Inhibin Resistance Is Associated with Aggressive Tumorigenicity of Ovarian Cancer Cells

Michael D. Steller, Tanya J. Shaw, Barbara C. Vanderhyden, and Jean-François Ethier

Centre for Cancer Therapeutics, Ottawa Regional Cancer Centre, and Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada

Abstract
Malignant ovarian epithelial tumors have been shown to have decreased inhibin production relative to activin production compared with normal ovarian surface epithelial (OSE) cells and nonmalignant ovarian tumors. Activin stimulates proliferation of many ovarian cancer cell lines. Inhibin antagonizes the action of activin, and inhibin-deficient mice develop gonadal tumors, suggesting that inhibin may be a tumor suppressor. However, its effects on OSE and ovarian cancer cells are unknown. We hypothesize that activin and inhibin are important regulators of biological activity in ovarian cancers. We found that inhibin A decreased murine OSE proliferation, whereas activin A had no effect. Activin A increased the proliferation of four of eight ovarian cancer cell lines (SKOV3, OCC1, OVCAR3, and A2780-s). Inhibin A decreased the proliferation of SKOV3, A2780-s, and OVCAR3 but had no effect on OCC1, ES-2, HEY, A2780-cp, and OVCA429 cells. When injected into nude mice, the inhibin-resistant cancer cell lines resulted in shorter survival time compared with the inhibin-responsive cells. Further investigations on SKOV3 and OCC1 cells showed that activin A increased invasion through Matrigel. Inhibin A decreased both basal and activin-induced proliferation and invasion of SKOV3 but had no effect on OCC1 cells. Reverse transcription-PCR analyses showed that the SKOV3 and OCC1 cells produced activin, but only SKOV3 produced inhibin. Analysis of the activin/inhibin signaling pathways indicated that Smad anchor for receptor activation was elevated in SKOV3 and OCC1 cells and that an up-regulation of the activin receptor expression may explain the inhibin resistance of OCC1 cells. Our results suggest that activin responsiveness may be gained during transformation of OSE cells and that inhibin resistance may contribute to the aggressive behavior of ovarian cancer cells. (Mol Cancer Res 2005;3(1):50–61)

Introduction
The clinical management of ovarian cancer is a therapeutic challenge. The disease is generally diagnosed at a very late stage, and despite significant effort to develop new therapies, the 5-year survival rate is still very low. Although the role of tumor suppressor genes and oncogenes is now clearer, the molecular biology of ovarian cancer is not completely understood. Ovarian tumors may arise from all types of ovarian cells, yet the majority seem to originate from the ovarian surface epithelium (OSE; ref. 1). It has been shown that improper regulation of factors controlling proliferation of normal ovarian cells, such as granulosa and epithelial cells, may lead to uncontrolled cell growth. Recent work on inhibin and activin may provide clues to ovarian cancer onset and progression. Activin and inhibin are produced by the ovary and are required for normal ovarian functions, such as the control of folliculogenesis where they act as paracrine factors. They are also required for the endocrine regulation of follicle-stimulating hormone secretion by the pituitary gonadotropes (2).

Activin is a secreted dimer protein composed of two β subunits covalently linked by a single disulfide bond. The predominant forms of the β subunit include βA and βB, which dimerize to form activins A, AB, and B. Activin stimulates the transcriptional activity of responsive genes by first binding to a complex of serine/threonine kinase membrane receptors. Four receptors are known to bind activin with high affinity (Alk2, Alk4, ActRII, and ActRIIB), although thus far only the last three have been shown to transduce an activin signal (2). Intracellular signaling proteins called Smad2 and Smad3 mediate the delivery of the signal from the membrane to the nucleus. Recent studies suggest that a protein named Smad anchor for receptor activation (SARA) facilitates the propagation of the Smad2 and Smad3 signals (3). SARA is associated with the internal membrane surface and serves as a docking protein to bring Smad2 and Smad3 near the receptor complex. SARA thus facilitates phosphorylation of these Smads by the kinase domain of type I receptor (3). Once phosphorylated, the affinity of the Smads for SARA is decreased, allowing dissociation of Smads from SARA as well as recruitment of Smad4. Subsequent nuclear transport of the Smad complex and transcriptional responses ensue. SARA is also shown to localize...
to early endosomes, and inhibition of endocytosis results in decreased Smad-dependent transcription (4-7). Thus, it is suggested that endocytosis is an important element in signal transduction pathways involving SARA.

The expression of the activin receptors has been shown in ovarian epithelial tumors (OET), suggesting that OET may be activin responsive (8-10). Consistent with this observation, activin stimulates the proliferation of many human ovarian cancer cell lines but not of normal OSE (11, 12).

Inhibin is an important regulator of activin actions. Inhibin is structurally related to activin and consists of an α subunit covalently linked to either one of the βA and βB subunits. Thus, the predominant forms of inhibin are inhibins A and B. Inhibin antagonizes activin actions by competing with activin for the binding of the activin type II receptors. This process requires the presence of the inhibin coreceptor, betaglycan, which increases the affinity of inhibin for the activin type II receptors (13). Recent studies have shown that modification of the inhibin functions may have a role in the development and progression of granulosa and ovarian epithelial cancers. The α-inhibin gene is described as a tumor suppressor gene in the mouse. Genetically modified female mice deficient in inhibin have unregulated gonadotropin levels leading to abnormal follicle growth, which is associated with sex cord-stromal tumor formation with 100% penetrance (14). In humans, however, the role of the α-inhibin gene is controversial because there is no evidence to support a role in tumor suppression.

In the ovary, activin and inhibin subunits are expressed mainly in granulosa cells (15) but are also detected in normal human OSE (hOSE; refs. 12, 16). Studies on diverse types of OET suggest that the inhibin α subunit expression is predominantly associated with nonmalignant tumors mainly of the mucinous type (17, 18). None of the malignant tumors in these studies expressed the inhibin α subunit. One study showed focal positive staining for the inhibin α peptide in the stromal components of serous tumors but not in the epithelial components (16). In contrast, the β subunits were detected in the majority of mucinous and serous tumors (nonmalignant and malignant; refs. 16, 17). Collectively, these results suggest that normal OSE and nonmalignant tumor cells produce inhibin and activin, but malignant OETs have decreased inhibin production relative to activin production. Accordingly, it was reported that activin was secreted by cultured primary OET (18) and human ovarian carcinoma (19). Inhibin levels declined after surgery, suggesting that tumors were the sites of production.

Other studies showed that women with OET with elevated serum inhibin A levels before surgery had a longer survival time relative to women without elevated levels (20). Similarly, patients with granulosa cell tumors expressing the inhibin α subunit had a better prognosis than those with inhibin immunonegative tumors (21). This contradicts the study of Frias et al. (22), which showed that high inhibin serum levels predict poor outcome for women with epithelial ovarian carcinoma. This discrepancy may be explained by the specificity of the methods for detecting inhibin. The inhibin α subunit is found within the circulation as an inhibin dimer or as an undimerized inhibin α subunit. In the first studies, the free α subunit was detected in addition to the inhibin dimer, whereas in the latter study only the inhibin dimer was measured. This suggests that the free α subunit, not the inhibin dimer, is associated with a better outcome for women with OET.

In an attempt to investigate the inhibin responsiveness of ovarian cancer cells, Fuller et al. (8) reported that betaglycan was detected in most primary granulosa cell tumors as well as in mucinous and serous cystadenocarcinomas, suggesting that these tissues maintain the potential to respond to inhibin. The relevance of these findings is not clear, because the role of inhibin in normal ovarian epithelial cells is not reported. The tumor suppressive effect of inhibin is not clearly shown in human ovarian cancers either.

We hypothesize that activin and inhibin are important regulators of biological activity in ovarian carcinomas. To test this hypothesis, we have examined the effects of activin and inhibin on the proliferative and invasive abilities of OSE and ovarian cancer cell lines. In addition, we have verified whether improper regulation of components of the activin signaling pathway is associated with the tumorigenicity of the SKOV3 and OCC1 cells.

### Results

**Effects of Activin A on Human Ovarian Cancer Cell Lines and Normal OSE Proliferation**

To assess the effects of activin A on OSE proliferation, various cell lines have been examined. Because primary cultures of hOSE were not easily obtained and proliferated slowly, results with the hOSE cells were limited. To compensate for this, an immortalized hOSE cell line, IOSE397, was used along with a primary culture of murine OSE (mOSE). Furthermore, a battery of ovarian cancer cell lines was examined for proliferation in response to activin A. These cells included SKOV3, OCC1, ES-2, OVCA429, OVCA37, HEY, A2780-s, and A2780-cp.

The ES-2, OVCA429, HEY, A2780-cp, hOSE, and mOSE cells did not show an increase in their rate of proliferation in response to 0.5 nmol/L activin A over the 72-hour experiment (Fig. 1A). In contrast, IOSE397 cells responded to activin A by increasing their proliferation. SKOV3, OCC1, OVCA37, and A2780-s cells all proliferated at a higher rate in response to activin A (P < 0.05). From this group, SKOV3 and OCC1 cells showed the greatest activin A–induced proliferation relative to untreated controls (1.28- and 1.61-fold for SKOV3 and OCC1 cells, respectively, versus 1.18- and 1.14-fold increases for A2780-s and OVCA37 cells, respectively).

**Antiproliferative Effects of Inhibin A on Human Ovarian Cancer Cell Lines and Normal OSE**

To evaluate the effect of inhibin A on proliferation of OSE and ovarian cancer cells, the same battery of cells used in the activin A experiments were incubated in the presence of 0.5 nmol/L inhibin A. Inhibin A had no significant effect on the proliferation of ES-2, A2780-cp, HEY, OVCA429, and OCC1 cells (Fig. 1B). In contrast, SKOV3 cells had a decreased
proliferation rate relative to basal levels when exposed to inhibin A (83% of untreated cells) as did OVCAR3 and A2780-s cells. Of note, similar effects were observed for inhibin-treated SKOV3 and OCC1 cells cultured in 0%, 0.1%, and 1% FCS-donor bovine serum (DBS; data not shown). hOSE cells seemed to have decreased proliferation when exposed to inhibin A, but this result did not reach statistical significance. Inhibin A had no effect on the proliferation rate of the IOSE397 cells. Conversely, the proliferation of mOSE cells exhibited a marked decreased (2.20-fold) in response to inhibin A.

SKOV3 and OCC1 cells were consequently selected for further study, as they showed the greatest activin A–induced proliferation. To assess whether the activin-induced proliferation of OCC1 and SKOV3 cells could be antagonized by inhibin A in the presence of increasing amounts of inhibin A (0, 0.1, 0.5, 1, 5, and 10 nmol/L) for 48 hours (Fig. 2). Activin-induced proliferation of OCC1 and SKOV3 cells over basal levels (1.27- and 1.37-fold, respectively). The SKOV3 cells were also highly responsive to the antagonistic effect of inhibin A. Addition of inhibin A, even at the lowest dose, resulted in complete abrogation of the activin-induced proliferation (P < 0.05). In contrast, the activin-induced OCC1 cell proliferation was not antagonized by inhibin A even with doses 20 times higher than the activin A concentration.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was done to determine the apoptotic effect of inhibin A and activin A on IOSE397, SKOV3, and OCC1 cells (data not shown). Each of these cell lines was tested under four conditions: without treatment, with
0.5 nmol/L activin A, with 0.5 nmol/L inhibin A, or with both activin A and inhibin A treatment. Whereas IOSE397 cells responded to 0.5 nmol/L activin A by undergoing apoptosis (45% of cells stained for TUNEL), inhibin A treatment completely reversed this trend back to basal levels (6% of cells stained for TUNEL). In contrast, neither inhibin A nor activin A induced apoptosis in the SKOV3 and OCC1 cells.

**Invasive Effects of Activin and Anti-invasive Effects of Inhibin on Human Ovarian Cancer Cell Lines**

The migration of SKOV3 and OCC1 cells through Matrigel over 48 hours was used to determine if activin A would induce or enhance invasion. The invasion of the OCC1 cells was increased in the presence of activin A (P < 0.05), but inhibin failed to block that augmentation (Fig. 3A). These assays also showed that activin A increased the invasion of SKOV3 cells (2.30-fold), whereas inhibin A reversed the effects of activin A (P < 0.05) in SKOV3 cells (Fig. 3B). Furthermore, inhibin A, when given alone, decreased the basal rate of invasion of SKOV3 cells (3.64-fold).

**Mouse Xenograft Models of Ovarian Cancer**

Xenograft models of CD-1 nude mice injected i.p. with ovarian cancer cells were analyzed to determine the

![Image](image-url)

**FIGURE 3.** Effects of activin A and inhibin A on the rates of migration of ovarian cancer cell lines through Matrigel. (A) OCC1 cells and (B) SKOV3 cells were plated in Matrigel-coated 24-well inserts in medium with 10% serum. Cells received no treatment, 0.5 nmol/L activin A, 0.5 nmol/L inhibin A, or both. Invading cells were counted after 48 hours. Columns, mean from three experiments in triplicate; bars, SE. Two-way ANOVA with Bonferroni t tests were done to determine significant differences indicated by different letters (P < 0.05).

---

### Table 1. Median Survival of Xenograft Mouse Models with Corresponding Activin and Inhibin Responsiveness of the Cell lines in vitro

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Median survival (d)</th>
<th>Activin responsive</th>
<th>Inhibin responsive</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCC1</td>
<td>15</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>ES-2</td>
<td>16</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>HEY</td>
<td>24</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>A2780-cp</td>
<td>24</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>OVCA429</td>
<td>62</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>A2780-s</td>
<td>46</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>SKOV3</td>
<td>&gt;105*</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>OVCAR3</td>
<td>&gt;150*</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Fraction of xenografts that developed tumors: 2/7 for SKOV3 and 0/6 for OVCAR3.

aggressiveness associated with the cell lines studied. The length of time mice survived postinjection defined the aggressiveness of each cell line. Table 1 shows the median survival of mice xenografted with the ovarian cancer cell lines along with the responsiveness of the same cell lines to activin A and inhibin A in vitro. Interestingly, the cell lines that were nonresponsive to inhibin A were on average more aggressive in our xenograft models than the cell lines that were inhibin-responsive (median survival 28.2 ± 8.7 versus 100.3 ± 30.1 days, respectively; P < 0.05).

### Expression Analysis of the Activin and Inhibin Subunits

To evaluate the production of activin and inhibin in the cells and any potential autocrine activity from activin and inhibin, the expression of activin and inhibin subunit mRNAs of normal hOSE, IOSE397, SKOV3, and OCC1 cells were analyzed using reverse transcription-PCR (RT-PCR; Fig. 4). All four cell types expressed both activin β subunits. Concerning the inhibin α subunit, the results showed that hOSE, IOSE397, and SKOV3 cells are capable of producing α subunit mRNA. Interestingly, OCC1 cells had undetectable levels of the α subunit mRNA at the RT-PCR level, suggesting that these cells are unable to produce inhibin.

![Image](image-url)

**FIGURE 4.** Expression analysis of inhibin α and β subunits mRNA in OSE cells and ovarian cancer cell lines. The α, β, and γ subunits mRNA from hOSE, IOSE397, SKOV3, and OCC1 cells were detected by RT-PCR. Amplified products were separated on agarose gels and visualized by ethidium bromide staining. +, samples that received reverse transcriptase (RT); –, samples that received no reverse transcriptase as negative controls. The experiment was repeated from three independent RNA samples for each cell line with the same results.
Expression Analysis of the Activin Receptors and Betaglycan

RT-PCR for the expression of the activin receptors was done to determine which cells were capable of responding to the activin and/or inhibin signal. hOSE, IOSE397, OCC1, and SKOV3 cells were shown to express mRNA transcripts for Alk2, Alk4, ActRII, and ActRIIB (Fig. 5A). To evaluate the activin receptor protein levels, Western blot analyses were done (Fig. 5B) along with densitometry (Fig. 5C). Unfortunately, hOSE data were unavailable for Western blots due to restrictions in the amount of sample available. As an alternative, IOSE397 cells were used as the model of normal OSE. Relative to IOSE397 cells, SKOV3 cells had 1.89-fold higher levels of Alk2 protein but 59% lower levels of Alk4. The levels of ActRII and ActRIIB in SKOV3 cells were similar to those observed in IOSE397 cells. In the OCC1 cells, all four types of activin receptor had higher levels relative to IOSE397 cells (1.88-, 1.67-, 3.94-, and 1.84-fold increases to Alk2, Alk4, ActRII, and ActRIIB, respectively). In fact, the OCC1 cells had even higher levels of Alk4, ActRII, and ActRIIB relative to the SKOV3 cells (2.65-, 4.36-, and 1.73-fold increases, respectively).

FIGURE 5. Expression analysis of activin receptors in OSE cells and ovarian cancer cell lines. A, ALK2, ALK4, ActRII, and ActRIIB mRNA from hOSE, IOSE397, SKOV3, and OCC1 cells were detected by RT-PCR. Amplification of ActRII was done on separate samples and is shown with the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. +, samples that received reverse transcriptase; −, samples that received no reverse transcriptase as negative controls. B, Immunodetection of activin receptors. Samples were separated by SDS-PAGE using 7.5% gels. Left, standard molecular weights. C, Relative levels of activin receptors were determined by densitometric analyses and normalized against α-tubulin levels. Columns, mean from three experiments; bars, SE. One-way ANOVA with Bonferroni t tests was done with significant differences (asterisks) relative to IOSE397 cells (P < 0.05).
Further results showed that hOSE, IOSE397, OCC1, and SKOV3 cells were capable of producing betaglycan mRNA (Fig. 6A). In agreement with the RT-PCR results, Western blots showed the presence of betaglycan protein in IOSE397, SKOV3, and OCC1 cells (Fig. 6B). The various molecular weight species of betaglycan detected by Western analysis correspond to different degrees of glycosylation of the molecule.

Expression Analysis of the Intracellular Components of the Activin Signaling Pathway

Deregulation of intracellular signaling components in the activin signaling cascade has the potential to affect the intensity of the activin signal; thus, the intracellular components of the activin signaling cascade were analyzed by RT-PCR starting with Smad2 to Smad4. SARA mRNA and protein levels were also determined.

The mRNAs encoding the Smads were detected in hOSE, IOSE397, OCC1, and SKOV3 cells (Fig. 7A). Based on the RT-PCR results, Smad3 was selected for further study based on its potentially elevated levels of expression in OCC1 and SKOV3 cells relative to hOSE cells. However, Western blots of Smad3 (Fig. 7B) yielded no appreciable differences among IOSE397, SKOV3, and OCC1 cells (Fig. 7C).

RT-PCR analysis showed that IOSE397, SKOV3, and OCC1 cells expressed SARA mRNA (Fig. 8A). SARA protein was detected in IOSE397, SKOV3, and OCC1 cells as two isoforms with molecular weight of ~200 and 220 (Fig. 8B). The specificity of these bands was confirmed by replacing the SARA antibody for normal IgG from nonimmunized goat (data not shown). Densitometric analysis showed that SKOV3 and OCC1 cells expressed 1.89- and 1.86-fold, respectively, higher levels of SARA protein relative to IOSE397 cells (Fig. 8C).

Discussion

Although the α-inhibin gene has been described as a potential tumor suppressor gene in the mouse, the effect of inhibin on human cancer cells is unknown. The results reported in this study help to elucidate the roles that inhibin A and activin A may play in ovarian tumorigenesis. Here, we report data demonstrating that inhibin and activin modulate ovarian cancer cell proliferation and invasiveness as well as data suggesting that deregulation of the responsiveness toward activin and inhibin may contribute to ovarian tumorigenicity.

Activin A Had No Effect on Proliferation of Primary OSE Cells but Increased the Proliferation of SKOV3 and OCC1 Cells

Because inhibin is a potent antagonist of activin, we first determined the activin effect on ovarian cancer cells. We also analyzed the effects of activin on normal OSE cells, because most OET cells are thought to derive from the OSE. Our results showed that activin A increased the proliferation of the ovarian cancer cell lines OVCAR3, A2780-s, SKOV3, and OCC1. We also showed that activin A did not affect the growth of mOSE and hOSE. Those results agree with previous studies demonstrating that activin stimulated the proliferation of many ovarian cancer cell lines (11) but not of normal hOSE (12).

In contrast with the lack of an activin effect on normal OSE cells, the proliferation of the immortalized OSE cell line IOSE397 is decreased in the presence of activin A. These cells responded to activin A by undergoing apoptosis as shown by TUNEL assay (data not shown). This phenomenon is noted previously with other hOSE cells immortalized with the SV40 large T antigen (9) and occurs with other cell types immortalized the same way. In fact, mOSE immortalized with the SV40 large T antigen have been shown to have decreased proliferation in response to activin² presumably due to increased apoptotic mechanisms. The SV40 large T antigen binds to members of the

Figure 6. Expression analysis of betaglycan in OSE cells and ovarian cancer cell lines. A. Beta-glycan mRNA from K562, hOSE, IOSE397, SKOV3, and OCC1 cells was detected by RT-PCR. The erythroleukemia cell line K562 was included as a positive control. +, samples that received reverse transcriptase; −, samples that received no reverse transcriptase as negative controls. B. Immunodetection of betaglycan. Samples were separated by SDS-PAGE using 7.5% gels. Left, molecular weight standards.
Rb family, such as pRb, p107, and p130, and override the Rb-mediated repression of E2F-dependent transcription, which results in S-phase progression (23). On the other hand, in HepG2 and B cells, activin increases the expression of p21/CIP1/WAF1, which results in the accumulation of hypophosphorylated Rb leading to G1 arrest and apoptosis (24). Because the activin pathway and SV40 large T antigen have opposite effects on the Rb pathway, it is possible that the induction of apoptosis in the IOSE397 cells is not due to an intrinsic response of these immortalized OSE cells to activin but to a blocking effect of activin on the action of the SV40 large T antigen. Therefore, the IOSE397 cells are not a good model to study the effect of activin and inhibin on normal OSE cell proliferation.

Inhibin A Decreased OSE Cells Growth

The growth of the mOSE cells is decreased substantially when incubated in the presence of inhibin A. Because no exogenous activin is added to the culture, we speculate that inhibin is antagonizing an endogenously expressed growth factor. However, mOSE cells are not responsive to activin stimulation, suggesting that either these cells are maximally stimulated by activin or an endogenous growth factor other than activin may be present. It has recently been shown that inhibin can be a potent antagonist of factors other than activin, such as the bone morphogenetic proteins (25), which can signal through the activin receptors. Although the expression of bone morphogenetic proteins has not been shown in mOSE cells, we can speculate that the regulation of proliferation by inhibin may occur by the antagonism of growth factors other than activin, such as bone morphogenetic proteins.

In contrast to its antiproliferative effect on mOSE, inhibin A does not decrease significantly the proliferation of the hOSE cells. Nevertheless, a trend toward decreased proliferation was observed. This discrepancy can perhaps be explained by the slow basal rate of proliferation of the hOSE cells, which had a doubling time of 72 hours. The proliferation assay was done over a period of 72 hours, so a longer time period may be required to observe any significant negative effect of inhibin on the proliferation of hOSE cells.

Activin A Stimulated SKOV3 and OCC1 Cells Invasion

Activin A stimulates the invasion rate of both SKOV3 and OCC1 cells. Although the mechanisms behind the observed activin-induced invasion are not explored in this study, it is possible that there is involvement of matrix metalloproteinases. Previously, it has been shown that activin A can stimulate both

![Image](image1.png)

**FIGURE 7.** Expression analysis of Smads in OSE cells and ovarian cancer cell lines. **A.** Smad2-Smad4 mRNA from hOSE, IOSE397, SKOV3, and OCC1 cells were detected by RT-PCR. +, samples that received reverse transcriptase; −, samples that received no reverse transcriptase as negative controls. **B.** Immunodetection of Smad3. Samples were separated by SDS-PAGE using 7.5% gels. **C.** Relative levels of Smad3 protein were determined by densitometric analyses and normalized against α-tubulin levels. Columns, mean from three experiments; bars, SE. One-way ANOVA was done with no significant differences detected (P < 0.05).
production and activity of matrix metalloproteinase-2 in mouse peritoneal macrophages (26). Furthermore, activin A specifically induces the early expression of matrix metalloproteinase-2 within villous cytotrophoblast cells of the first trimester placenta and stimulates the outgrowth of cytotrophoblast cells into the surrounding matrix (27). Matrix metalloproteinase-2 has been implicated previously in the invasion of ovarian cancer cells (28). For example, transforming growth factor-β1 stimulates the secretion of matrix metalloproteinase-2 and the invasive behavior in SKOV3 cells (28). Thus, although activin A is implicated with invasive properties, this is the first time that exogenous activin A is shown to increase invasiveness of ovarian cancer cells.

Differential Response of Ovarian Cancer Cells toward Inhibin A

This study shows for the first time that inhibin A decreases proliferation and invasion in an ovarian cancer cell line (i.e., SKOV3 cells). By RT-PCR, we showed that SKOV3 cells express both activin β subunits, which is consistent with previous studies that show that SKOV3 cells secrete activin (11). In fact, the SKOV3 cells may produce high levels of activins containing the βB subunit (activin B or activin AB), which is in agreement with previously published data that indicate elevated mRNA expression of the βB subunit (11).

The decrease of SKOV3 basal proliferation by exogenous inhibin A may then be explained by the inactivation of endogenous activin-induced growth. In contrast, the OCC1 cells are resistant to inhibin inhibition even when exposed to high doses of inhibin A.

Up-Regulation of the Activin Signaling Pathway in SKOV3 and OCC1 Cells

To understand why the ovarian cancer cells studied have different responses to activin A and inhibin A from their putative parental OSE cells, expression of the activin and inhibin signaling components was analyzed. Our results indicated an up-regulation of SARA protein in ovarian cancer cell lines (SKOV3 and OCC1) relative to IOSE397 cells. To our knowledge, this is the first time an increased amount of SARA has been shown in ovarian cancer cells relative to normal hOSE. A wealth of evidence implicates SARA as a facilitator of the transforming growth factor-β/activin signaling transduction pathway (3, 29, 30). We thus speculate that the higher levels of SARA in OCC1 and SKOV3 cells may give these cells a proliferative advantage when exposed to activin. However, some controversy exists as to the true nature of SARA in the transforming growth factor-β/activin signal transduction pathways. One study shows that overexpression of SARA in T cells treated with transforming...
growth factor-β is associated with a dose-dependent decrease in cotransfected reporter gene expression (31), whereas another shows that SARA overexpression has no effect in Mv1Lu cells (3).

The inhibin resistance of OCC1 cells is not due to the absence of betaglycan because these cells express the betaglycan protein. The presence of betaglycan in the ovarian cancer cell lines studied herein agrees with the detection of betaglycan mRNA in ovarian tumors (8). The OCC1 cells also show higher protein levels of ActRII and ActRIIB relative to the IOSE397 cells. Up-regulation of ActRIIB was reported previously in ovarian cancer cell lines that proliferate in response to activin A, such as OVCAR3 cells (10, 12). Therefore, we postulated that the inhibin resistance of the OCC1 cells may be attributed to the increased levels of the activin receptors. In this scenario, the high number of activin receptors along with an up-regulation of SARA renders OCC1 cells highly responsive to activin. Consequently, the number of betaglycan molecules may not be sufficient for inhibin to compete with activin for the binding of the overabundant activin receptors. This would efficiently nullify the inhibitory effect of inhibin on activin stimulation in OCC1 cells. Accordingly, Lebrun and Vale (32) have shown that overexpression of ActRII in K562 cells renders these cells inhibin resistant. However, we cannot rule out possibilities of inactivating mutations in betaglycan or the loss of function of unknown genes involved in a putative inhibin signaling pathway as the cause of OCC1 resistance to inhibin.

**OCC1 Cells Do Not Express the Inhibin α Subunit**

The results showed that the OCC1 cell line had undetectable levels of the α subunit mRNA. Because the OCC1 cells express the β subunits, these cells may not produce any inhibins and may only produce activins. Therefore, the OCC1 cells do not possess an inhibin autocrine regulation of the activin signaling cascade. Because more aggressive forms of OET are associated with a lack of inhibin production coupled with greater activin production, it is reasonable to suggest that the OCC1 cells represent a later and more aggressive stage of ovarian cancer than SKOV3 cells.

Accordingly, the OCC1 cells show a higher tumorigenicity index than the SKOV3 cells in our xenograft tumor mouse models. The median survival of mice injected with OCC1 cells is only 15 days compared with 105 days for the mice injected with SKOV3 cells. In fact, in this study, the ovarian cancer cell lines that were nonresponsive to inhibin A were more aggressive than the cell lines that were inhibin responsive, suggesting that development of inhibin resistance contributes to the ability of these cells to form tumors.

**Conclusion**

This is the first time that the proliferation and invasiveness of ovarian cancer cells have been shown to decrease on exposure to exogenous inhibin. We also showed that inhibin-resistant ovarian cancer cell lines are more aggressive in mouse xenograft models. Because those models used mice of reproductive age with intact ovaries, it is possible that the circulatory inhibin will explain the difference in aggressiveness between the inhibin-responsive and the inhibin-resistant ovarian cancer cell lines. It is interesting to note that the occurrence of OET is considerably higher in postmenopausal women where circulating levels of inhibin are low, probably due to a decline in follicle numbers (33), and where the activin A levels show a 2-fold increase (34). The circulating levels of inhibin in postmenopausal women may thus be too low to have a tumor suppressor effect on the inhibin-responsive OET. It is therefore possible that inhibin or inhibin-like drugs will be a viable treatment for women with inhibin-responsive ovarian cancers.

**Materials and Methods**

**Culture of Cell Lines**

The following cell lines were used in this study: OCC1, a clear cell carcinoma cell line obtained from M. Skinner (Boston, MA); SKOV3, OVCA 429, HEY, and OVCAR3, ovarian carcinoma cell lines obtained from G. Mills (Houston, TX); K562, a human erythroleukemia cell line obtained from H. Atkins (Ottawa, Ontario, Canada); IOSE397, a SV40 large T antigen immortalized hOSE cell line obtained from N. Auersperg (Vancouver, British Columbia, Canada); ES-2, a clear cell carcinoma cell line obtained from J. Bell (Ottawa, Ontario, Canada); and A2780-s and A2780-cp, obtained from M. Molepo (Ottawa, Ontario, Canada).

For maintenance, SKOV3 cells were cultured in DMEM (Life Technologies, Burlington, Ontario, Canada) with 10% of a heat-inactivated 1:3 mixture of FCS (CanSera, Rexdale, Ontario, Canada) and DBS. A2780-s and A2780-cp cells were cultured similarly to the SKOV3 cells with 1% nonessential amino acids (Life Technologies) added to the culture. OCC1, OVCAR3, and HEY cells were cultured in αMEM with 10% FCS-DBS. ES-2 and OVCAR429 cells were cultured similarly but had 1% nonessential amino acids added to the culture. K562 cells were cultured in RPMI 1640 (Life Technologies) with 10% FCS-DBS, whereas the IOSE397 cells were cultured in a 1:1 mixture of 199 and MCDB105 media (Sigma, St. Louis, MO) with 10% FCS-DBS and 1% nonessential amino acids. The tumorigenicity and histopathologic characterizations of the ovarian cancer cell lines used in this study have been done previously and confirmed (35).

**Isolation and Culture of Normal Mouse and hOSE Cells**

Primary mOSE cells were collected and cultured as described previously (36). Normal hOSE cells were obtained by firm scraping of the OSE at the time of oophorectomy for gynecologic reasons other than ovarian pathology and were cultured as described by Tonary et al. (37).

**cDNA Synthesis and RT-PCR**

Total RNA was prepared from cells harvested when they reached 80% confluence with the use of the RNAeasy Mini Kit system (Qiagen, Mississauga, Ontario, Canada) from three independent samples for each cell line. A DNase treatment and removal system, DNA free (Ambion, Austin, TX), was used on the total RNA. RNA (1.5 μg) was reverse transcribed into cDNA by use of random primers and SuperScript III reverse transcriptase (Invitrogen, Burlington, Ontario, Canada).
according to manufacturer’s instructions. Aliquots (2 μL) of the cDNA were then PCR amplified in a 50 μL volume using Taq polymerase (Invitrogen) according to manufacturer’s instructions to determine the presence or absence of a specific mRNA. For each amplification, 40 cycles were done. Sequence-specific primers for the amplification of the activin and inhibin subunits (βA, βB, and α subunits), the activin receptors (ALK2, ALK4, ActRII, and ActRIIB), the intracellular components of the cascade termed Smads (Smad2-Smad4), SARA, and the inhibin coreceptor (betaglycan) are shown in Table 2. K562 cells, an erythroleukemia cell line, were used as a positive control for betaglycan. PCR products (25 μL) were analyzed by agarose (1.5%) gel electrophoresis and visualized by ethidium bromide staining. The sizes of the visualized bands were estimated by comparison with DNA molecular weight markers (Invitrogen).

Western Blot Analyses

Protein lysates were extracted from cells harvested at the time they reached 80% confluency using standard protocols (38). Protein samples were then treated with a protease inhibitor cocktail (Complete Mini, Roche, Laval, Quebec, Canada). Protein content was quantified by spectroscopy using the cocktail (Complete Mini, Roche, Laval, Quebec, Canada). Protein samples were then treated with a protease inhibitor time they reached 80% confluency using standard protocols.

Western blotting was performed according to standard protocols. Protein lysates were extracted from cells harvested at the time they reached 80% confluency using standard protocols (38). Protein samples were then treated with a protease inhibitor cocktail (Complete Mini, Roche, Laval, Quebec, Canada). Protein content was quantified by spectroscopy using the cocktail (Complete Mini, Roche, Laval, Quebec, Canada). Protein samples were then treated with a protease inhibitor time they reached 80% confluency using standard protocols.

Table 2. Primer Specifications and Size of Amplified Fragments

<table>
<thead>
<tr>
<th>Components</th>
<th>Primer sequences</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>βA subunit</td>
<td>5'-CCCTTGCTTGCTGAGGAGATTTCC</td>
<td>329</td>
</tr>
<tr>
<td>βB subunit</td>
<td>5'-GCCCTCCCTCCAAGGTTCACTTC</td>
<td>315</td>
</tr>
<tr>
<td>α subunit</td>
<td>5'-CCGAGGCGGTTCTCGGAAATTC</td>
<td>773</td>
</tr>
<tr>
<td>Activin receptors</td>
<td>ALK2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-TGTTGCTTACATGATTGCTTCCC</td>
<td>361</td>
</tr>
<tr>
<td></td>
<td>5'-GGAAATAGAGGCCCACTCTCTAA</td>
<td>395</td>
</tr>
<tr>
<td></td>
<td>5'-TCTGCCTGGTCTGCTGACCA</td>
<td>352</td>
</tr>
<tr>
<td></td>
<td>5'-AGCAGGGGAGAGTTGGT</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>5'-AACAGGGGAGAGTTGGT</td>
<td>201</td>
</tr>
<tr>
<td>ActRIIB</td>
<td>5'-TGCAGAGGGACAGCTGTGAGATC</td>
<td></td>
</tr>
<tr>
<td>Betaglycan</td>
<td>5'-CCCTCCAATCGAAGAAGCCCAT</td>
<td>572</td>
</tr>
<tr>
<td></td>
<td>5'-CTGGAGAAGAGGATTGAGGATGTA</td>
<td></td>
</tr>
<tr>
<td>Intracellular components</td>
<td>Smad2</td>
<td>389</td>
</tr>
<tr>
<td></td>
<td>5'-AAGAGGCCTTGGTCCTACTGCTGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GAGACGACATCCAAAGGACAATTGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-CCAGCCATGCTGCTACTC</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>5'-AGCGAGGCTGCTGCTCCTCAAGATC</td>
<td>473</td>
</tr>
<tr>
<td></td>
<td>5'-CTGGACTTGGTCAGAGCAGATC</td>
<td>366</td>
</tr>
<tr>
<td></td>
<td>5'-TCTCTCTCTTCTGCTGCTGAGG</td>
<td></td>
</tr>
<tr>
<td>Internal control</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>720</td>
</tr>
<tr>
<td></td>
<td>5'-AGCCACATGCTGCTGACACACTATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-AAGGAGGAGCATGCTGAGCTG</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: For each component, the sense primer is shown on the first line followed by the antisense primer on the second line.
were subsequently treated with 10% serum with or without 0.5 nmol/L activin A (R&D Systems) and/or 0.5 nmol/L inhibin A (DSL, Webster, TX). The media and treatment were renewed daily. Cells were counted daily in triplicate for three independent experiments from days 0 to 3 using a Coulter Z1 particle counter (Coulter, Miami, FL). The optimal activin A concentration used in this study was determined from different preliminary dose-response studies. Briefly, a concentration range of up to 10 nmol/L activin A was tested in previous studies using the Lj/T2 cells transfected with two different activin-responsive reporter constructs (GRAS-PRL-lux and oFSHf; ref. 39) or Chinese hamster ovary cells transfected with the activin-responsive construct p3TP-lux (data not shown) as well as in preliminary dose-response proliferation studies on SKOV3 and OCC1 cells (data not shown). Those studies indicated that 0.5 nmol/L activin A was the lowest concentration capable of inducing the maximal activity. We determined previously that inhibin A antagonized 0.5 nmol/L activin A in the Lj/T2 cell system (39) and in the preliminary dose-response proliferation study on SKOV3 cells (data not shown) with an IC_{50} of ~0.15 nmol/L. Therefore, 0.5 nmol/L inhibin A was used in the current study and considered to be sufficient to show an activity on inhibin-responsive cell lines.

TUNEL Staining and Apoptosis in Response to Activin and/or Inhibin Treatments

IOSE397, SKOV3, and OCC1 cells were first washed thrice with PBS. Cells were then fixed for 1 hour with freshly prepared 4% paraformaldehyde in PBS (pH 7.4). Samples were again washed with PBS in triplicate, and 0.1% Triton X-100 in 0.1% sodium citrate was applied to the cells for 2 minutes on ice to allow permeabilization. Labeling with TUNEL was conducted as per the In situ Cell Death Detection Kit, Fluorescein (Roche). After washing thrice with PBS, the area around the samples was dried. Samples were treated with 50 μL TUNEL reaction mixture and coverslipped. The samples were then incubated in a humidified atmosphere for 1 hour at 37°C in the dark. After rinsing thrice with PBS, samples were directly analyzed by fluorescence microscopy. This process was repeated for three experiments in triplicate.

Invasion Assays in Response to Activin and/or Inhibin Treatments

Falcon cell culture inserts (VWR, Mississauga, Ontario, Canada) were first placed into the wells of a 24-well plate. Matrigel (Becton Dickinson, Oakville, Ontario, Canada) diluted five times with serum-free medium was added to the interior of the insert and allowed to solidify overnight at 37°C. DMEM + 10% FCS-DBS (100 μL) was added to the insert interior and allowed to equilibrate for 1 hour. SKOV3 or OCC1 cells were seeded at 5 x 10^5 cells/mL (100 μL) inside the insert. An additional 700 μL DMEM plus 10% FCS-DBS were added to the lower chamber of the well. Following a 48-hour incubation, noninvading cells on the surface of the Matrigel-coated membrane were removed by scraping with a cotton swab. Cells that migrated through the Matrigel and the pores of the underlying membrane were fixed for 30 minutes in 10% buffered formalin and stained with 1% toluidine blue in 10% buffered formalin for 3 hours. Following several washes with PBS, the stained cells were manually counted in triplicate for three independent experiments.

Xenograft Mouse Models of Ovarian Cancer

For each ovarian cancer cell line, 1 x 10^7 cells were resuspended in 500 μL PBS and injected i.p. (day 0) into 6- to 8-week-old female CD-1 nude mice (Charles River Laboratories, Wilmington, MA) and animals were monitored for tumor formation until humane end points were reached. After 150 days, the experiment was terminated. Cell line aggression was inferred based on the median survival of the mice. Whereas seven and six mice were injected for the study of SKOV3 and OVCAR3 cells, respectively, three mice were injected for the study of the remaining ovarian cancer cell lines.

Statistical Analyses

For the proliferation and the invasion assays, all cell counts were expressed as the mean ± SE values of at least three independent experiments done in triplicate. The probability of significant differences on comparison of only two groups was determined by Student’s t test (two-tailed) with significance at P < 0.05. When multiple treatment groups were analyzed, statistical comparisons were made by one-way or two-way ANOVA as specified. A Bonferroni t tests was used to determine significance (P < 0.05) between specific treatment groups when whole group differences were detected by ANOVA.

Acknowledgments

We thank Drs. M. Skinner, G. Mills, H. Atkins, N. Auersperg, J. Bell, and M. Molpe for sharing their cell lines; K. Clarke for collection of the mOSE cells; and E.A. MacDonald, in collaboration with the Division of Gynecologic Oncology at the Ottawa Hospital, for collection of hOSE cells.

References


Inhibin Resistance Is Associated with Aggressive Tumorigenicity of Ovarian Cancer Cells

1 Gynecological Cancer Research award from the Mitchell Family Fund, National Ovarian Cancer Association and Cancer Care Ontario (J-F. Ethier), National Cancer Institute of Canada (B.C. Vanderhyden), and Betty Irene West Canadian Institute of Health Research doctoral research award (T.J. Shaw).

Michael D. Steller, Tanya J. Shaw, Barbara C. Vanderhyden, et al.