination chemotherapy using MIS and cytotoxic agents, the MIS effect on primary human tumors in vitro, and on previously implanted growing established cell lines or primary tumors in immunodeficient mice, and the response of both the tumor and host to long-term MIS treatment. The favorable response of a non-Mullerian tumor to MIS broadens the spectrum of tumors potentially treatable with the biological modifier. It is attractive to suggest that the common theme underlying the favorable response to MIS of germ cell, genital tract, and neural crest derivatives may be their migratory characteristics. This observation will stimulate further study of MIS against metastasizing tumors.

Table 2 Cell cycle kinetics of OM431 cell line in monolayer growth

<table>
<thead>
<tr>
<th>Density</th>
<th>G0 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer, 24 h</td>
<td>56.2</td>
<td>42.1</td>
<td>1.7</td>
</tr>
<tr>
<td>MIS, 24 h</td>
<td>86</td>
<td>10.1</td>
<td>3.9</td>
</tr>
</tbody>
</table>

*P. L. Hudson et al., manuscript in preparation.*
while in the second assay, the animals received 0.001 and 1.69 ± 0.43 (P < 0.005) for the average MIS serum levels of the MIS-treated group received 44.7 μg MIS over 8 days respectively, for the controls, versus 1.68 ± 0.09 (P < 0.01).


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