Targeting Aldehyde Dehydrogenase Cancer Stem Cells in Ovarian Cancer


Abstract

Aldehyde dehydrogenase-1A1 (ALDH1A1) expression characterizes a subpopulation of cells with tumor-initiating or cancer stem cell properties in several malignancies. Our goal was to characterize the phenotype of ALDH1A1-positive ovarian cancer cells and examine the biological effects of ALDH1A1 gene silencing. In our analysis of multiple ovarian cancer cell lines, we found that ALDH1A1 expression and activity was significantly higher in taxane- and platinum-resistant cell lines. In patient samples, 72.9% of ovarian cancers had ALDH1A1 expression in which the percentage of ALDH1A1-positive cells correlated negatively with progression-free survival (6.05 vs. 13.81 months; P < 0.035). Subpopulations of A2780cp20 cells with ALDH1A1 activity were isolated for orthotopic tumor–initiating studies, where tumorigenicity was approximately 50-fold higher with ALDH1A1-positive cells. Interestingly, tumors derived from ALDH1A1-positive cells gave rise to both ALDH1A1-positive and ALDH1A1-negative populations, but ALDH1A1-negative cells could not generate ALDH1A1-positive cells. In an in vivo orthotopic mouse model of ovarian cancer, ALDH1A1 silencing using nanoliposomal siRNA sensitized both taxane- and platinum-resistant cell lines to chemotherapy, significantly reducing tumor growth in mice compared with chemotherapy alone (a 74%–90% reduction; P < 0.015). These data show that the ALDH1A1 subpopulation is associated with chemoresistance and outcome in ovarian cancer patients, and targeting ALDH1A1 sensitizes resistant cells to chemotherapy. ALDH1A1-positive cells have enhanced, but not absolute, tumorigenicity but do have differentiation capacity lacking in ALDH1A1-negative cells. This enzyme may be important for identification and targeting of chemoresistant cell populations in ovarian cancer. Mol Cancer Ther; 9(12): 3186–99. ©2010 AACR.

Introduction

Ovarian cancer was expected to be diagnosed in 21,550 women in 2009 and take the lives of 14,600 women (1). Although ovarian cancer is among the most chemo-sensitive malignancies at the time of initial treatment (surgery and taxane/platinum-based chemotherapy), most patients will develop tumor recurrence and succumb to chemoresistant disease (2). An understanding of the mechanisms mediating survival of subpopulations of ovarian cancer cells is necessary to significantly improve outcomes in this disease.

In many malignancies, a subpopulation of malignant cells termed cancer stem cells or tumor-initiating cells has been hypothesized to represent the most tumorigenic and treatment-resistant cells within a heterogeneous tumor mass. Defined by their enhanced ability to generate murine xenografts and give rise to heterogeneous tumors that are composed of both tumor-initiating cell and non-tumor-initiating cell populations, these cells may also be more chemoresistant and depend on unique biological processes compared with the majority of tumor cells (3, 4). In ovarian cancer, many of these properties have been identified in populations of CD44/c-kit–positive cells (5), CD133-positive cells (6–8), and Hoechst-excluding cells (the side population; ref. 9).

Among several markers that have been used to identify cancer stem cells, aldehyde dehydrogenase-1A1...
(ALDH1A1) has been a valid marker among several malignant and nonmalignant tissues (10–20). It holds the attractive distinction of not only being a potential marker of stemness but potentially playing a role in the biology of tumor-initiating cells as well (10). ALDH1A1, 1 of 17 ALDH isoforms, is an intracellular enzyme that oxidizes aldehydes, serving a detoxifying role, and converts retinol to retinoic acid, mediating control on differentiation pathways. The ALDH1A1 population defines normal hematopoietic stem cells, being used to isolate cells for stem cell transplants in patients. Using the ALDEFLUOR assay, a functional flow cytometric assay that identifies cells with active ALDH1A1, tumor-initiating cell-enriched populations have been identified in multiple malignancies (20), including breast (11–14), colon (15, 16), pancreas (17), lung (18), and liver (19). Whether or not the ALDH1A1-active population is enriched for tumor-initiating cells has not been demonstrated for ovarian cancer. More importantly, although ALDH1A1 is implicated in chemoresistance pathways, it is not known whether targeting ALDH1A1 can sensitize resistant cells to chemotherapy and therefore represent a potential target for cancer stem cell–directed therapy. We sought to characterize expression of ALDH1A1 in ovarian cancer cell lines and patient samples, determine whether it contains tumor-initiating cell properties, and examine whether targeting ALDH1A1 sensitizes cells to chemotherapy in both in vitro and in vivo ovarian cancer models.

Materials and Methods

Cell lines and culture

The ovarian cancer cell lines SKOV3ip1, SKOV3-TRip2, HeyA8, HeyA8MDR, A2780ip2, A2780cp20, IGROV-AFI, and IGROV-ep20 (21, 22) were maintained in RPMI-1640 medium supplemented with 15% fetal bovine serum (HyClone). SKOV3TRip2 [taxane-resistant, a kind gift of Dr. Michael Seiden (23)] and HeyA8MDR were maintained with the addition of 150 nmol/L of paclitaxel. The HIO-180 SV40-immortalized, nontumorigenic cell line derived from normal ovarian surface epithelium was a kind gift of Dr. Andrew Godwin. All cell lines were routinely screened for Mycoplasma species (GenProbe detection kit) with experiments done at 70% to 80% confluent cultures. Purity of cell lines was confirmed with STR genomic analysis, and cells used were always less than 20 passages from the stocks tested for purity.

Whole genomic analysis

RNA was extracted from 3 independent collections of SKOV3ip1 and SKOV3TRip2 cells at 80% confluence with the RNaseasy Mini kit (Qiagen). It was subjected to microarray analysis using the Illumina HumanRef-8 Expression BeadChip, which targets ~24,500 well-annotated transcripts. Microarray data were normalized by the cubic-spline method (24) using the Illumina BeadStudio software. The significance of differentially expressed genes was determined by Student’s t test followed by correction for false discovery (25). A heat map was generated using Cluster 3.0 and Java TreeView software. The array data have been registered with GEO (accession #GSE23779) for public access.

Western blot analysis

Cultured cell lysates were collected in modified radiomunoprecipitation assay lysis buffer with protease inhibitor cocktail (Roche) and subjected to immunoblot analysis by standard techniques (26) using anti-ALDH1A1 antibody (BD Biosciences) at 1:1,000 dilution overnight at 4 °C, or anti–β-actin antibody (Sigma Chemical) at 1:2,000.

Immunohistochemical staining and clinical correlations

Immunohistochemical (IHC) analysis was done on formalin-fixed, paraffin-embedded samples, using standard techniques (26). For ALDH1A1, antigen retrieval was in citrate buffer for 45 minutes in an atmospheric pressure steamer, using anti-ALDH1A1 antibody (BD Biosciences) at 1:500 dilution in Cyto-Q reagent (Innovex Biosciences) overnight at 4 °C. Primary antibody detection was with Mach 4 HRP polymer (Biocare Medical) for 20 minutes at room temperature, followed by diaminobenzidine incubation. After IHC staining, the number of tumor cells positive for ALDH1A1 was counted and expressed as a percentage of all tumor cells by an examiner blinded to clinical outcome. Patient samples were categorized as having low (<1%), intermediate (1%–20%), or high (21%–100%) ALDH1A1 expression. The IHC analysis was done on samples collected at primary debulking surgery on 65 untreated patients with stage III–IV, high-grade papillary serous adenocarcinoma; with institutional review board approval, clinical information was collected. Progression-free and overall survival were plotted with the Kaplan–Meier method for patients in each group of ALDH1A1 expression and compared with the log-rank statistic by using PASW 17.0.

For dual staining of ALDH1A1 and CD68 (for macrophages), staining for ALDH1A1 was done first as previously, followed by exposure to anti-CD68 antibody (1:4,000; Dako) and goat anti-mouse-AP (Jackson ImmunoResearch). AP was developed with Feringi Blue chromagen kit (Biocare Medical). For dual staining of ALDH1A1 and hypoxic tumor regions, mice bearing SKOV3TRip2 xenografts were injected with 60 mg/kg of Hypoxyprobe-1 reagent (HPI, Inc.). Tumor sections in FFPE were subjected to antigen retrieval as above, followed by exposure to fluorescein isothiocyanate (FITC)-conjugated anti-hypoxyprobe-1 mouse antibody (1:50) overnight at 4 °C. This was detected with HRP-conjugated anti–FITC antibody (1:500, Jackson ImmunoResearch) and DAB resolution. Endogenous murine
IgG was then blocked with anti-mouse IgG F(ab’)2 fragments (Jackson ImmunoResearch), and ALDH1A1 stained as above using AP-conjugated anti-mouse IgG and Ferangi Blue chromagen.

ALDEFLUOR assay and tumorigenicity in limiting dilutions

Active ALDH1A1 was identified with the ALDEFLUOR assay according to manufacturer’s instructions (StemCell Technologies). The ALDH1A1-positive population was defined by cells with increased FITC signal, with gates determined by diethylaminobenzaldehyde (DEAB)-treated cells (DEAB being an inhibitor of ALDH1A1 activity). For tumorigenicity experiments, the ALDEFLUOR-positive population from A2780cp20 cells were sorted with a FACS Aria II flow cytometer (BD Biosciences) and reanalyzed to confirm at least 95% positivity. Collected cells were washed and resuspended in Ca2+- and Mg2+-free Hanks’ balanced salt solution (HBSS; Gibco) and injected intraperitoneally into NOD-SCID mice in limiting dilutions. Mice were followed for 1 year or until tumors formed, then sacrificed and tumor confirmed histologically. For flow cytometric analysis of these tumors, xenografts were dissociated mechanically with a scalpel, passed through a 70-μm filter to collect single-cell suspensions, with the remaining clumped cells incubated in 0.5 mg/mL of collagenase and 0.0369 mg/mL of hyaluronidase (Calbiochem) for 30 minutes at 37°C. These chemically digested cells were again filtered through a 70 μm filter, added to the initial collection and subjected to the ALDEFLUOR assay. ALDEFLUOR-positive cells or negative cells were then injected into additional mice (n = 5) to examine maintenance of tumorigenicity.

Primary xenograft development

With institutional IRB and IACUC approval, excess of freshly collected omental metastases from advanced stage ovarian cancer patients were acquired after tissue required for diagnosis and management had been sequenced. 3 to 4-mm3 sections were cut and implanted subcutaneously on the dorsal aspect of NOD-SCID mice. Adjacent sections were submitted for histologic analysis to confirm tumor. Tumors were measured in 2 dimensions twice per week. After progressive growth was noted, mice with formed tumors were treated with vehicle or cisplatin (7.5 mg/kg weekly by intraperitoneal administration). Mice were treated for 8 weeks and then sacrificed, and tumors were harvested.

SiRNA downregulation in vitro

To examine downregulation of ALDH1A1 with siRNA, cells were exposed to 2.5 μg/mL of control siRNA (target sequence 5’-AATTCTCCGAACGTGTCACGT-3’; Sigma), or 1 of 3 tested ALDH1A1-targeting constructs (SASI_Hs01_00244055, 00244056, or 00303091; Sigma), at a 1:3 siRNA (μg) to Lipofectamine 2000 (μL) ratio. Lipofectamine 2000 and siRNA were incubated for 20 minutes at room temperature, added to cells in serum-free RPMI to incubate for 6 hours, followed by the addition of 15% FBS/RPMI thereafter. Transfected cells were grown at 37°C for 48 to 72 hours and then harvested for Western blot.

Assessment of cell viability with chemotherapy IC50 and cell-cycle analysis

To a 96-well plate, 2,000 cells per well were exposed to increasing concentrations of docetaxel or cisplatin in triplicates. Viability was assessed by 2-hour incubation with 0.15% MTT (Sigma) and spectrophotometric analysis at OD450 (optical density at 450 nm). For effects of siRNA on IC50, cells were incubated with siRNA for 24 hours in 6-well plates and then replated in 96-well plates, and chemotherapy was administered after 12 hours to allow attachment. IC50 was determined by finding the dose at which the drug had 50% of its effect and calculated by the following equation: IC50 = [(OD450max − OD450min)/2] + OD450min. Test of synergy was according to the Loewe additivity model (27) and calculated by the following equation: combination index (CI) = [D1/Dc1] + [D2/Dc2] (where CI of 1 suggests an additive effect, >1 suggests synergy, and <1 suggests antagonism). For cell-cycle analysis, cells were transfected with siRNA as described previously for 72 hours, trypsinized, washed in PBS, and fixed in 75% ethanol overnight. Cells were then centrifuged, washed twice in PBS, and reconstituted in PBS with 50 μg/mL of propidium iodide. Propidium iodide fluorescence was assessed by flow cytometry, and percentage of cells in each cycle was calculated by the cell-cycle analysis module for FlowJo.

Orthotopic ovarian cancer model and in vivo delivery of siRNA

For orthotopic therapy experiments using ovarian cancer cell lines, female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute and cared for in accordance with guidelines of the American Association for Accreditation of Laboratory Animal Care. For all in vivo experiments, trypsinized cells were suspended in HBSS and 106 cells injected intraperitoneally into 40 mice per experiment. After 1 week, mice were randomized to: a) control siRNA/DOPC, b) control siRNA/DOPC plus chemotherapy, c) ALDH1A1-targeting siRNA/DOPC, or d) chemotherapy plus ALDH1A1-targeting siRNA/DOPC. SiRNA/DOPC dose was 5 μg twice per week in a volume of 100 μL intraperitoneally. Chemotherapy doses were docetaxel 35 μg intraperitoneally weekly for SKOV3Trp2, or cisplatin 160 μg intraperitoneally weekly for A2780cp20. Mice were treated for 4 weeks before sacrifice and tumor collection. SiRNA was incorporated into 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) neutral nanoliposomes as previously described (28), lyophilized, and reconstituted in 0.9% saline for administration.
Statistical analysis

Comparisons between treatment groups of tumor weight was carried out with the 2-tailed Student’s t test, if tests of data normality were met. Those represented by alternate distribution were examined by Mann–Whitney U statistic. Differences between groups were considered statistically significant at P < 0.05. The number of mice per group (n = 10) was chosen as directed by a power analysis to detect a 50% decrease in tumor growth with β error of 0.2. Progression-free and overall survival in patients with 3 categories of ALDH1A1 staining were compared by plotting with the Kaplan–Meier method and assessing for statistical differences with the log-rank statistic, using PASW 17.0 software.

Results

Expression profiling of chemoresistant ovarian cancer cell lines

To discover genes mediating taxane resistance, expression profiling of parental SKOV3ip1 and taxane-resistant SKOV3TRip2 cells was done with microarray analysis using the Illumina HumanRef-8 Expression BeadChip. The SKOV3TRip2 cell line was previously generated through progressive exposure to paclitaxel (designated SKOV3TR; 23) and then passaged intraperitoneally in mice for 2 generations to select populations with enhanced tumorigenicity. Similarly, SKOV3ip1 were derived from SKOV3 parental cells to select for cells with enhanced tumorigenicity. We found 34 genes to be upregulated more than 10-fold in SKOV3TRip2 (Fig. 1), among which was ALDH1A1, with a 92.7-fold increase (P = 0.0025). Twenty genes were more than 10-fold increased in SKOV3ip1. SKOV3TRip2 cells were confirmed to have approximately 3,000-fold increased resistance to docetaxel, as measured by MTT IC₅₀ (62.5 nmol/L vs. 0.02 nmol/L; Fig. 2A).

ALDH1A1 expression in ovarian cancer cell lines

To confirm an increase in ALDH1A1 expression/activity in SKOV3TRip2 and examine expression in other ovarian cancer cell lines, 4 pairs of parental and chemoresistant cell lines were examined: SKOV3ip1/SKOVS3TRip2; HeyA8/HeyA8MDR (multidrug resistant); A2780ip2/A2780cp20 (10-fold increased cisplatin resistance); and IGROV-AF1/IGROV-cp20 (5-fold increased cisplatin resistance). In addition, an immortalized, non-transformed cell line derived from normal ovarian surface epithelium, HIO-180, was examined. We found that expression of total ALDH1A1, as measured by Western blot analysis, was in each case higher in the chemoresistant cell line, with the exception of HeyA8/HeyA8MDR, in which ALDH1A1 was low to absent in cells but not in the parental cell line (Fig. 2C). Furthermore, the strong shift in fluorescent signal in some cells suggests that there was not simply a general increase in expression in all cells but rather separate populations of ALDH1A1-negative and -positive cells. This was confirmed by immunohistochemistry, which showed distinct populations of ALDH1A1-positive or -negative cells in A2780cp20 and SKOV3TRip2 cells but not in the parental A2780ip2 and SKOV3ip1 cells in culture (Fig. 2D). Finally, we observed that this heterogeneous profile was maintained in tumors. After intraperitoneal injection of SKOV3TRip2 cells into nude mice and collection of the resulting orthotopic tumor implants, IHC staining of for ALDH1A1 showed both positive and negative ALDH1A1 subpopulations (Fig. 2E). To examine whether this heterogeneity in expression was due to differential expression in hypoxic regions, a tumor-bearing mouse was injected with hypoxyprobe reagent and sacrificed after 30 minutes. The tumor was stained with ALDH1A1 and antihypoxyprobe antibody. We found that the ALDH1A1-positive cells were not preferentially localized to hypoxic regions in the tumor, with only 1.5% of ALDH1A1-positive cells concurrently positive for hypoxyprobe and only 3.3% of hypoxyprobe-positive cells also positive for ALDH1A1 (P < 0.01; Fig. 2F).

ALDH1A1 expression in human ovarian cancer specimens

To determine the pattern of ALDH1A1 expression and possible correlations with chemoresistance in patients, we next examined ALDH1A1 expression in 65 untreated, high-grade papillary serous stage III–IV ovarian cancer patient specimens (patient characteristics in Table 1). We found a wide range of expression patterns (Fig. 3A). There was no ALDH1A1 in tumor cells in 27.1% of samples. ALDH1A1 expression was noted in 1% to 20% of cells in 44% of tumors, representing the largest cohort of expression patterns. As in xenografts from cell lines, expression was typically strong in some cells and negative in others, signifying distinct heterogeneity in the tumor. There was no distinct histologic pattern to the location of the positive cells (such as around vasculature or on the leading edge of the tumor), but positive cells did tend to cluster together. The remaining tumors (28.9%) all had between 21% and 100% staining, with 10% of all patients having strong ALDH1A1 expression in nearly 100% of their tumor cells. To confirm that ALDH1A1 expression was not being mistakenly identified in tumor-infiltrating macrophages, several snap-frozen samples were dual stained for ALDH1A1 and CD68. Although images are not as detailed as those from paraffin-embedded samples, dual staining clearly shows that the majority of macrophages (blue) are ALDH1A1 negative and therefore the heterogeneous ALDH1A1 positivity in tumors is not simply due to detection of macrophage infiltration (Fig. 3B).
Figure 1. Comparison of whole genome expression profiling between SKOV3TRip2 and SKOV3ip1 cell lines. Total RNA from the SKOV3TRip2 and SKOV3ip1 cell lines were subjected to whole genome expression profiling using the Illumina platform. The genes with a greater than 10-fold increase in SKOV3TRip2 are shown in red, whereas those with a greater than 10-fold increase in SKOV3ip1 are shown in green. FC, fold change.
To determine whether ALDH1A1 expression correlated with clinical outcomes, we compared progression-free survival and overall survival from patient samples described earlier (and in Table 1) in cohorts with no ALDH1A1 expression, 1% to 20% expression, and greater than 20% expression, as this grouping allowed similar}

**Correlation of ALDH1A1 expression with clinical outcomes**

To determine whether ALDH1A1 expression correlated with clinical outcomes, we compared progression-free survival and overall survival from patient samples described earlier (and in Table 1) in cohorts with no ALDH1A1 expression, 1% to 20% expression, and greater than 20% expression, as this grouping allowed similar
numbers between groups. Patients with greater than 20% ALDH1A1-positive cells had a shorter median progression-free survival (6.1 months) than those with 1% to 20% ALDH1A1-positive cells (8.2 months) or those with no ALDH1A1-positive cells (13.8 months), which was statistically significant according to the log-rank test (P = 0.035; Supplementary Fig. 1). Overall survival, which reflects resistance to multiple chemotherapeutic agents used in the recurrent setting, showed a trend toward a poor outcome with increasing ALDH1A1 expression (median overall survival 1.09 vs. 1.84 vs. 2.32 years), but the trend was not statistically significant (P = 0.33; Supplementary Fig. 1).

**Preferential survival of ALDH1A1-positive cells with cisplatin treatment**

To determine whether the ALDH1A1-positive cells have preferential survival in the tumor microenvironment with platinum treatment, we established mouse xenografts from primary patient samples by subcutaneously implanting a freshly collected tumor specimen into NOD-SCID mice. A subcutaneous rather than orthotopic model was used so that tumor growth and response could be accurately measured. Once tumors were established and growing, and achieved a size of approximately 1 cm³, intraperitoneal administration of 7.5 μg/kg of cisplatin weekly was initiated whereas only vehicle was administered to controls (Fig. 3D). When tumors grew to a size of 2 cm³ in controls, having remained stable with cisplatin treatment, they were harvested and sections stained for ALDH1A1 expression. Baseline expression of ALDH1A1 in the implanted tumor was seen in approximately 1% of cancer cells and similar levels were found in growing xenografts in untreated mice (Fig. 3E). A significant increase in the percentage of ALDH1A1-positive cells was, however, noted in cisplatin-treated xenografts to 38% (P < 0.001; Fig. 3E). Consistent with this, the ALDEFLUOR assay on the dissociated tumor showed that 0.6% of cells from untreated tumors were ALDEFLUOR positive whereas 17.6% of cells from cisplatin-treated tumors were ALDEFLUOR positive. Because the treated xenograft in this case did not regress, but rather remained stable in size, cisplatin exposure may have induced ALDH1A1 expression in surviving cells in addition to preferential killing of ALDH1A1-negative cells.

**Tumor-initiating capacity of ALDH1A1-positive ovarian cancer cells**

In breast and other cancers, the ALDH1A1-active cancer cells have been shown to represent a tumor-initiating population (10–19). To determine whether this were the case in ovarian cancer, we sorted ALDH1A1-positive and -negative populations from the A2780cp20 cell line using the ALDEFLUOR assay and injected cells intraperitoneally into NOD-SCID mice in limiting dilutions to determine tumor-initiating potential. As summarized in Table 2, ALDEFLUOR-positive cells exhibited increased tumorigenic potential, with 100% tumor initiation after the injection of 100,000, 25,000, or 5,000 cells, and 1 tumor was established after the injection of 1,000 cells. ALDEFLUOR-negative cells could form tumors, although at a lower rate: 2 of 5 mice formed tumors after the injection of 25,000 or 100,000 cells and no tumors formed after the injection of 5,000 or 1,000 cells. Mice were followed for 1 year after injection and thorough necropsies were performed in remaining mice to confirm that tumors failed to develop. The TD50, or dose of cells required to permit tumor formation in 50% of animals, was 50-fold lower with ALDEFLUOR-positive cells. Perhaps, more striking was the makeup of these tumors. One requirement of a tumor-initiating population is that it has the capacity to give rise to heterogeneous tumors, composed of both stem cell and non–stem cell populations, therefore...
demonstrating multipotent differentiation potential. This was noted in tumors that formed after the injection of ALDEFLUOR-positive cells. In all 16 of these tumors, a strongly positive ALDH1A1 population was noted in the minority of the sample, on average 4.7% of the tumor (range 2.4%–6.1%; Fig. 4A). However, no ALDEFLUOR-positive cells were found in the tumors that formed after the injection of ALDH1A1-negative cells (Fig. 4B). This was confirmed by the IFIC analysis (Fig. 4C and D). This argues against the idea that tumors are formed because of contamination with ALDEFLUOR-positive cells or that ALDH1A1 expression is simply induced by the tumor microenvironment regardless of the capacity of the cells.

This difference in the capacity to generate ALDEFLUOR-positive cells was also noted in vitro. SKOV3TRip2 cells sorted into ALDEFLUOR-positive and -negative populations were cultured separately, and the ALDEFLUOR assay was done on the different populations at 24, 48, and 72 hours (Fig. 4E and F). Of the ALDEFLUOR-positive cells, the population gradually reverted to 75.3%, 54.2%, and 51.4% ALDEFLUOR-positive cells, respectively, for each time point. However, the ALDEFLUOR-negative cells could not produce any ALDEFLUOR-positive cells.

To confirm that the ALDEFLUOR-positive cells from tumors maintained tumorigenicity, these populations...
were sorted and reinjected intraperitoneally into mice and continued to form tumors at 100% rate with 25,000 cells injected. However, ALDEFLUOR-negative cells from the tumors forming after ALDEFLUOR-negative cells were injected did not form tumors. Taken together, these studies show that ALDEFLUOR-positive cells have increased but not absolute tumorigenicity, but they do have a differentiation capacity and maintenance of the tumorigenic phenotype that is absent in ALDEFLUOR-negative cells.

In an effort to determine whether ALDEFLUOR-positive cells, freshly collected from ovarian cancer patients, have similar tumorigenicity, we have sorted ALDEFLUOR-positive and -negative cells from 5 separate ovarian cancer patients, dissociating tumors metastatic to the omentum at the time of primary debulking surgery. In this cohort, 1.5% to 17.8% of cells were ALDEFLUOR positive. A total of 25,000 ALDEFLUOR-positive cells, 100,000 ALDEFLUOR-negative cells, or 100,000 unsorted cells were injected intraperitoneally into 5 mice per group per patient. Unfortunately, no tumors formed in any mice, highlighting the difficulty of tumorigenicity studies in primary ovarian cancer samples dissociated to single cell suspensions.

To preliminarily determine whether there is an overlap between the ALDEFLUOR-positive population and other...
markers of putative stem cells in ovarian cancer, these 5 samples were also profiled for CD44, c-kit, and CD133. We were not able to identify a convincing positive c-kit population from any sample. CD133-positive cells made up an average of 3.1% of total tumor cells (range, 0.6%–5.7%) and were greater than 80% of ALDEFLUOR-positive cells in all 5 samples (mean, 86.7%; range, 81.5%–100%). CD44 was more commonly expressed, representing an average of 45.7% of tumors (but with a very broad range of 2.4%–98.2%). Of the CD44-positive cells, 75.4% were also ALDEFLUOR-positive (range, 46.6%–88.8%). Similarly, the SKOV3TRip2 line has 82% CD44-positive cells, and of these, 74% were ALDEFLUOR positive. Although a great number of samples will need to be examined to fully delineate whether multiple marker-positive cells can more accurately define the most pure tumorigenic cell, there is certainly overlap in marker expression. There are both double-positive CD44/ALDEFLUOR and CD133/ALDEFLUOR-positive populations that may prove more discerning as cancer stem cell populations, and ongoing studies could assess this distinction. Interestingly, the A2780cp20 cell line is completely negative for CD44 and the HeyA8 cell line is negative for ALDH1A1/ALDEFLUOR, despite the fact that both are highly tumorigenic. This highlights the fact that these cannot be the sole mediators of tumorigenicity in mice.

**Downregulation of ALDH1A1 sensitizes ovarian cancer cells to chemotherapy**

Given the association of ALDH1A1 expression with chemoresistant cell lines and a decreased progression-free survival in ovarian cancer patients, we asked whether downregulation of ALDH1A1 could sensitize resistant cells to chemotherapy. Two different siRNA constructs were identified that reduced ALDH1A1 expression by greater than 80% (Fig. 5A). Reduction in the ALDEFLUOR population was confirmed (Fig. 5B). SKOV3TRip2 or A2780cp20 cells were exposed to ALDH1A1-targeting siRNA (ALDH1A1 siRNA) or control siRNA for 24 hours before replating and adding increasing concentrations of docetaxel or cisplatin, respectively. Cell viability 4 days after the addition of chemotherapy was assessed with the MTT assay. In SKOV3TRip2 cells, siRNA-ALDH1A1 alone reduced viability by 49% (Fig. 5C; $P < 0.001$). Downregulation of ALDH1A1 also reduced the docetaxel IC₅₀ from 178 to 82 nmol/L. In A2780cp20, the effects of ALDH1A1 downregulation alone were modest (Fig. 5D; reduced viability by 15.9%, $P = 0.040$) but sensitization to cisplatin was considerable, with a decrease in the IC₅₀ from 5.1 to 2.0 µmol/L. Tests for synergy suggest moderate synergy in each cell line (CI = 0.82 for SKOV3TRip2 and 0.75 for A2780cp20). The contrasting effects of ALDH1A1-siRNA alone are consistent with the number of ALDH1A1-active cells in these cell lines, with SKOV3TRip2 cell lines having 50% to 60% of ALDEFLUOR-positive cells and A2780cp20 having just 2% of 3%. To determine how ALDH1A1 downregulation alone may affect cell growth, cell-cycle analysis was done in a separate experiment. We found that ALDH1A1 downregulation induced an accumulation of SKOV3TRip2 cells in S and G₂ phases ($P < 0.001$; compared with control siRNA) but had only minimal effects on the cell cycle of A2780cp20 cells (Fig. 5E).

There are no known inhibitors of ALDH1A1 for *in vivo* studies. Therefore, we used a method for delivery of siRNA *in vivo*, using DOPC nanoparticles. We and others (28–32) have previously shown delivery of siRNA incorporated into DOPC nanoparticles to the tumor parenchyma with subsequent target downregulation. In this study, nude mice were injected intraperitoneally with either SKOV3TRip2 or A2780cp20 cells and randomized to 4 treatment groups to begin 1 week after cell injection: a) control siRNA in DOPC, delivered intraperitoneally twice per week; b) docetaxel 35 mg, delivered intraperitoneally weekly (for SKOV3TRip2 model) or cisplatin 160 µg, delivered intraperitoneally weekly (for A2780cp20 model); c) ALDH1A1-siRNA in DOPC, intraperitoneally twice per week; or d) ALDH1A1-siRNA in DOPC plus docetaxel (for SKOV3TRip2) or cisplatin (for A2780cp20). After 4 weeks of treatment, mice were sacrificed and total tumor weight recorded. The IHC analysis confirmed reduced ALDH1A1 expression with ALDH1A1-siRNA/DOPC treatment compared with controls but not with chemotherapy alone (Supplementary Fig. 2; too little tissue was available to examine with the ALDEFLUOR assay). In SKOV3TRip2 xenografts (Fig. 5F), there was a nonsignificant reduction of 37.0% in tumor growth with docetaxel treatment ($P = 0.17$) and of 25.0% with ALDH1A1 siRNA treatment ($P = 0.38$) compared with control siRNA/DOPC. The observation that ALDH1A1 downregulation alone significantly decreased SKOV3TRip2 growth *in vitro* but was less pronounced *in vivo* suggests that tumor microenvironment factors such as supporting stromal cells may be able to protect cells from ALDH1A1 depletion. However, the combination of ALDH1A1 siRNA and docetaxel resulted in significantly reduced growth by 93.6% compared with control siRNA ($P < 0.001$), by 89.8% compared with docetaxel plus control siRNA ($P = 0.003$), and by 91.4% compared with ALDH1A1 siRNA ($P = 0.002$). In A2780cp20 (Fig. 5G), there was a similar nonsignificant reduction of 43.9% in tumor weight with cisplatin alone ($P = 0.32$) and of 57.0% with ALDH1A1 siRNA treatment ($P = 0.19$). These effects may be even less significant than the mean tumor weights suggest, given the presence of 2 especially large tumors in the control siRNA group. However, again combined therapy showed a sensitization to chemotherapy with ALDH1A1 siRNA, with combination therapy reducing growth by 85.0% compared with control siRNA ($P = 0.048$), by 73.4% compared with cisplatin plus control siRNA ($P = 0.013$), and by 65.3% compared with ALDH1A1 siRNA alone ($P = 0.039$). Given the minimal effects of each single agent and the consistent finding of significant improvement with combined therapy, these data suggest a synergy between...
ALDH1A1 downregulation and both taxane and platinum chemotherapeutic agents, though formal dose-finding experiments would be required to definitively prove synergy.

Discussion

We have found that ALDH1A1 expression and activity are increased in chemoresistant ovarian cancer cell lines.
and in in situ primary ovarian cancer xenografts treated with cisplatin. Expression of ALDH1A1 is frequent in ovarian tumors, and patients with low ALDH1A1 expression levels have a more favorable outcome than those with more ALDH1A1-positive cells. ALDEFLUOR-positive cells have increased (but not absolute) tumorigenicity compared with ALDEFLUOR-negative cells and have a differentiating capacity that is not present in the ALDEFLUOR-negative population. Most important, downregulation of ALDH1A1 expression sensitized normally chemoresistant tumors to both doctaxel and cisplatin both in vitro and in an orthotopic mouse model of ovarian cancer.

The search for tumor-initiating cells in ovarian cancer has resulted in findings that the CD44+/c-kit+ population has an approximately 5,000-fold increase in tumorigenicity, with tumors forming after the injection of as few as 100 cells from primary tumor, xenograft, or spheroid formation has resulted in observations that the CD44+/c-kit+ population is highly tumorigenic. However, Tanei et al., who studied tissue obtained before and after neoadjuvant chemotherapy, found that despite a positive response to treatment, the proportion of CD44+/CD24− population was unchanged. In these samples, however, the ALDH1A1-positive population was significantly increased (34).

ALDH1A1 has previously been proposed to play a role in chemoresistance, having been noted to be higher in proteomic profiling of IGROV platinum-resistant ovarian cancer cells (35), in genomic profiling of multidrug-resistant gastric carcinoma (36), and in cells resistant to cyclophosphamide (37, 38), oxazaphosphorines (39), and now docetaxel and cisplatin. ALDH1A1 oxidizes many intracellular aldehydes into carboxylic acids (40), detoxifying many of the free oxygen radicals generated by chemotherapeutic agents. It stands to reason that a stem cell population should be resistant to multiple chemotherapeutic
agents rather than being specific to one class. This also follows clinically, in that most ovarian cancer patients who develop resistance to platinum agents have resistance to multiple agents (2). ALDH1A1 has been shown to be associated with BRCA1 in breast cancer, in that knockdown of BRCA1 increases the ALDEFLUOR population and ALDEFLUOR-positive cells preferentially contain BRCA1 loss of heterozygosity (41). These findings could also be important to BRCA-mediated ovarian cancer. Despite this body of evidence for the importance of ALDH1A1, it is not fully understood whether any of the additional ALDH1 isoforms are important to stem cell biology. In our study, ALDH1A1 can be specifically identified with isotype-specific antibodies (as used for the IHC analysis and Western blotting). However, the more important and consistently used identifier of a stem cell population is the ALDEFLUOR assay, which, although primarily dependent on ALDH1A1, may also identify ALDH1A2 and ALDH1A3 isotypes (42) and unpublished data by Stem Cell Technologies. As a therapeutic agent, we have seen positive effects by targeting ALDH1A1 with siRNA, but to maximize the efficacy of therapeutics, the contribution of these additional isotypes will need to be defined with additional studies.

Although our finding of a poor outcome in patients with high ALDH1A1 expression agrees with similar investigations in breast cancer (12, 13) and ovarian cancer (20), one interesting report found that a high ALDH1A1 expression level actually confers a positive prognosis in ovarian cancer (43). This cohort also contained patients with absent, scattered, and diffuse staining. However, this cohort included patients with stage I and II disease and low-grade tumors, and ALDH1A1 expression was higher in these patients [confirming findings from a previous report (44)]. Furthermore, with multivariate analysis, only stage correlated with survival; ALDH1A1 expression no longer predicted outcomes. In ovarian cancer, there is a well-recognized dichotomy in carcinogenesis and pathobiology (45), whereby low-grade tumors (which are more often diagnosed at stage I or II) are paradoxically more chemoresistant but have prolonged survival due to slow growth. Given these collective data, and the several mechanisms by which ALDH1A1 has been shown to contribute to chemoresistance, it may be that ALDH1A1 is more frequently expressed in low-grade tumors but participates in chemoresistance to both high-grade and low-grade subtypes.

We have shown that the ALDH1A1-positive population has properties of cancer stem cells, is associated with taxane and platinum resistance, and can be resensitized to chemotherapy with downregulation of ALDH1A1 in vitro and in vivo. Therefore, ALDH1A1 is not just a marker of an aggressive population but also a mediator of the phenotype and a viable target for therapy. As better models are developed to more purely define the true chemoresistant population in de novo patient tumors, the ALDH1A1 population, either alone or in combination with other markers and mediators of resistance, may represent a population that must be targeted to achieve increased response rates and survival in ovarian cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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