Cleavage of Müllerian Inhibiting Substance Activates Antiproliferative Effects in Vivo

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ABSTRACT

Müllerian inhibiting substance (MIS), an inhibitor of growth and development of the female reproductive ducts in male fetuses, requires precise proteolytic cleavage to yield its biologically active species. Human plasmin is now used to cleave and, thereby, activate immunoaffinity-purified recombinant human MIS at its monobasic arginine-serine site at residues 427–428. To avoid the need for exogenous enzymatic cleavage and to simplify purification, we created an arginine-arginine dibasic cleavage site (MIS RR) using site-directed mutagenesis to change the serine at position 428 (AGC) to an arginine (cGC). The mutant cDNA was then stably transfected into a MIS-responsive ocular melanoma cell line, OM431, followed by cloning for amplified expression to test its biological activity in vitro and in vivo.

Media from each clone were assayed for production of MIS RR by a sensitive ELISA for holo-MIS, and high- and low-producing clones were selected for further study. Media from the highest MIS RR producer caused Müllerian duct regression in an organ culture bioassay. Other transfections were done with an empty vector (pcDNA1 Neo) or a construct lacking the leader sequence and thus failing to secrete MIS, to serve as controls. The OM431 clones containing the MIS RR mutant were growth inhibited in monolayer culture. The high- and low-producing MIS RR OM431 clones, along with transfected OM431 controls, were injected into the tail veins of immunosuppressed SCID mice for in vivo analyses. Four to 6 weeks later, pulmonary metastases were counted in uniformly inflated lungs. OM431 clones containing the more easily cleaved MIS RR displayed a significant dose-dependent reduction in pulmonary metastases when compared to the lungs of animals given injections of OM431 clones containing empty vector, leaderless MIS, or wild-type MIS that requires activation by plasmin cleavage. Since the purification protocol of MIS RR is less complicated than that for wild-type MIS, which requires subsequent enzymatic activation, MIS RR can be used for scale-up production with increased yields for further therapeutic trials against MIS-sensitive tumors.

INTRODUCTION

MIS, initially described by Jost (1–3) as the “Müllerian inhibitor,” is secreted by the fetal testis and, early in male development, causes regression of the Müllerian ducts, the precursors for the Fallopian tubes, uterus, and the upper third of the vagina. MIS is secreted by Sertoli cells of embryonic, perinatal, and adult testis (4–6) and by the granulosa cells of the preadolescent and adult ovary (7–9). MIS is reported to play a role in inhibition of maturation of oocytes (10, 11) and spermatogonia (12). It may also be influential in pulmonary development (13) and has been implicated in testicular descent (14).

In addition to inhibiting normal growth and development, MIS has antiproliferative effects on tumors of Müllerian duct origin (15–18) as well as on OM431, a human ocular melanoma of neural crest derivation (19–21). Candidate type I (22) and type II (23, 24) MIS receptors recently have been cloned, but convincing saturable binding of MIS to these cloned products has yet to be demonstrated. Although the candidate type I receptor is more widespread and may be shared among other members of the TGF-β family (25, 26), the type II receptor is highly expressed in the testis and ovary (23, 24).

MIS is a Mr 140,000 glycosylated disulfide-linked homodimer (27, 28) which has certain similarities to other members of the TGF-β superfamily (29). Proteolytic cleavage is required to generate the biologically active carboxy-terminal fragment (30) which is required for Müllerian duct regression and which shares homology with processed TGF-β. In vivo, cleavage occurs at a monobasic Arg(427)-Ser(428) site (30, 31) by an unidentified endogenous enzyme. Unlike MIS, TGF-β and other members of the superfamily contain a tetrabasic cleavage site that is more efficiently processed (32). MIS is produced in our laboratory predominantly in a noncleaved form from transfected amplified DHFR-deficient, CHO cells (29) in which monobasic cleavage is inefficient and variable. After immunoaffinity purification of the secreted recombinant human...
MIS, plasmin cleavage followed by molecular sieve chromatography in acid has been used to isolate the purified active carboxy terminus (30, 31). Alternatively, plasmin cleavage of holo-MIS without subsequent chromatography is used to prepare samples in which amino- and carboxy-terminal fragments remain in noncovalent association (33).

MIS, as a mediator of regression of a fetal organ system and as an inducer of programmed cell death (34, 35), is a paradigm for mechanistic studies and for development as a tumor suppressor chemotherapeutic agent. In the selection of an effective therapeutic agent, specificity for the target tumor, the appropriate stage of the cell cycle, molecular stability, efficacy, toxicity, route of delivery, the state of the patient, and drug/drug compatibility are all factors that must be considered. Potentially, one may use as the therapeutic agent, the activated ligand, its receptor, a downstream factor in its pathway of activation, a product activated by the inhibitor, or an activating or inhibiting transcription factor. In the case of MIS, the ligand has been cloned for some time. However, its therapeutic efficacy was variable, depending on the serendipitous degree of activation of holo-MIS at its monobasic cleavage site in the engineered Chinese hamster cell of production. Therefore, to explore alternatives of producing a more efficacious and practical therapeutic agent, we explored methods of more reliably and reproducibly cleaving MIS, created the appropriate cleavage constructs, and tested them for their ability to produce MIS capable of inhibiting tumor growth.

Site-directed mutagenesis using the Kunkel method of M13 mutagenesis (36) was used to convert the monobasic cleavage site of MIS to a dibasic (RR) cleavage site and to create, as a control, a leaderless MIS protein that should be translated, but not secreted by the cell. The MIS RR and leaderless MIS mutant cDNAs and empty vector were transfected into MIS-responsive ocular melanoma cells, OM431. Biological activity of the modified MIS protein secreted by transfected OM431 cells was assessed by Müllerian duct regression in an organ culture (37), using concentrated culture media and immunofluorescence-purified MIS RR. Once biological activity was confirmed, the antiproliferative efficacy was studied in cells transfected with MIS RR in constructs in vitro and in vivo in metastasis assays.

MATERIALS AND METHODS

Modification of MIS. Using the Kunkel method of site-directed mutagenesis (36) human MIS cDNA was excised from the pD1 plasmid (38) and subcloned into polyclonal Amp (Invitrogen) using HindIII and NotI restriction endonuclease sites in the polylinker. The MIS cDNA was then subcloned into the M13mp19 replicative form using HindIII and XbaI sites in the polylinker. A deoxyuracil-containing single-stranded template was generated by transformation of CJ236 (dut-, ung-) with M13mp19/hMIS and purification of the phage DNA. Mutagenic oligonucleotides were designed with unique restriction enzyme sites to generate (a) a leaderless MIS that would be translated but not secreted and (b) a dibasic RR cleavage site (see below); the mutagenic oligonucleotides were then annealed to the deoxyuracil-containing template. Second-strand synthesis was performed and the double-stranded product transformed into XL1 Blue Escherichia coli that results in degradation of the wild-type deoxyuracil-containing strand and replication of the mutagenic strand. Colonies were then selected and mini-prep replicative form DNA tested by restriction enzyme digest for presence of the mutation. The mutant hMIS cDNAs were then subcloned into PUC18 using the HindIII/XbaI sites, and the presence of the mutations was further confirmed by sequencing. The mutagenic oligonucleotides (lower case) in the antisense orientation are as follows:

**Leaderless MIS:**

le: `{Kozak Box} HindIII

5'-TGGCTCTTCTGCTCTGATGGCCATCGTCCCAAGGCTAGCCC

CCAGGCGACG-3'

**MIS RR:**

le: `{Arg} ApaLI

5'-TGGCCCGCGCAGGCGCGCTGGACGACGCGGCGCG-3'

Cell Lines. Several different human ocular melanoma cell lines had been established (39); when tested for growth inhibitory response, OM431 was best inhibited by MIS (21). OM431 cells were grown in the alpha modification of MEM with ribonucleosides (alpha-MEM+) and 5% MIS-free female FCS. The wild-type human MIS cDNA, the MIS RR mutant, and the leaderless MIS mutant were subcloned into pcDNAI Neo (Invitrogen) using the HindIII and NotI sites in the polylinker. The completed constructs as well as the empty vector control were stably transfected into OM431 using the calcium phosphate transfection method. Clonal populations were isolated under G418 (Geneticin) selection.

MIS Analysis. A MIS ELISA (40) was used to detect the comparative secretion of MIS by wild-type MIS, MIS RR, or leaderless MIS clones. All clones were grown in alpha-MEM+ with 5% female FCS and 0.5 mg/ml Geneticin (Sigma). Samples of culture media were collected for ELISA analysis either after the clones had been in culture from 4 to 7 days and reached confluency, or every day in experiments that assessed the monoclonal growth of the clones. There was no detectable MIS in the culture media obtained from either the leaderless MIS/OM431 clones, except when excess cell lysis occurred and low levels of MIS could be detected (5 ng/ml), or the empty vector-transfected OM431 clones, which were also assayed by Southern blot for presence of the neomycin-resistance gene. High- and low-MIS RR-producing clones were identified by the MIS ELISA based on protein levels per million cells at confluence. Protein gel electrophoresis of immunooaffinity-purified MIS RR and wild-type MIS was performed on a 15% acrylamide gel at 30 mA for 1 h. The gel was then stained with Coomasie blue overnight and destained in 20% methanol/10% acetic acid. Regeneration of the Müllerian duct in response to different doses of MIS RR and wild-type holo-MIS was demonstrated in an organ culture bioassay (37) in which urogenital ridges from 14.5-day timed pregnant Holtzman rat fetuses were harvested and placed for 3 days at 37°C in organ culture dishes on agar over Cambridge Medical Research Laboratory 1066 media containing 10% female FCS. The urogenital ridges were then oriented, fixed, stained with hematoxylin and eosin, and serially sectioned. Regression of the Müllerian duct was graded using a morphological scale (37).
Monolayer Growth. Monolayer growth of cells transfected with the different constructs was determined by comparing cell numbers obtained on different days after seeding P100 Petri dishes (Falcon) with the same initial cell number. Prior to plating the clones for the experiments, the OM431-transfected clones were washed in HBSS, trypsinized, and seeded at a high density, and allowed to grow for 2 days. This was repeated to ensure that the cells were in logarithmic growth. The cells were then seeded in a similar manner and allowed to grow to density arrest over 4 days to assure exposure to secreted MIS; the cells were then trypsinized and further resuspended in fresh media after centrifugation to remove the trypsin. The cells were counted using a Coulter counter, resuspended to a concentration of 14,000 cells/ml, and recounted to verify the accuracy of the dilution. Transfected cells (140,000) were plated in P100 dishes and the cells in 2–5 plates counted for each clone on days 1, 2, 4, and 6 to assess growth. All clones were grown in α-MEM, α-MEM+, 10% female FCS (with no exogenous MIS as doe-

Lung Metastasis Assay. Cells were grown and cycled as described above, trypsinized, counted, and resuspended to a concentration of 400,000 cells/ml. Female SCID (41) mice were obtained from the Massachusetts General Hospital Radiation Biology Department where all work was done in accordance with a protocol approved by the Massachusetts General Hospital Animal Care Institutional Review Board. Using sterile technique, the tail veins of these mice were washed with alcohol followed by sterile mineral oil, and then injected with 0.25 ml of each of the transfected OM431 clones using tuberculin syringes and a 27-gauge needle, and the animals were returned to their cages. Four to 5 weeks later the lungs were harvested, inflated uniformly with 4% paraformaldehyde at 20 cm H2O pressure, and embedded in paraffin. Central coronal 8-mm sections were cut and stained with hematoxylin and eosin. Using the AppleSc carving program, three central coronal lung sections from each mouse were scanned and analyzed for pulmonary metastases after screening by light microscopy to eliminate areas of hemorrhage. Comparisons are of area occupied by tumor to that occupied by the total inflated lung expressed as a ratio, using the ImageGraphics software (version 1.37). Values for pulmonary tumor area ratio are expressed as the mean ± SE. Reduction in percentage of tumor area occupied by MIS RR cells was analyzed statistically with post hoc testing by ANOVA using the "SuperANOVA" software package from ABACUS concepts. A P of < 0.05 was considered to be statistically significant.

RESULTS

Prior to determining the biological activity of the mutant MIS RR protein, production was measured by ELISA in eight MIS RR OM431 clones. Samples of culture media were obtained when cells reached near confluency. Although all clones secreted MIS RR, two were selected for further evaluation. A high MIS RR OM431 clone that produced an average of 6.61 μg MIS RR/1,000,000 cells (n = 10) after 6–7 days in culture was compared to a low-producing clone that made 1.59 μg MIS RR/1,000,000 cells (n = 9) over the same time period. The

ELISA of media samples for the empty vector OM431 clone could detect no MIS (n = 10).

When purified to homogeneity by immunoaffinity chromatography, MIS RR protein exhibited 70-, 55-, and 12.5-kDa protein bands, visualized after Coomassie blue staining of polyacrylamide electrophoresis gels, indicating that reproducibly a considerable degree of cleavage of MIS had occurred (Fig. 1). The biological activity of MIS RR in causing regression of the Mullerian ducts was determined in the standard rat urogenital ridge assay. The dose response of a representative immunoaffinity-purified MIS RR preparation showed partial (0.15 pg/ml) to complete (0.24 pg/ml) regression of Mullerian ducts, similar to that caused by wild-type MIS.

Both the OM431/MIS RR high-producing (n = 8) and low-producing (n = 6) clones were significantly growth inhibited (P < 0.05) in vitro compared to the empty vector OM431 clone (n = 10) (Fig. 2A). In these experiments the MIS RR OM431 high-producing clone produced a mean of 8.7 μg MIS

Fig. 1 Polyacrylamide gel electrophoretic analysis of MIS RR is representative of the degree of endogenous cleavage of MIS RR resulting in the production of 55- (large arrow) and 12.5-kDa (small arrow) fragments separated on these reducing gels and stained with Coomassie brilliant blue (Lane 2). Intact 70-kDa holo-MIS RR monomer appears above the 55-kDa band (Lane 2). The position of the prestained molecular weight markers is given on the left in Lane 1.
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on MIS secretion. Clonal cells transfected with empty vector presented as mean ± SE.

Cell counts were normalized to the empty vector as a control, and high-producing clone grew significantly less than the low-producing (n = 5, ♦), which does not secrete MIS, grew equally well, whereas growth of the MIS RR high-producing clonal cells (n = 5, ▲) was significantly inhibited (*, P < 0.0002) after day 4 of incubation.

Fig. 2 A, MIS RR inhibits OMM431 monolayer cell growth. High (n = 8, □) and low (n = 6, □) MIS RR-producing OMM431 clones were both significantly growth inhibited (P < 0.01) relative to OMM431 clonal cells transfected with empty vector alone (n = 10, □). Furthermore, the MIS RR high-producing clone grew significantly less than the low-producing clone (P < 0.02). In each case, cells were counted on day 6 of culture. Cell counts were normalized to the empty vector as a control, and presented as mean ± SE. B, inhibition of OMM431 cell growth dependent on MIS secretion. Clonal cells transfected with empty vector (n = 5, □) or with the leaderless MIS mutant (n = 5, ♦), which does not secrete MIS, grew equally well, whereas growth of the MIS RR high-producing clonal cells (n = 5, ▲) was significantly inhibited (*, P < 0.0002) after day 4 of incubation.

RR/10⁶ cells and the low-producing clone generated a mean of 2.2 μg MIS RR/10⁶ cells. Monolayer growth of the MIS RR OMM431 high-producing clone was also significantly inhibited compared to the growth of the MIS RR clone (P < 0.05) producing lower levels of MIS. Secretion of MIS appears to be required for its biological activity in these cells since the proliferation of an OMM431 cell line transfected with the leaderless MIS mutant, which lacks a signal peptide, was not significantly different from the OMM431 clone transfected with empty vector (n = 5) but was significantly greater (P < 0.05) than the MIS RR high-producing clone (n = 5, Fig. 2B).

MIS RR-transfected OMM431 cells were significantly growth inhibited in vivo. Representative mid-sagittal lung sections from mice given injections of the OMM431 clone producing high levels of MIS RR show a significant reduction in total percentage area of lung occupied by metastases compared to either OMM431 cells containing the MIS RR low producer or the leaderless MIS clones (Fig. 3). Growth of tumors in all animals is compared in Table 1. The OMM431/MIS RR high-producer clonal cells resulted in fewer metastases as compared to the clones transfected with the low MIS producer (P < 0.002), empty vector (P < 0.01), or leaderless MIS (P < 0.02) constructs.

DISCUSSION

In the male fetus, MIS induces regression of the Müllerian ducts, preventing the formation of the Fallopian tubes, uterus, and upper third of the vagina (1–4). This inhibitory effect of MIS on normal development provoked an early interest in the effect of MIS on tumors arising from Müllerian duct precursor cells. Previously, we showed that MIS has an antiproliferative effect on established and primary endometrial and ovarian carcinoma cells in vitro (15–18). Subsequently, an inhibitory effect of MIS was observed on the human ocular melanoma cell line, OMM431, of neural crest derivation (19–21). Based on observed autocrine inhibitory effects on granulosa cell growth and progesterone production (42, 43) and paracrine inhibitory effects on oocytes (10, 11) and spermatogonia (12), as well as the pattern of mRNA expression of candidate MIS receptors (22–24), we suspect that germ cell and granulosa cells may be additional therapeutic targets. In order to consider in vivo tests of efficacy against such a wide range of human tumors, we anticipated the need to identify a reliable and stable MIS ligand and to streamline its production.

MIS requires proteolytic processing to generate the biologically active carboxy terminus which has TGF-β homology (31). Immunoaffinity-purified recombinant human MIS, as currently generated from transfected CHO cells (29), is, however, only partially cleaved. We previously demonstrated that the antiproliferative effect of wild-type MIS on ocular melanoma (21) varies with the amount of cleavage present during production and purification (30). Similar variability was seen when wild-type MIS preparations were assessed for ability to inhibit autophosphorylation of the epidermal growth factor receptor, i.e., increased cleavage resulted in enhanced inhibition of autophosphorylation.°° The specific endogenous monobasic cleavage enzyme responsible for this activity in the Sertoli cells of the testis or in target tissues has not yet been identified; however, it is clear that such cleavage is essential for bioactivity (30). A more efficient TGF-β-like dibasic R427-R428 cleavage site was created to replace MIS R427-S428 as a strategy to facilitate cleavage of MIS, and the present experiments were designed to assess the effect of this modification on the antiproliferative activity of MIS.

Culture media from stably transfected MIS RR OMM431 cells were concentrated and tested in the organ culture Müllerian duct regression bioassay (37) to assess biological activity of the mutant protein. Once biological activity was confirmed, culture media from the MIS RR OMM431 high-producing clone were immunoaffinity purified and, on gel electrophoresis, found to be the cleaved MIS species. Small quantities of this immunoaffin-


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Lung metastases were inhibited in animals given injections of transfected OM431 cells. Lungs from SCID mice given injections 4 weeks earlier with either 100,000 cells of leaderless MIS (A), MIS RR low-producing (B), or high-producing (C) OM431 clones, uniformly inflated with 20 cm H₂O pressure at the time of harvest, were embedded in paraffin, sectioned, and H & E stained. Compared to leaderless MIS and MIS RR low-producing OM431 clones, the lungs from the SCID mice given injections of the MIS RR OM431 high-producing clone showed the least number of metastases.

### Table 1: MIS RR results in significant reduction in metastases of OM431 cells

<table>
<thead>
<tr>
<th>OM431 Clone</th>
<th>Mean % tumor area: experiment 1</th>
<th>Mean % tumor area: experiment 2</th>
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<tbody>
<tr>
<td>Empty vector</td>
<td>20.473 ± 2.365</td>
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<tr>
<td>(n = 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaderless MIS</td>
<td>17.420 ± 4.378</td>
<td>18.590 ± 2.746</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>MIS RR high producing</td>
<td>1.696 ± 0.732</td>
<td>1.039 ± 0.256</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>MIS RR low producing</td>
<td>6.816 ± 4.378</td>
<td>14.854 ± 2.729</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 8)</td>
<td></td>
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</tbody>
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* The mean percentage of tumor area ± SE of lungs from animals given injections of the OM431 MIS RR high-producing clone indicated that this clonal line produced the least number of metastases when compared to MIS RR low-producing, empty vector (n = 7, P < 0.0028), and leaderless MIS (n = 3, P < 0.0096 and n = 10, P < 0.0001) OM431 clones.

MIS RR results in significant reduction in metastases of OM431 cells.

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CHO cells as well as a novel yeast. Purified MIS RR protein can then be compared with wild-type MIS for specific activity and stability, and production of the most effective ligand scaled up for use in in vivo animal trials and eventually in human clinical trials against MIS-sensitive tumors preselected by the presence of MIS receptor. Meanwhile a search is underway to uncover the endogenous enzyme that cleaves MIS, with the expectation that cotransfecting its gene with the MIS gene may yield the most efficient production of cleaved and hence activated MIS.

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