Insulin Receptor Substrate-1 Is an Important Mediator of Ovarian Cancer Cell Growth Suppression by All-trans Retinoic Acid

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Abstract

There is a need to identify more effective drugs for the treatment of ovarian cancer as it is the leading cause of death among gynecologic tumors. All-trans retinoic acid (ATRA), a natural retinoid, arrests the growth of CA-OV3 ovarian carcinoma cells in G0-G1. Because the insulin-like growth factor-I receptor has been implicated in the proliferation of various tumors, we investigated its potential role in the suppression of ovarian cancer cell growth by ATRA. Our studies revealed that insulin receptor substrate-1 (IRS-1) protein levels decrease in CA-OV3 cells on ATRA treatment, whereas no differences in IRS-1 levels were seen in the ATRA-resistant SK-OV3 cells. Moreover, CA-OV3 clones overexpressing IRS-1 were growth inhibited less by ATRA, whereas SK-OV3 clones in which levels of IRS-1 were reduced by expression of antisense IRS-1 became sensitive to growth inhibition by ATRA treatment. Studies to determine the mechanism by which ATRA reduced IRS-1 expression showed that ATRA altered steady-state levels of IRS-1 mRNA and the stability of IRS-1 protein. Finally, the role of IRS-1 as a potential molecular target of ATRA in ovarian tumors was assessed by immunohistochemistry in an ovarian cancer tissue array. Compared with normal ovary, the majority of malignant epithelial ovarian tumors overexpressed IRS-1. Thus, there seems to be a correlation between IRS-1 expression and malignancy in ovarian tumors. Our results suggest that IRS-1 is in fact an important growth-regulatory molecule that can be a potential effective target for chemotherapeutic intervention with growth-suppressive agents, including retinoids. [Cancer Res 2007;67(19):9266–75]

Introduction

Ovarian cancer is the most lethal tumor of the female genital tract accounting for ~55% of gynecologic cancer deaths in the United States (1). It is estimated that 200,000 cases of ovarian cancer will be diagnosed worldwide per year with ~115,000 deaths (2). The high mortality rate is attributed to the fact that ovarian cancer is generally detected at an advanced stage when the disease is widespread. This is because early-stage ovarian cancer is often asymptomatic. Therefore, despite aggressive debulking surgery followed by chemotherapy, the overall prognosis for these patients remains poor (3). Taking this into account, it is clear that an effective alternative therapy for ovarian cancer is needed. There are several ongoing clinical trials sponsored by the National Cancer Institute that use retinoids in cancer therapy.5

Retinoids, a group of natural and synthetic derivatives of vitamin A, have been shown to play an important role in regulating many essential body functions, including cellular growth and differentiation. In addition, various retinoids are effective chemotherapeutic and chemopreventive agents for several human cancers. Currently, retinoids are used for the treatment of acute promyelocytic leukemia and head and neck cancers (4, 5). Reports from several laboratories including ours have shown that retinoids inhibit the growth and cell cycle progression of ovarian carcinoma cell lines (4). Our studies have focused on the effect of all-trans retinoic acid (ATRA) treatment on two ovarian carcinoma cell lines, CA-OV3 and SK-OV3. Our results show that SK-OV3 cells are resistant to the growth-inhibitory effects of ATRA, whereas CA-OV3 cells are sensitive. ATRA has been shown to block CA-OV3 cell cycle progression in the G1 phase (6–8). Additional studies have shown that ATRA treatment leads to an increase in protein levels of Rb2/p130, a member of the retinoblastoma family of proteins that controls G1 checkpoint progression. We have also reported that ATRA suppresses the growth-stimulatory activity of various purified growth factors, including insulin-like growth factor (IGF)-1 (4, 9).

In the past few years, the type I IGF receptor (IGF-IR), its ligands, and components of the IGF-I signal transduction pathway have emerged as distinct players in cancer biology. IGF-IR plays an important role in mitogenesis, transformation, and protection from apoptosis, leading to sustained cell proliferation of cancer cells (10–12). IGF-IR has been implicated in the development of several cancers, including ovarian cancer (13, 14). Increased levels of IGF-I and IGF-IR have been found in various ovarian cancer cell lines and in primary ovarian cancer tissues (14, 15).

The IGF-IR is a tyrosine kinase receptor that is activated following ligand binding. The autophosphorylated receptor binds to and phosphorylates tyrosine residues of several adaptor molecules, such as the insulin receptor substrates (IRS-1 to IRS-6) and Src homology and collagen (Shc). IRS-1 is the prototype member of the IRS family and, along with IRS-2, is widely expressed in various tissues, including the ovary and mammary gland. IRS-1 has >20 tyrosine phosphorylation sites in its COOH terminus and phosphorylated IRS-1 and Shc serve as docking molecules for several Src homology 2 domain-containing proteins. IRS-1 binds to the p85 regulatory subunit and activates phosphatidylinositol 3-kinase, which in turn activates protein kinase B (Akt), a pathway known to prevent apoptosis and mediate cellular proliferation. Tyrosine-phosphorylated Shc binds Grb2/Sos-activating Ras and

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5 http://www.clinicaltrials.gov/
the mitogen-activated protein kinase (MAPK) pathway, which is also active in mitogenesis. Although these two well-established pathways have different downstream effectors, they are not independent of each other. For example, IRS-1 can also bind Grb2 and activate the MAPK pathway (16, 17).

There is increasing evidence in breast cancer models that IRS-1 is the main adaptor molecule activated by IGF-IR and plays a role in cell proliferation. IRS-2 on the other hand is thought to regulate cell motility and plays a role in metastasis (18, 19). Several studies have shown that retinoids inhibit breast cancer cell growth through interference in the IGF-IR signaling pathway. Inhibition of IGF-1-stimulated growth, increased production of IGF-binding protein 3 and, recently, down-regulation of Akt pathway due to degradation of IRS-1 (20, 21) are some of the mechanisms by which retinoids inhibit growth of breast cancer cells.

We were therefore interested in investigating if retinoids could suppress the growth of ovarian tumor cells by altering this important growth-regulatory pathway. Our results show that treatment with ATRA decreases IRS-1 protein in CA-OV3 cells but not in SK-OV3 cells. We show that ATRA decreased the mRNA and protein stability of IRS-1 and increased its ubiquitination and proteasomal degradation in CA-OV3 cells. Furthermore, modulation of IRS-1 levels in the two cell lines altered their response to ATRA treatment. We also show by immunohistochemistry the up-regulation of IRS-1 in ovarian malignancies of epithelial origin compared with normal ovary in a tissue microarray of ovarian cancer. Our results suggest that IRS-1 is in fact an important growth-regulatory molecule in ovarian cancer cells and can serve as a potential target for chemotherapeutic intervention with retinoids.

Materials and Methods

Cell culture. The human ovarian adenocarcinoma cell lines CA-OV3 and SK-OV3 were obtained from the American Type Culture Collection. Cells were cultured as described previously (6–8).

Antibodies and reagents. The following primary antibodies were used for Western blot analysis: anti-IRS-1, anti-IRS-2, and anti-phosphorylated Ser1161/1162 IRS-1 (where the immunizing sequence corresponds to the region containing mouse Ser1161/human Ser1162 of IRS-1) antibodies obtained from Upstate Technology; anti-phosphorylated Tyr781 and anti-phosphorylated Ser636/639 IRS-1 antibodies obtained from Cell Signaling Technology; anti-Shc antibody obtained from BD Biosciences; and anti-ubiquitin, anti-actin, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies obtained from Santa Cruz Biotechnology. The secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-rabbit antibody obtained from Amersham Biosciences and HRP anti-goat antibody obtained from Pierce Biotechnology. The anti-IRS-1 antibody from Upstate Biotechnology was used for the immunoprecipitation and anti-IRS-1 antibody from Santa Cruz Biotechnology was used for Western blotting after immunoprecipitation. G418 from Alexis Biochemicals was dissolved in water and used at a concentration of 800 μg/mL. The proteasomal inhibitor clasto-lactacystin β-lactone (CLBL) was purchased from Calbiochem, dissolved in DMSO, and used at a concentration of 10−6 mol/L. Cycloheximide was purchased from Sigma Life Sciences, dissolved in ethanol, and used at a concentration of 10 μg/mL. ATRA was a generous gift from Hoffman La Roche and used at a concentration of 10−8 mol/L in ethanol to treat cells under subdued light on days 0, 3, and 6.

Western blotting and immunoprecipitation. After treatment with ethanol or ATRA for 24, 48, and 72 h, whole-cell extracts were prepared and processed for Western blotting and immunoprecipitation as described (22).

Reverse transcription-PCR analysis. mRNA was obtained from ATRA-treated and control CA-OV3 cells at 12, 18, 24, 48, and 72 h using the RNAzol B protocol (Tel-Test). Reverse transcriptions were done using the RT-for-PCR kit from Clontech. PCR was carried out as per the manufacturer’s protocol with the exception of the use of [32P]dATP (1,000 Ci/mmol; Perkin-Elmer Life Sciences) as described (6–8). Primers were as follows: IRS-1, 5′-GTCTTACCCGCAACACAGAGT-3′ (forward) and 5′-CCCTTGACAAATCTCGTCTATG-3′ (reverse); IRS-2, 5′-ACCTGGGATGTATCTATGC-3′ (forward) and 5′-AAAGGGTTTGGATGGAAG-3′ (reverse); and IGF-IR, 5′-ACTCTGCCCCGCAATCTCA-3′ (forward) and 5′-CCCTTCTCTGCCGCACTACTA-3′ (reverse). GAPDH primers supplied with the cDNA synthesis kit were used as loading control. The steady-state mRNA levels of IRS-1, IRS-2, and IGF-IR were analyzed by phosphorimager and quantitated using OptiQuant software. GAPDH mRNA levels were used for normalization. The amount of mRNA at time 0 is set to 100%. The levels of mRNA following ATRA treatment for the various time points are expressed as % of control.

Stable transfection. Stable transfection of cells was done using the calcium phosphate method. CA-OV3 cells were transfected with an IRS-1 full-length mouse cDNA and cloned in the sense orientation in pRC vector. SK-OV3 cells were transfected with an IRS-1 mouse cDNA cloned in the antisense orientation in pRC vector. The plasmid constructs were a kind gift from Dr. Eva Surnasz (Temple University, Philadelphia, PA). Cells were also transfected with empty pCDNA3 vector to use as a control. All the plasmids encode for G418 resistance and contain a constitutive cytomegalovirus promoter. Medium containing fresh drug was added every 3 days. Isolated colonies of resistant cells were selected −2 weeks after transfection.

Growth on monolayer. Growth of cells on monolayer was determined by direct cell counting under the microscope using 0.4% trypan blue (Life Technologies) at a ratio of 1:1.

Growth in soft agar. Anchorage-independent growth was determined by using a growth in soft agar assay as described (6–8). After 15 days, each plate was examined microscopically and ≥100 colonies were screened to determine the number of colonies >50 μm in size.

Protein stability assay. Protein stability was determined at 0, 4, 8, 16, 24, 32, and 40 h after addition of cycloheximide as described previously (22). Data are represented as % IRS-1 protein remaining in ethanol- and ATRA-treated plates with the 0-h cycloheximide time point set to 100%. The half-life (t1/2) of IRS-1 protein was calculated using the regression program of Microsoft Excel 2003.

Immunohistochemistry of ovarian cancer tissue arrays. An ovarian cancer tissue array slide (Ish Abliss AccuMax array) and two breast cancer tissue array slides used as positive and negative controls were used for immunohistochemistry using the avidin-biotin-peroxidase methodology according to the manufacturer’s instructions (Vektor Laboratories). In the negative control, the primary antibody was omitted.

Statistical analysis. Statistical analysis was done using the InStat software package from GraphPad. Unpaired Student’s t test was used to compare the growth of transfected clones to the wild-type cells. Paired Student’s t test was used to compare the mRNA levels of IRS-1 following ATRA treatment for the various time points. Differences having a P value of <0.05, <0.01, <0.001, or <0.0001 are reported as statistically significant and are designated with the symbols *, **, *** and ****, respectively.

Results

ATRA treatment decreases IRS-1 levels in CA-OV3 ovarian carcinoma cells. We first examined the protein levels of IRS-1, IRS-2, and Shc, the key adaptor molecules in the IGF-1 signaling pathway following treatment with either ATRA or ethanol, which was used as a control. As shown in Fig. 1A, treatment of CA-OV3 cells with 10−6 mol/L ATRA for various lengths of time resulted in a large decrease in IRS-1 protein levels by 72 h. However, no differences in protein levels of IRS-2 or Shc were observed on ATRA treatment. On the other hand, as seen in Fig. 1B, treatment of SK-OV3 cells with ATRA did not result in a change in protein levels of IRS-1, IRS-2, or Shc.

Modulation of IRS-1 expression affects the sensitivity of ovarian carcinoma cells to ATRA treatment. Because we
observed a decrease in IRS-1 levels in CA-OV3 cells following ATRA treatment, our next goal was to determine if modulating the levels of IRS-1 would alter their sensitivity to ATRA. We hypothesized that if down-regulation of IRS-1 was an essential factor in mediating suppression of ovarian tumor cell growth by ATRA, overexpression of IRS-1 in CA-OV3 cells would cause them to become more resistant to growth inhibition by ATRA treatment. To test this hypothesis, stably transfected CA-OV3 cells overexpressing full-length mouse IRS-1 cDNA cloned in the sense orientation were selected with G418. We chose several positive clones with different levels of IRS-1 protein expression and examined their response to ATRA treatment. Clone 18 had the highest amount of IRS-1 expression (Fig. 2A, top).

The graph in Fig. 2A compares the effect of ATRA treatment on growth of sense IRS-1–overexpressing CA-OV3 (CAsIRS1) clones 18, 20, 24, and 29 with the wild-type CA-OV3 cells and an empty vector clone. The results show that, although the wild-type and the empty vector control CA-OV3 cells exhibited ~35% inhibition in growth following 5 days of ATRA treatment, the CAsIRS1 clones were significantly resistant to ATRA being inhibited only approximately 10% to 20%. After 7 days of ATRA treatment, CAsIRS1 clones 18, 20, and 24 continued to show significantly less (~20–35%) growth inhibition compared with wild-type and empty vector control CA-OV3 cells (~55–60%), whereas CAsIRS1 clone 29 exhibited ~60% growth inhibition, which is comparable with the wild-type and empty vector control CA-OV3 cells. These results clearly indicate that overexpression of IRS-1 induces the CA-OV3 cells to become less sensitive to growth inhibition by ATRA following 5 days of treatment. However, the resistance to growth inhibition is not retained in some clones, such as clone 29, on longer treatment. A likely explanation is that treatment for longer periods leads to a decrease in both exogenous and endogenous IRS-1 in some CAsIRS1 clones. It can be seen in Fig. 2B that this is the case for clone 29.

We also used growth in soft agar as a second assay to determine the effect of overexpression of IRS-1 on the growth of CAsIRS1 clones compared with the controls following ATRA treatment. As shown previously in our laboratory, we observed a marked decrease in the colony formation and the colony size of CA-OV3 cells following treatment with ATRA for 15 days (7). This decrease in growth was dose dependent, with maximum inhibition at 10⁻⁶ mol/L ATRA treatment. On the other hand, CAsIRS1 clone 18 had larger and a higher number of colonies with ATRA treatment. Whereas the colony formation at 10⁻⁶ mol/L ATRA treatment was only moderately higher compared with the wild-type CA-OV3 cells, at the lower concentrations of 10⁻⁷ and 10⁻⁸ mol/L ATRA, the number of larger colonies was higher compared with the CA-OV3 cells (Fig. 2C). Figure 2D shows that the results of the growth in soft agar assay for various CAsIRS1 clones and the controls were comparable with our observation from the growth in monolayer experiments. The soft agar assay clearly showed that the IRS-1–overexpressing clones were more resistant to ATRA-mediated growth inhibition. An interesting additional observation was that, although there were differences between the clones in the number of large colonies formed at 10⁻⁶ mol/L ATRA treatment, all of the IRS-1–overexpressing clones formed colonies comparable in size at 10⁻⁷ and 10⁻⁸ mol/L ATRA concentrations. This showed that the CAsIRS1 clones were all resistant to the growth-inhibitory effect of ATRA at the lower concentrations (10⁻⁷ and 10⁻⁸ mol/L).

Because maintenance of IRS-1 levels in CA-OV3 cells resulted in induction of resistance to ATRA growth suppression, we next hypothesized that reduction of IRS-1 levels in ATRA-resistant SK-OV3 cells might induce sensitivity to ATRA growth inhibition. Thus, we next tested the effect of IRS-1 knockdown on the growth of SK-OV3 cells following ATRA treatment. Stably transfected SK-OV3 cells expressing full-length antisense mouse IRS-1 (SKasIRS1) were selected with G418 and single-cell clones were screened for the knockdown of IRS-1 expression. SKasIRS1 clones 14, 18, and 19 expressed reduced levels of IRS-1 protein (Fig. 2E, top). The graph in Fig. 2E shows that, whereas the wild-type and empty vector control SK-OV3 cells were completely resistant to ATRA treatment at days 5 and 7, the antisense clones exhibited modest but significant (~15–20%) growth inhibition following ATRA treatment for 7 days. These results clearly show that down-regulation of IRS-1 is important for the ATRA-mediated growth inhibition of ovarian carcinoma cells.

**Decrease in IRS-1 protein following ATRA treatment is due to reduction in IRS-1 mRNA levels and decreased protein stability.** Next, we wished to investigate the mechanism by which ATRA treatment results in a decrease in IRS-1 protein levels. First, we did [³²P]dATP-labeled reverse transcription-PCR (RT-PCR) to

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**Figure 1.** IRS-1 protein levels are decreased following ATRA treatment of CA-OV3 cells. A, CA-OV3 cells were untreated (UT) or treated with either ethanol (E) or 10⁻⁶ mol/L ATRA (RA) and Western blot analysis was done as described in Materials and Methods. Actin or GAPDH was used as controls for protein loading. B, Western blot analysis of SK-OV3 cell lysates. All data are representative of three independent experiments.
Figure 2. Modulation of IRS-1 levels alters ATRA-mediated growth inhibition of ovarian carcinoma cells. A, wild-type CA-OV3 cells, empty vector clone, and IRS-1–overexpressing clones 18, 20, 24, and 29 (protein levels shown in top) were treated with either 10⁻⁶ mol/L ATRA or ethanol and direct cell counts were done using trypan blue. Data are representative of three or more independent experiments. B, Western blot analysis of CA-OV3 cells, empty vector clone, and CAsIRS1 clones for IRS-1 following treatment with ATRA or ethanol. GAPDH was used as a loading control. C, 10⁴ CA-OV3 cells, empty vector clone, and CAsIRS1 clones 18, 20, 24, 25, and 13 were treated with either ethanol or varying concentrations of ATRA (10⁻⁶, 10⁻⁷, and 10⁻⁸ mol/L) and soft agar assay was done as described in Materials and Methods. Photomicrographs show growth in soft agar of colonies of CA-OV3 cells and CAsIRS1 clone 18 treated with ethanol and different concentrations of ATRA as visualized under the microscope using ×10 magnification after 15 d. Bar, 100 μm. D, quantitation data for growth in soft agar expressed as the % of control (ethanol) of colonies >50 μm counted under the microscope for CA-OV3, empty vector clone, and CAsIRS1 clones 18, 20, 24, 25, and 13. All data are representative of three independent experiments. E, wild-type SK-OV3 cells, empty vector clone, and antisense IRS-1 clones 14, 18, and 19 (protein levels shown in top) were treated with either 10⁻⁶ mol/L ATRA or ethanol for up to 7 d and direct cell counts were done using trypan blue. For bar graphs, data are representative of three independent experiments.
determine if the decrease in IRS-1 protein was due to a decrease in the amount of IRS-1 mRNA. It is clear from gels in Fig. 3A that there indeed is a time-dependent decrease in the mRNA levels of IRS-1 with ATRA treatment of CA-OV3 cells. Interestingly, mRNA levels of IRS-2 were also decreased following ATRA treatment. In contrast, we observed no significant change in IGF-IR mRNA levels with ATRA treatment. It is clear from the graph showing the quantification of the gels that IRS-1 mRNA levels decreased significantly by 48 h following ATRA treatment, whereas IRS-2 mRNA levels decreased rapidly and remain at the reduced level throughout the time following ATRA treatment. IGF-IR mRNA levels remain constant throughout the 72 h of treatment with ATRA.

Next, we wished to determine if posttranscriptional/posttranslational mechanisms might also be responsible for the reduction in IRS-1 levels following ATRA treatment. Previous work from our laboratory has shown that ATRA acts via posttranslational mechanisms to alter the levels of cell cycle regulatory proteins, such as Rb2/p130 and p27 (22, 23). Figure 3B shows that IRS-1 protein persists longer in ethanol-treated CA-OV3 cells compared with ATRA-treated cells. The graph shows that the $t_{1/2}$ of IRS-1 decreases from $>40$ h to $\sim 24$ h in ATRA-treated CA-OV3 cells. This suggests that the decrease in IRS-1 protein following ATRA treatment involves posttranslational mechanisms in addition to transcriptional regulation and may result in a modification in its degradation pathway. In contrast to the wild-type CA-OV3 cells, IRS-1 protein in the CasIRS1 clone 18 (Fig. 3C) remains the same in both ethanol- and ATRA-treated samples.

**ATRA increases the ubiquitination of IRS-1 in CA-OV3 cells.** To ascertain the posttranslational mechanism responsible for the degradation of IRS-1 in CA-OV3 cells following ATRA treatment, we examined the ubiquitin proteasome system. Figure 4A shows that the decrease in IRS-1 protein observed following ATRA treatment for 72 h is abrogated by treatment of cells with the proteasome inhibitor CLBL. CLBL, a nonreversible inhibitor of the proteasome, is the active metabolite of the proteasome inhibitor lactacystin (22). We next wanted to determine the extent of ubiquitination of IRS-1 following ATRA treatment of CA-OV3 cells. Immunoprecipitation with IRS-1 and subsequent Western blot for ubiquitin were
Figure 4. ATRA affects ubiquitination and phosphorylation of IRS-1 protein in CA-OV3 cells. A, Western blot analysis of IRS-1 protein levels in CA-OV3 cells treated with ethanol or ATRA for 2 d followed by treatment with or without 10⁻⁶ mol/L of proteasome inhibitor CLBL for 1 d. B, ubiquitination of IRS-1 increases following ATRA treatment. CA-OV3 cells were treated with 10⁻⁶ mol/L ATRA or ethanol for 2 d followed by a 24-h treatment with CLBL. Cells were harvested and immunoprecipitation (IP) was done for IRS-1. Western blot (IB) was then done with an anti-ubiquitin (Ub) antibody. IRS-1 Western blot was done to verify the amount of immunoprecipitated IRS-1 protein. C, CA-OV3 cells were treated with either ethanol or 10⁻⁶ mol/L ATRA for 0, 24, 48, and 72 h. Western blots were done on whole-cell lysates using an antibody specific for phosphorylation at Ser³¹² (immunizing sequence corresponds to Ser³⁰⁷ in mice and Ser³¹² in humans) and Ser⁶³⁶/⁶³⁹ of IRS-1. Total IRS-1 levels were also determined and GAPDH was used as the loading control. D, CA-OV3 cells were treated with ethanol or 10⁻⁶ mol/L ATRA for 72 h. Cells were harvested and immunoprecipitation was done with an antibody against IRS-1 (Upstate Biotechnology). Western blot was then done using an anti-phosphotyrosine (pY) antibody. The total IRS-1 levels were verified by Western blotting with anti-IRS-1 antibody (Santa Cruz Biotechnology) after stripping the membrane.

Discussion

In this study, we investigated the molecular mechanisms by which ATRA blocks the IGF-IR signaling pathway, leading to growth inhibition of CA-OV3 ovarian carcinoma cells. Our results
show that (a) ATRA decreased the protein and mRNA levels of IRS-1 in the ATRA-sensitive CA-OV3 cells, whereas IRS-1 protein levels were not affected by ATRA in the resistant SK-OV3 cells; (b) overexpression of IRS-1 in CA-OV3 cells rendered them resistant to ATRA-mediated growth inhibition; (c) knockdown of IRS-1 levels in SK-OV3 cells rendered them moderately sensitive to ATRA treatment; (d) ATRA decreased the stability of IRS-1 protein in CA-OV3 cells with a corresponding increase in ubiquitination and proteasomal degradation; (e) the tyrosine phosphorylation of IRS-1 was decreased, whereas the serine phosphorylation was maintained by ATRA treatment of CA-OV3 cells; and (f) IRS-1 is highly expressed in malignant ovarian cancer tissues, especially the epithelial tumors, suggesting a correlation between IRS-1 expression and malignancy.

We observed a decrease in IRS-1 protein and mRNA levels following ATRA treatment of CA-OV3 cells. The activity of IRS-1 was also decreased as seen by its reduced tyrosine phosphorylation on treatment with ATRA. Many studies have shown that IRS-1 by itself is oncogenic and can induce malignant transformation (18). In addition, certain oncoproteins, such as SV40 T antigen, bind to IRS-1 and require IRS-1 tyrosine phosphorylation for mitogenic and transforming activity (25). IRS-1 is overexpressed in various cancers, such as hepatocellular carcinoma, pancreatic cancer, and breast cancer. IRS-1 was constitutively active and phosphorylated in a broad range of human tumors, including breast cancer, leiomyoma, Wilms’ tumor, rhabdomyosarcoma, liposarcoma, leiomyosarcoma, and adrenal cortical carcinoma. Blocking the constitutively activated IRS-1 with a dominant-negative IRS-1 dramatically reduced cancer cell growth in breast cancer and hepatocellular carcinoma (26, 27). It has also been shown that IRS-1 gene transcription can be enhanced by estrogen in breast cancer cells and in the mouse mammary gland (28). Our study shows similar results where IRS-1 expression is seen in the ethanol-treated control CA-OV3 and SK-OV3 ovarian carcinoma cells. However, the ATRA-sensitive CA-OV3 cells show reduced IRS-1 levels and activity on ATRA treatment, suggesting that it might play a role in the growth inhibition of these cells. Our results, however, vary slightly from a similar study looking at the ATRA-mediated growth inhibition of MCF-7 breast cancer cells in that they observed no changes in mRNA levels, although a reduction in total phosphorylated and tyrosine-phosphorylated protein levels was seen (19). This suggests that, in spite of the same response mediated by retinoid treatment, the mechanisms responsible for this response may vary in different cell models.

The involvement of IRS-1 in the ATRA-mediated growth inhibition of CA-OV3 cells is supported by our findings that

![Image of ovarian tumors](image-url)

Figure 5. Immunohistochemical detection of IRS-1 in ovarian tumors. Expression of IRS-1 in ovarian cancer tissue microarray with sections of normal ovary (A); serous adenocarcinoma, mucinous adenocarcinoma, and clear cell carcinoma (B); transitional cell carcinoma, endometrioid carcinoma, and Brenner tumor (C); and yolk sac tumor, granulosa cell tumor, and serous papillary cystadenoma (D).
constitutive overexpression of IRS-1 in CA-OV3 cells renders them somewhat resistant to ATRA treatment. The role of IRS-1 as a growth-regulatory molecule is further reiterated by the growth inhibition seen following ATRA treatment of SK-OV3 cells expressing antisense IRS-1. Several studies using breast cancer models have shown a role for IRS-1 in cell proliferation. It was shown that overexpression of IRS-1 in MCF-7 human breast cancer cells resulted in autocrine growth that was partially estrogen independent (29). On the other hand, down-regulation of IRS-1 by small interfering RNA (siRNA) in MCF-7 cells blocked IGF-I-mediated.

### Table 1. Staging and IRS-1 expression in ovarian cancer tissue microarray

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<th>Stage (age)</th>
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<td>Normal ovary</td>
<td>−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Endometrioid carcinoma</td>
<td>−</td>
<td>IA (34)</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Endometrioid carcinoma</td>
<td>+</td>
<td>IIIB (66)</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Endometrioid carcinoma</td>
<td>++</td>
<td>IA (43)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Endometrioid carcinoma</td>
<td>++</td>
<td>IIIC (45)</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Mucinous cystadenoma</td>
<td>−</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Brenner tumor</td>
<td>+++</td>
<td>39</td>
<td>Positive in epithelial cells</td>
</tr>
<tr>
<td>31</td>
<td>Brenner tumor</td>
<td>++++</td>
<td>IA (87)</td>
<td>Stroma negative</td>
</tr>
<tr>
<td>32</td>
<td>Brenner tumor</td>
<td>−</td>
<td>IA (50)</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Brenner tumor</td>
<td>++</td>
<td>IA (46)</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Mucinous cystadenoma</td>
<td>−</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>Yolk sac tumor/endodermal</td>
<td>+</td>
<td>IIIC (29)</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Yolk sac tumor</td>
<td>+</td>
<td>IIIC (11)</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>Yolk sac tumor</td>
<td>+</td>
<td>IA (12)</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Serous papillary cystadenoma</td>
<td>−</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>Fibroma</td>
<td>−</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Granulosa cell tumor</td>
<td>+</td>
<td>IA (43)</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>Granulosa cell tumor</td>
<td>+++</td>
<td>IA (55)</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Granulosa cell tumor</td>
<td>++</td>
<td>IC (80)</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>Serous papillary cystadenoma</td>
<td>−</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>Fibroma</td>
<td>−</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>Dysgerminoma</td>
<td>−</td>
<td>IC (32)</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Dysgerminoma</td>
<td>−</td>
<td>IIIC (11)</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>Dysgerminoma</td>
<td>−</td>
<td>IC (19)</td>
<td></td>
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<tr>
<td>48</td>
<td>Serous papillary cystadenoma</td>
<td>−</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>Corpus luteum</td>
<td>+++</td>
<td></td>
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</tr>
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</table>

NOTE: The histologic pattern, expression of IRS-1 by immunohistochemistry (weak positivity to strong positivity denoted as +, ++, and +++), and the stage and age of the patient at the time of tumor or tissue removal are shown in the table.
and estrogen-mediated cell growth and resulted in cell death (30). Recently, a study showed that lowering IRS-1 levels by siRNA not only resulted in the down-regulation of Akt and induction of apoptosis in MCF-7 cells but also enhanced taxol-induced cell death (31). Two recent studies further confirm the distinct roles of IRS-1 and IRS-2 in the pathogenesis of breast cancer. The loss of IRS-1 enhanced metastasis but not proliferation due to a compensatory increase in IRS-2 levels (32). In T47D-YA breast cancer cells that lack IRS expression, overexpression of IRS-1 resulted in increased proliferation, whereas overexpression of IRS-2 resulted in increased motility (33). Our results also show that IRS-1 plays a role in cell proliferation of ovarian cancer cells and is similar to the results obtained by del Rincon et al. (20) showing that IRS-1-overexpressing MCF-7 cells were resistant to the growth-inhibitory effects of ATRA.

We have also shown that ATRA mediates the decrease in IRS-1 levels by reducing protein stability, which is the result of an increase in the degradation of the protein by the ubiquitin proteosomal pathway. This suggests that ATRA also alters IRS-1 levels in CA-OV3 cells by posttranscriptional/posttranslational mechanisms. Our laboratory and others have shown that ATRA acts by either increasing or decreasing the proteasome-dependent degradation of several cell cycle regulatory proteins, including Rb2/p130 (22), cyclin D1 (34), p27 (35) cyclin-dependent kinase 4 (36), Skp2 (37), and promyelocytic leukemia/retinoic acid receptor-α (38). Several studies have shown that IRS-1 protein levels are regulated by IGF-I and insulin via the ubiquitin/26S proteasome pathway (39–41). Our results are consistent with previous studies reported by del Rincon et al. (21) showing that ATRA degrades IRS-1 in MCF-7 breast cancer cells via the ubiquitin proteosomal pathway. As mentioned earlier, IRS-1 can be activated by tyrosine kinase-mediated phosphorylation of numerous tyrosine residues. However, the ability of IRS-1 to instigate downstream signaling and subsequent cellular functions is under negative feedback control by several mechanisms. Importantly, IRS-1 contains >30 serine phosphorylation sites that are direct targets of serine/threonine kinases that inhibit IRS-1 tyrosine phosphorylation. Serine phosphorylation of IRS-1 can interfere with its function by targeting it for inactivation and/or proteasomal degradation (24). Our results indicate that ATRA may affect serine phosphorylation of IRS-1 as seen by the maintenance of phosphorylated Ser312 IRS-1 levels in spite of decreased total IRS-1 levels. Studies have shown that phosphorylation of Ser312 disrupts IRS-1 function by inhibiting interactions between the IRS-1 phosphotyrosine-binding domain and upstream receptors such as IGF-IR (42). Consequently, IRS-1 is not phosphorylated on tyrosine residues, hence blocking downstream signaling complexes. This in essence shuts off IRS-1-mediated signaling. The inflammatory cytokine tumor necrosis factor-α has been shown to inactivate IRS-1 through a JNK-mediated phosphorylation of Ser317, leading to insulin resistance (43).

(44) identified 18 PKCδ serine/threonine phosphorylation sites on IRS-1 and showed that, when Ser312 along with Ser323 and Ser374 were mutated to alanines, the phosphorylation of IRS-1 by activated PKCδ was significantly decreased. The phosphorylation of Ser312 in CA-OV3 cells observed following ATRA treatment in our study could be through activation of kinases such as JNK, IKK, or PKCδ. Thus, decoding the mechanisms of negative feedback regulation via IRS-1 serine phosphorylation mediated by ATRA could prove to be a critical step to fully understanding the role of IRS-1 in ovarian cancer.

Finally, our immunohistochemical studies of an ovarian cancer tissue array containing different phenotypes of ovarian tumors revealed that IRS-1 is highly expressed in malignant epithelial ovarian tumors. This is consistent with our studies in CA-OV3 and SK-OV3 cells, which are cell lines derived from serous adenocarcinomas and are epithelial in nature. Although there is a clear distinction in expression of IRS-1 between normal and benign tumors versus malignant tumors, there does not seem to be a correlation between the stage of the cancer and expression of IRS-1. This is not surprising as immunohistochemical studies done using other cell models show similar discrepancies. Koda et al. (45) recently showed that IRS-1 is expressed in primary breast cancer and metastases and that levels of IRS-1 correlated with poor differentiation (grade 3) and positive lymph node status. Interestingly, in estrogen receptor (ER)-positive tumors, IRS-1 levels correlated positively with proliferation as determined by Ki67 staining, but in ER-negative breast tumors, IRS-1 correlated negatively with proliferation. Another recent study in human breast cancer showed that nuclear IRS-1 levels were low in normal mammary epithelial cells and higher in grade 2 ductal carcinoma and lobular carcinoma and were associated with more differentiated cancer (46). Yet, another study has shown that IRS-1 levels are decreased in differentiated breast tumors (47). In contrast, IRS-1 expression is decreased in 44% of non–small cell lung tumors, and the loss of IRS-1 expression was more in larger tumors (48). A potential explanation for the apparent discrepancy among these IRS-1 expression studies may be that the overall expression of IRS-1 may not actually reflect its functional status.

In conclusion, our study has shown that IRS-1 is an important growth-regulatory molecule in the ATRA-mediated growth inhibition of ovarian carcinoma cells and hence could be a potential chemotherapeutic target.

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