Müllerian Inhibiting Substance Inhibits Ovarian Cell Growth through an Rb-independent Mechanism*

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Müllerian inhibiting substance (MIS), a transforming growth factor-β family member, causes regression of the Müllerian duct in male embryos. MIS overexpression in transgenic mice ablates the ovary, and MIS inhibits the growth of ovarian cancer cell lines in vitro, suggesting a key role for this hormone in postnatal development of the ovary. This report describes a mechanism for MIS-mediated growth inhibition in both a human epithelial ovarian cancer cell line and a cell line derived from normal ovarian surface epithelium, which is the origin of human epithelial ovarian cancers. MIS-treated cells accumulated in the G1 phase of the cell cycle and subsequently underwent apoptosis. MIS up-regulated the cyclin-dependent kinase inhibitor p16 through an MIS type II receptor-mediated mechanism and inhibited growth in the absence of detectable or inactive Rb protein. Prolonged treatment with MIS down-regulated the Rb-related protein p130 and increased the Rb family-regulated transcription factor E2F1, overexpression of which inhibited growth. These findings demonstrate that p16 is required for MIS-mediated growth inhibition in ovarian epithelial cells and tumor cells and suggest that up-regulation of E2F1 also plays a role in this process.

MIS, a member of the TGF-β family of hormones, induces regression of the epithelial-mesenchymal unit of the Müllerian duct in the embryonic urogenital ridge in males. In the absence of MIS, differentiation of the Müllerian duct into the uterus, fallopian tubes, and upper vagina in female embryos occurs autonomously (1). The 140-kDa MIS homodimer is enzymatically cleaved into two distinct fragments. The carboxy-terminal fragment composed of a dimer with subunits of Mr 12,500 retains bioactivity, whereas a noncleavable mutant is devoid of biological function (2). MIS is produced at high levels by Sertoli cells of the testis even after the regression of the Müllerian duct and decreases at adolescence. In females, it is synthesized by granulosa cells of the ovary. Measurement of circulating serum MIS levels in females indicates that MIS in females is produced postnatally, increases at the onset of puberty, and is undetectable at menopause (3, 4). It is hypothesized that binding of MIS ligand to the MIS type II receptor, a serine threonine kinase (5–7), leads to heterodimerization with a type I receptor, initiating a signaling cascade.

The MIS type II receptor gene contains 11 exons and encodes a 63-kDa protein, which is expressed at very high levels in the uterus, testis, and ovary (5–7). Male mice that lack both alleles of the MIS type II receptor have a persistent Müllerian duct, which differentiates into a uterus and oviducts, a phenotype reminiscent of MIS ligand null mice (8). Imbeaud et al. (9) have identified MIS type II receptor mutations in male patients with Persistent Müllerian Duct syndrome, which reaffirms the developmental significance of its expression in humans. Transgenic female mice that overexpress MIS ligand, demonstrate complete ablation of the ovary, along with undeveloped uterus and oviducts (10). In male mice overexpression of MIS ligand leads to feminized genitalia, undescended testes, and a poorly differentiated Wolffian duct (10). Thus, regulation of MIS levels in the serum probably plays an important role in development of the ovary and masculinization of male reproductive organs.

In addition to its significant role in sexual development, several lines of evidence suggest that MIS is a multifunctional hormone (11–13). Because epithelial ovarian tumors originate from the surface epithelium of the ovary, which is also the origin of the Müllerian duct (14, 15), the effect of MIS on the growth of ovarian cancer cells has been an area of intense study. MIS inhibits the growth of human ovarian cancer cell lines and single cell suspensions derived from solid tumors or ascites from ovarian cancer patients in nonadherent colony inhibition assays (16, 17). Recently, Masiakos et al. (18) correlated MIS-mediated growth inhibition with expression of MIS type II receptor in primary ovarian epithelial cells derived from cancer patients.

To uncover the molecular mechanism by which MIS inhibits the growth of ovarian cancer cells, we analyzed the MIS-sensitve human ovarian cancer cell line OVCAR 8, which expresses significant levels of MIS type II receptor (18). Because cyclin-dependent kinase inhibitors (CDKI) play a key role in modula-
tion of growth (19, 20), we first examined MIS-mediated CDKI regulation during growth inhibition. CDKIs are a family of molecules that inhibit the kinase activity of cyclin/cyclin-dependent kinase (CDK) complexes, whose targets such as the retinoblastoma family gene products, Rb, p107, and p130 modulate cell cycle progression (20–22). There are two classes of CDKIs based on their specificity toward CDKs. The CIP family, which includes p21, p27, and p57, has a broader specificity toward CDKs than do members of theINK4 family, which includes p15, p16, p18, and p19 and specifically inhibits the kinase activity of CDK4 and CDK6 (20). The p16 locus 9p21 is mutated or silenced in a variety of human cancers and cell lines, and it encodes for two unrelated polypeptides p16 and p14ARF (23, 24). Although several reports describe aberrant regulation of p16 expression in human cancers and cell lines, very few growth inhibitory molecules induce p16 expression during growth inhibition (25). Overexpression of p16, in addition to interrupting cell cycle progression, also regulates apoptotic cell death (26–30). Although the effects of p16 on the cell cycle are mediated predominantly through inhibition of Rb, it is also known to influence apoptosis and senescence through pathways that are independent of Rb (26, 31). In this report we demonstrate that MIS-mediated inhibition of ovarian cancer cell growth is manifested by a block in cell cycle progression and apoptosis. The growth inhibitory effects of MIS are mediated through specific induction of p16 protein expression and via regulation of p130 and E2F1 in the absence of detectable levels of Rb.

**EXPERIMENTAL PROCEDURES**

**Cell lines and Reagents—**OVCAR 8 and COS cells were grown in Dulbecco's modified Eagle's medium and 10% fetal bovine serum. Human ovarian surface epithelial cells HOSE 6-3 (a gift from Dr. Samuel Mok, Brigham and Women's Hospital) were grown in a 1:1 mixture of M199 (Sigma) and MDCB 105 (Sigma) media supplemented with 10% fetal bovine serum. Dominant negative MIS type II receptor (DN-MIS type IIR) expressing OVCAR 8 clones were generated by cotransfecting 0.5 μg of hygromycin resistance plasmid and 10 μg of CMV-FLAG-tagged rat dominant negative MIS type II receptor construct using 9 μl of FUGENE 6 (Roche Molecular Biochemicals). Binding studies with biotinylated MIS were done as described previously (18).

**Antibodies and Western and Northern Blot Analyses—**The rabbit MIS type II receptor antibody was generated by injecting animals with the peptide CGTDFCNANYSHLPPSG, which corresponds to amino acids 111–127 of the receptor. The antibody was purified over a Protein A-Sepharose column before use. Mouse monoclonal antibodies against p16 (JC6), p57 (KP10), p21 (CP36), and E2F1 (KH95) were a kind gift of Samuel Mok, Brigham and Women's Hospital) were grown in a 1:1 mixture of M199 (Sigma) and MDCB 105 (Sigma) media supplemented with 10% fetal bovine serum. Dominant negative MIS type II receptor antibody (DN-MIS type IIR) expressing OVCAR 8 clones were generated by cotransfecting 0.5 μg of hygromycin resistance plasmid and 10 μg of CMV-FLAG-tagged rat dominant negative MIS type II receptor construct using 9 μl of FUGENE 6 (Roche Molecular Biochemicals). Binding studies with biotinylated MIS were done as described previously (18).

**RESULTS**

**Expression of MIS Type II Receptor Protein and MIS Ligand Binding in OVCAR 8 Cells—**The growth of the human ovarian cancer cell line OVCAR 8, which expresses MIS type II receptor mRNA, is inhibited following the expression of bioactive MIS ligand (18). Western analysis using a rabbit MIS type II receptor antiserum demonstrated the presence of the 63-kDa endogenous receptor protein in OVCAR 8 cells (Fig. 1A). Preimmune rabbit serum confirmed the specificity of anti-MIS type II receptor antibody. Antibody specificity was also confirmed using COS cells transfected with a CMV-driven FLAG-tagged rat MIS type II receptor construct (32).

OVCAR 8 cells bound biotinylated MIS specifically with a concentration required to reach half saturation of approximately 12 nM, a measure of the dissociation constant of the MIS-receptor complex (Fig. 1B). Total binding of MIS-biotin approached saturation at a concentration of 75 nM and was specifically competed with a 10-fold molar excess of unconjugated MIS. These values were comparable to the Kd and saturating concentration of MIS seen in several ovarian cancer cell lines that express the MIS type II receptor (18).

**MIS Induces Apoptosis of OVCAR 8 Cells—**We previously demonstrated that stable expression of a CMV-driven MIS construct in OVCAR 8 resulted in 87% inhibition of drug-resistant colony growth compared with cells transfected with the vector or the leaderless inactive form of MIS (18) (see below). Fluorescence-activated cell sorting (FACS) was performed to determine whether inhibition of OVCAR 8 cell growth by MIS correlated with perturbation in the cell cycle. A 14–18% increase in the G1 phase was observed after 48 h of treatment with MIS (Fig. 2A). A difference in cell number became evident after 4–7 days of MIS treatment with increasing numbers of dying, poorly adherent cell bodies. Annexin V-FITC staining was used to detect early stage apoptosis in cells treated with MIS. Annexin V binds to phosphatidyl serine with high affinity and is translocated from the inner surface of the plasma membrane to the outside after initiation of apoptosis in most cell types. A progressive increase in annexin V-
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**Fig. 1.** OVCAR 8 cells express the MIS type II receptor. **A**, total protein lysates (100 μg) from OVCAR 8 cells were analyzed by immunoblotting with rabbit anti-MIS type II receptor antibody or rabbit preimmune serum. **B**, specific binding of biotinylated MIS to OVCAR 8 cells. Increasing concentrations of MIS-Biotin incubated with OVCAR 8 cells resulted in increasing shifts in mean fluorescence per cell. The composite figure for total and specific binding (±S.E.) from at least two experiments normalized against background fluorescence is shown. The dissociation constant, $K_d$, of ~12 nM was calculated at half-saturation of MIS specific binding.

staining with prolonged exposure of OVCAR 8 cells to MIS was observed; a 2-fold increase in annexin V-positive cells seen after 2 days of MIS treatment increased to 3–4 and 4- to 6-fold after 4 and 7 days, respectively (Fig. 2B). Furthermore, electrophoretic analysis of DNA from dying cells revealed fragmentation of chromatin into a nucleosomal ladder characteristic of apoptosis (Fig. 2C). This suggested that MIS-mediated growth inhibition of OVCAR 8 cells resulted from a block in cell cycle progression and ensuing cell death.

**MIS-induced p16 Expression Is Mediated through the Endogenous MIS Type II Receptor**—Regulation of cyclin-dependent kinase inhibitors (CDKI), a family of molecules that inhibits the kinase activity of cyclin/cyclin-dependent kinase (CDK) complexes, plays a key role in proliferation, differentiation, and apoptosis (20). Therefore we tested whether MIS affects the expression of CDKIs. Western blot analysis was performed using protein lysates obtained from OVCAR 8 cells treated with MIS for various periods of time. Fig. 3A shows that treatment with 35 nM MIS up-regulates the expression of p16 protein as early as 30 min, which persists for up to 24 h. Interestingly, the p16 protein in MIS-treated cells also appeared to have a slightly reduced mobility compared with that in untreated cells. A similar experiment performed to determine the lowest dose of MIS required to up-regulate p16 showed that a concentration as low as 7 nM (1 μg/ml) was sufficient (Fig. 3A, lower panel). No change in levels of p27 or p57 was detected. A 2-fold induction of p21 protein and mRNA was observed in several experiments (Fig. 3B). Northern blot analysis of total RNA isolated from cells treated with MIS for increasing periods of time indicated that the increase in p16 protein was not due to elevated p16 mRNA levels (Fig. 3C, upper panel). p16 turnover calculated from cycloheximide-Western blot analysis demonstrated the half-life of p16 protein to be ~10 h. Treatment with MIS slightly prolonged the half-life of p16 to >10 h (Fig. 3C, lower panel). However, this increase in stability of p16 protein does not fully account for the increase in p16 protein, which is observed within 30 min of MIS treatment. It is thus likely that MIS increases the translation of p16 mRNA. This is consistent with an increase in p16 protein during squamous differentiation through mechanisms such as protein stability and increased translational activity (33). TGF-β, which induces p15, p21, and p27 to inhibit the growth of different cell types (34–36), failed to elevate p16 expression in OVCAR 8 cells but up-regulated the level of p21 protein (Fig. 3D, upper and lower panels). This suggests that the signaling pathway utilized by MIS is distinct from that of TGF-β.

A kinase-defective, dominant negative rat MIS type II receptor was stably transfected into OVCAR 8 cells to determine whether the increase in p16 protein is in fact mediated through the endogenous MIS type II receptor. Western blot analysis using an anti-FLAG antibody demonstrated the expression of the kinase-defective receptor transgene in two OVCAR 8 cell clones (Fig. 3E, upper panel). Failure of MIS to increase p16 in these cells indicated that this effect is mediated by the endogenous MIS type II receptor (Fig. 3E, lower panel). Both clones demonstrated increased binding of biotinylated MIS compared with vector-transfected cells (data not shown).

**MIS-induced p16 Abrogates Growth**—To determine whether up-regulation of p16 is responsible for MIS-induced inhibition of OVCAR 8 cell growth, colony inhibition assays were performed. As shown previously, stable expression of MIS in OVCAR 8 cells inhibited the growth of drug-resistant colonies by 75% compared with cells transfected with vector construct. The importance of p16 up-regulation in MIS-mediated growth inhibition is demonstrated by the ability of antisense p16 to rescue cells from MIS-mediated growth inhibition. Expression of antisense p16 alone did not increase colony numbers, suggesting that abrogation of MIS-mediated growth inhibition was not due to the enhanced proliferative potential of cells expressing it (Fig. 4A). The ability of antisense p16 to block the translation of p16 protein was demonstrated by transient transfection of sense and antisense p16 constructs into COS cells. The presence of the antisense p16 construct inhibited the translation of p16 protein derived from a p16 expression construct (Fig. 4B). The ability of p16 to inhibit the growth of OVCAR 8 cells was further demonstrated by expressing a CMV-driven p16 construct in OVCAR 8 cells. As seen in Fig. 4C, expression of p16 inhibited drug-resistant colony growth by 80% compared with cells transfected with the vector.

**Effects of MIS on a Cell Line Derived from the Surface epithelium of Normal Ovary**—Approximately 80–90% of ovarian cancers originate from the surface epithelium of the ovary and
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### Table A

| Experiment | Treatment | Time | Cell fraction (%) |
|------------|-----------|------|------------------|
|            |           |      | G1   | S   | G2/M |
| 1          | —  | 0hr  | 49.9 | 25.9 | 24.2 |
|            | —  | 48hr | 52.4 | 22.8 | 24.8 |
|            | +MIS | 48hr | 63.5 | 21.9 | 14.6 |
| 2          | —  | 0hr  | 47.7 | 27.0 | 25.2 |
|            | —  | 48hr | 54.5 | 25.3 | 20.2 |
|            | +MIS | 48hr | 62.0 | 20.5 | 17.5 |
| 3          | —  | 0hr  | 43.2 | 31.0 | 25.8 |
|            | —  | 48hr | 52.5 | 23.5 | 24.0 |
|            | +MIS | 48hr | 60.2 | 19.3 | 20.6 |

### Diagram B

**DAY 2**

- MIS

+ MIS

**DAY 4**

- MIS

+ MIS

**DAY 7**

- MIS

+ MIS

### Table C

| Sample | % Apoptotic |
|--------|-------------|
| -MIS   | 0.49        |
| +MIS, #1 | 1.15      |
| +MIS, #2 | 1.13      |
| +MIS, #3 | 1.07      |

| Sample | % Apoptotic |
|--------|-------------|
| -MIS   | 1.24        |
| +MIS, #1 | 4.28      |
| +MIS, #2 | 3.86      |
| +MIS, #3 | 3.33      |

| Sample | % Apoptotic |
|--------|-------------|
| -MIS   | 0.76        |
| +MIS, #1 | 4.87      |
| +MIS, #2 | 4.25      |
| +MIS, #3 | 3.42      |

**600 bp**
FIG. 3. MIS type II receptor mediated induction of p16 protein. A, MIS induces p16. Upper panel, MIS (35 nM)-treated OVCAR 8 protein lysates (100 µg) were immunoblotted with mouse anti-p16 antibody. Lower panel, protein lysates (100 µg) from cells treated for 6 h with increasing concentrations of MIS were probed with anti-p16 antibody. An equal amount of protein was used in each lane. B, Western blot analysis of total protein isolated from OVCAR 8 cells treated with 35 nM MIS for indicated periods time using anti-p21, p27, and p57 antibodies. C, Upper panel, MIS does not induce p16 mRNA. 10 µg of total cellular RNA isolated from OVCAR 8 cells treated with 35 nM MIS was probed with human p16 cDNA. Lower panel, p16 immunoblot of cellular lysates from untreated and MIS-treated (24 h) OVCAR 8 cells after inhibition of new protein synthesis with cycloheximide (CHX). D, TGF-β induces p21 but not p16 in OVCAR 8 cells. Total protein isolated from OVCAR 8 cells treated with 200 pM TGF-β was analyzed using anti-p16 and anti-p21 antibodies. E, OVCAR 8 cells were stably transfected either with vector (Vector) or a kinase-defective, FLAG-tagged rat MIS type II receptor mutant (R2DN3 and R2DN4). Upper panel, immunoblot of total protein (100 µg) isolated from Vector, R2DN3, and R2DN4 cells with mouse anti-FLAG antibody. Lower panel, p16 induction is mediated by endogenous MIS type II receptor. p16 expression in OVCAR 8 cell clones with Vector, R2DN3, and R2DN4 treated with 35 nM MIS for 24 h.
malignant ovarian epithelial tumors have the potential to differentiate into epithelium similar to that of the Mullerian duct (14, 15). Thus we tested the effects of MIS on HOSE 6-3, a cell line derived from normal human ovarian surface epithelium. These laser-dissected cells were immortalized with the human papilloma virus (HPV) E6 and E7 oncoproteins for maintenance in vitro (37). Western blot analysis of proteins derived from HOSE 6-3 cells demonstrated the expression of MIS type II receptor (Fig. 5A). A 2- to 4-fold increase in annexin V-positive cells was seen in HOSE 6-3 cells treated with MIS for 4 days, indicating that MIS induces programmed cell death in these cells (Fig. 5B). Furthermore, MIS treatment of HOSE 6-3 cells for 24 h induced p16 protein expression (Fig. 5C). These observations suggested that MIS-mediated induction of p16 and growth regulation is also operational in the surface epithelial cells of normal ovary from which ovarian cancers derive.

**MIS Regulates the Expression of E2F1**—p16 has also been shown to modulate downstream pathways, which involve Rb, p107, p130, and members of the E2F family of transcription factors (20, 38, 39). OVCAR 8 cells did not express Rb protein when tested by either immunofluorescence (Fig. 6A, upper panel) or Western blot (Fig. 6A, lower panel) suggesting that the inhibitory effect of p16 on OVCAR 8 cell growth is mediated via an Rb-independent mechanism. Furthermore, MIS-mediated growth inhibition of HOSE 6-3 cells, immortalized with E7 and E6 oncoproteins (37), which inactivate Rb and p53, respectively, also indicates that MIS functions through a Rb, p53-independent pathway. Western blot analysis was performed to determine whether MIS regulates the expression of other members of the pocket protein family. Exposure of OVCAR 8 cells to MIS for 4 days down-regulated p130 protein levels by an estimated 5-fold with no detectable change in p107 protein (Fig. 6B, upper panel). Difference in p130 expression could not be detected before 3 days of treatment in repeated experiments (data not shown). This suggests that the effect of MIS on p130 expression is indirect. TGF-β treatment of OVCAR 8 cells, however, had no effect on either p107 or p130 protein expression (Fig. 6B, lower panel). Although TGF-β induced p21 expression, TGF-β treatment of OVCAR cells did not result in either an increase in the G1 phase of the cell cycle or apoptosis.

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**Fig. 4.** P16 induction is required for MIS-mediated inhibition of growth. A, equal numbers of OVCAR 8 cells were stably transfected with 0.5 ug of hygromycin resistance plasmid and 20 ug of CMV-vector, 10 ug of CMV-driven K2, 10 ug of K2 + 10 ug of CMV-antisense p16 (as-p16), or 10 ug of as-p16 DNA. DNA was equalized with CMV-vector DNA. Colonies, which grew in medium containing 100 ug/ml hygromycin for 3 weeks, were stained with crystal violet. The number of drug-resistant colonies in each plate is represented as percentage survivors. Colonies in plates transfected with vector was set at 100%. A representative experiment is shown in the bottom panel. B, antisense p16 ablates translation of p16 protein. Lysates from COS cells transiently transfected with 0.1 ug of CMV-driven sense or 3 ug of antisense p16 constructs, or cotransfected with 2.9 ug of antisense and 0.1 ug of sense p16 constructs, were immunoblotted with an antibody to p16. Position of the p16 protein is indicated. C, p16 inhibits growth of OVCAR 8 cells. Cells stably transfected with 0.5 ug of hygromycin resistance plasmid and either 10 ug of vector or p16 expression constructs were analyzed as described above.

**Fig. 5.** MIS induces p16 expression in a normal human ovarian surface epithelial cell line (HOSE 6-3). A, expression of MIS type II receptor in HOSE 6-3 cells. 100 ug of total protein from HOSE 6-3 cells was probed for expression of MIS type II receptor protein. 100 ug of protein lysate from OVCAR 8 cells was used as positive control. A parallel blot probed with the preimmune serum for antibody specificity is shown. B, quantification of cells undergoing apoptosis. HOSE 6-3 cells were treated with 35 nM MIS for 4 days, stained with annexin V-FITC and PI, and analyzed by FACS. A representative experiment is shown in the left panel. Zones A and B represent live and early apoptotic cells, respectively. The right panel shows the fold increase in annexin V-positive, early apoptotic cells following 4 days in culture in the absence or presence of MIS (n = 3). C, MIS induces p16 expression in HOSE 6-3 cells. Cells were treated with 35 nm MIS for 24 h, and 100 ug of total protein was analyzed by Western blot using mouse anti-p16 antibody. Positions of the molecular weight markers, MIS type II receptor, and p16 protein are indicated.
This corroborates reports that demonstrate that effects of TGF-β on growth are mediated predominantly through Rb. The ability of pRB, p107, and p130 to regulate the cell cycle depends on their capacity to associate and regulate the activity of cellular partners, notably the E2F family of transcription factors. TGF-β treatment has been shown to repress the levels of E2F1 mRNA and protein (40, 41). Thus we investigated whether MIS modulates the expression of E2F1 in OVCAR 8 cells. MIS induced E2F1 protein following 4 days of treatment (Fig. 6C). As with p130, no change in levels was observed up to 3 days, suggesting that the effect of MIS on the expression of E2F1 is indirect. Interestingly, induction of p16, which occurred as early as 30 min of MIS treatment, persisted for up to 7 days (Fig. 6D). TGF-β, which has been shown to down-regulate the level of E2F1 mRNA and protein (40, 41), had no
Effect on the expression of E2F family members in OVCAR 8 cells (data not shown).

Overexpression of E2Fs, notably E2F1, has been shown to induce apoptosis in several cell systems (39, 42). Thus we investigated whether expressing high levels of E2F1 would inhibit the growth of OVCAR 8 cells. Indeed, stable overexpression of E2F1 in OVCAR 8 cells resulted in 60% reduction in colony numbers compared with cells transfected with the vector alone (Fig. 6E) suggesting that the induction of apoptosis observed in OVCAR 8 cells might be the result of E2F1 up-regulation. Thus treatment of OVCAR 8 cells with MIS inhibits growth through a complex regulation of the cell cycle regulatory proteins.

Discussion

The role of MIS in the dissolution of the Müllerian duct in male embryos is well established. However, its role in female sexual development is not well understood. The complete ablation of ovaries of transgenic mice that overexpress MIS (10) suggests that MIS plays a key role in the postnatal development of the ovary. Masaikos et al. (18) recently demonstrated that MIS inhibits the growth of human ovarian cancer cell lines and primary tumor cells derived from patients with ovarian cancer. We now identify a molecular mechanism responsible for MIS-mediated ovarian cancer cell growth inhibition using both the human epithelial ovarian cancer cell line OVCAR 8, and a cell line, HOSE 6-3, derived from the normal surface epithelium of the ovary, which is the origin of human epithelial ovarian cancers.

Growth inhibition of ovarian epithelial cells by MIS is manifested by an increase in the G1 phase of the cell cycle and programmed cell death. As with effects of cytokines such as TGF-β and interferons on other cell types, MIS-mediated growth inhibition of cell lines derived from the ovary correlated with an up-regulation of CDK1. However, unlike TGF-β, which induces p15, p21, and p27 during growth inhibition (34–36), MIS specifically increased the level of p16 protein. Thus these two cytokines, which belong to the same family, appear to use distinct molecular signals to inhibit cell growth. The importance of MIS-mediated induction of p16 protein in growth inhibition is demonstrated by the reversal of this process upon abrogation of p16 translation.

A major mechanism by which CDKIs alter cell cycle distribution is by preventing the phosphorylation of Rb (20). However, growth inhibitory responses to CDKIs may also involve Rb-independent mechanisms that are mediated through other Rb family members, p130 and p107 (see below), as well as other cell cycle regulatory molecules. Zhu et al. (43, 44) demonstrated that each member of the Rb family can display cell cycle effects that are cell type-specific. In addition, both p27 and p21 can block proliferation through Rb-independent mechanisms (45). The ability of p16 to inhibit the proliferation of HeLa cells in which Rb is rendered inactive by HPV-E7 also supports the existence of an alternate pathway (26). Rb protein expression was not detectable in OVCAR 8 by standard techniques, and MIS treatment did not result in discernible levels of Rb. Therefore, p16 inhibits OVCAR 8 cell proliferation (Fig. 4C) via an Rb-independent pathway. However, we cannot rule out the possibility that these cells express very low amounts of Rb protein. The inhibitory effects of MIS-induced p16 protein on the growth of HOSE 6-3 cells, in which expression of HPV-E7 during immortalization (37) functionally inactivated Rb (46), also supports the contention that its effects are Rb-independent. MIS treatment of OVCAR 8 cells, however, did lower the level of p130, an Rb-related protein. Because the decrease in p130 occurred after 4 days of MIS treatment, it probably results from an indirect mechanism. Unlike Rb, the level of p130 does not remain constant during the cell cycle (47). Although hyperphosphorylation of p130 at certain residues has been shown to decrease its stability (48–50), we could not detect a decrease in p130 protein mobility reflective of hyperphosphorylation. Thus, prolonged MIS treatment may influence transcription, translation, or the stability of the p130 gene product.

p16, besides imposing a block in the cell cycle, has been implicated in the control of apoptosis through an unknown mechanism in many cell systems. Overexpression of p16 protects neuronal cells from cyclin D1-induced apoptosis (51) and acute lymphoblastic leukemia cells from dexamethasone-induced cell death (28). Conversely, p16-induced apoptosis was demonstrated in several cell systems, including ovarian cancer cell lines (26, 27, 29). Infection of human ovarian cancer cell lines, OVCAR 420 and SKOV 3, with p16 containing adenoviral vector results in G1 arrest and subsequent apoptosis (26, 27). It is unclear whether p16-induced apoptosis requires the presence of functional Rb (26). Based on these observations and the ability of antisense p16 to abrogate MIS-induced growth inhibition, we speculate that the initiation of MIS-induced apoptosis of ovarian cancer cells requires up-regulation of endogenous p16 protein expression. The fact that p16 was induced by MIS within 30 min of treatment and the finding that cell death in both OVCAR 8 and HOSE cells was prominent only after 4 days suggest that the effect of MIS-induced p16 on apoptosis is indirect. Such delayed apoptotic effects of p16, p18, and p27 overexpression have been reported in A549, HeLa, SKOV-3, and MTA1A2 cells (26).

MIS increased the level of E2F1 protein in OVCAR 8 cells, another observation that reflects a mechanism different from TGF-β, which decreases E2F1 mRNA and protein during growth inhibition (40, 41). The increase in E2F1 by MIS may result from a decrease in p130 and reversal of E2F4- or E2F5-p130-mediated repression of the E2F1 promoter (31, 52). In addition to facilitation of cell cycle progression, E2F1 also induces apoptosis in several cell systems (42, 53–55). E2F1 null mice exhibit an increase in mature lymphocytes and a broad range of tumors, suggesting a role in the regulation of apoptosis (56, 57). Interestingly, these tumors include those of the ovary and uterine horn (57), both of which express the MIS type II receptor (7). The apoptotic effect of E2F1, which is independent of its ability to activate transcription but requires DNA binding, can be inhibited by Rb (58, 59). The growth inhibitory effect of E2F1 overexpression on OVCAR 8 cell growth thus may be enhanced by the lack of Rb expression in this cell line.

In summary, MIS-mediated inhibition of ovarian cancer cell growth results in the accumulation of cells in the G1 phase of the cell cycle and delayed apoptosis. We speculate that elevated levels of p16 regulate these processes indirectly through p130 and E2F1. Low or absent p16 expression attributed to homozygous deletions, missense mutations, or hypermethylation of the promoter (60–63) was found in 31–36% of primary epithelial ovarian cancers of serous, endometrioid, and mucinous origin. This suggests an important role for p16 in regulating the growth of epithelial cells lining the surface of the ovary, from which 90% of ovarian cancers originate (14, 15). Thus up-regulation of p16 protein by either gene transfer technique or MIS treatment could offer therapeutic benefit in treatment of ovarian cancer patients.

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