Antisense Oligonucleotide Targeting of Raf-1: Importance of Raf-1 mRNA Expression Levels and Raf-1-Dependent Signaling in Determining Growth Response in Ovarian Cancer

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ABSTRACT

Purpose: We sought to identify determinants of growth response to the Raf-1-targeted antisense oligonucleotide (ASO; ISIS 5132) using a large panel of ovarian cancer cell lines.

Experimental Design: First- (ISIS 5132) and second-generation (ISIS 13650) anti-Raf 1 ASOs were compared with control oligonucleotides. Growth was assessed by cell counts; apoptosis was assessed by poly(ADP-ribose) polymerase cleavage; and cell cycle analysis was assessed by flow cytometry. Protein expression was detected by Western blot analysis, and mRNA expression was detected by quantitative reverse transcription-PCR. Raf-1 kinase activity was detected by anti-Raf-1 immunoprecipitation, followed by myelin basic protein phosphorylation.

Results: A panel of 15 ovarian cancer cell lines was used to model a range of growth responses to ASOs targeting Raf-1 mRNA. Growth inhibition varied from 10% to >90% inhibition. Growth inhibition was associated with increased apoptosis and accumulation of cells in the G2-M and S phases of the cell cycle. Growth response was not related to level of Raf-1 protein expression, Raf-1 kinase activity, intracellular ASO uptake, or degree of Raf-1 protein inhibition. However, ASO growth response was associated with a high proportion of Raf-1 mRNA [relative to total (i.e., Raf-1 + A-Raf + B-Raf) Raf mRNA] and significantly higher Raf-1 kinase activity induction following growth factor (transforming growth factor α) stimulation in the cell lines consistent with dependency of these cell lines on Raf-1.

Conclusions: These data indicate that ovarian cancers demonstrate differential sensitivity to ASOs targeted against Raf-1, and target expression levels and degree of utilization of Raf-1 signaling are implicated. Clinically sensitive tumors could feasibly be identified.

INTRODUCTION

One of the key pathways regulating mammalian cell growth is the extracellular signal-related kinase (ERK) signal pathway, which relays extracellular signals to the nucleus via a cascade of specific phosphorylation events involving Ras, Raf, mitogen-activated protein (MAP)/ERK kinase (MEK), and ERK (1, 2). The Raf family of serine/threonine kinases consists of Raf-1 (or c-Raf), A-Raf, and B-Raf, which share highly conserved amino-terminal regulatory regions and a COOH-terminal catalytic kinase domain (3). All of the three Raf isoforms are widely expressed, although protein levels vary greatly depending on tissue type (4). Of the three Raf isoforms, most biochemical studies have focused on Raf-1. Inactive Raf-1 is normally cytosolic but translocates to the plasma membrane on binding to Ras (5). Additional phosphorylation events, most notably at serine-338 and tyrosine-341 residues, then are required for complete Raf-1 activation (3). Once activated, Raf-1 will phosphorylate MEK, which in turn activates ERK (1, 2).

The use of antisense oligonucleotides (ASOs) to target destruction of key mRNAs is increasingly considered for the management of cancer (6, 7). Current targets in clinical trials include bcl-2, BCR-ABL, Ha-Ras, c-myc, protein kinase C, protein kinase A, p53, MDM2, and Raf-1 (6, 7). ISIS 5132 is an ASO designed to target Raf-1, which not only decreases Raf-1 mRNA and protein expression but also reduces cellular proliferation in a variety of cell types (8–10). We have shown previously that expression of Raf-1 protein is a negative prognostic factor in ovarian cancer because high levels correlate with poor survival and serous histology (10). Furthermore, ISIS 5132 inhibits growth of ovarian cancer cells in vitro and in vivo (10). Therefore, reducing Raf-1 protein levels by means of targeted ASOs may have a therapeutic role in the management of ovarian cancer. Clinical trials using ISIS 5132 have demonstrated that the ovarian cancer serum marker CA125 can be reduced markedly in ovarian cancer patients (11), and significant reduction in Raf-1 mRNA in peripheral blood mononuclear cells is achievable (12, 13). It is likely that selection of patients most likely to respond to treatment will be necessary if such compounds are to have any future clinical role, and tumors whose growth is reliant on Raf-1 are likely to be the most sensitive. In this study we have used a large series of ovarian cancer cell lines as a model reflecting the heterogeneity of Raf expression and sought to identify determinants of response and dependency in ovarian cancers.

MATERIALS AND METHODS

Cell Lines. PEO1, PE01(TOP), PE04, PE06, PE014, and PE016 were established as described previously (14); SKOV-3
and CaOV3 were obtained from American Type Culture Collection (Manassas, VA); OVCAR3, OVCAR4, and OVCAR5 were obtained from Dr. T. C. Hamilton (Fox Chase Institute, Philadelphia, PA); and 41M, 59M, A2780, and OAW42 were obtained from the European Tissue Collection (Porton Down, United Kingdom). All of the cells were grown routinely as monolayer cultures in RPMI 1640 media supplemented with 10% heat-inactivated FCS and 100 IU/ml penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37°C. Growth-inhibition experiments were set up using log-phase cells seeded into 24-well tissue culture plates (optimized around 1 x 10^4 in 1 ml) and incubated to reach 40–60% confluence 2–3 days before exposure. Cells for flow cytometric analysis were set up similarly in 60-mm diameter Petri dishes.

**Antisense Oligonucleotides.** ASOs of two chemical classes were investigated. The first-generation fully modified phosphorothioate (ISIS 5132; Ref. 8) and second-generation 2′-methoxymethoxy phosphorothioate (ISIS 13650) Raf-1 ASOs (sequence, TCCGCCCTGTGACATGCATT) were synthesized by ISIS Pharmaceuticals (Carlsbad, CA) as described previously, along with the control second-generation mismatch oligonucleotide ISIS 16971 (sequence, TCACATTGGCGCTTAGCCGT).

**Growth Assays.** Cells within individual wells of a 24-well plate were washed with PBS before addition of Optimem media (250 μl) containing Lipofectin (Life Technologies, Rockville, MD; 6 μg/ml). ASOs and mismatch oligonucleotide were added from 50 μM stock solutions. Cells were incubated at 37°C for 3 h, washed with PBS, and then fed with RPMI 1640 plus 10% FCS and 100 IU/ml penicillin/streptomycin. At designated times, cells were trypsinized and counted by cell counter.

**Western Blot Analysis.** Following exposure to oligonucleotides, cells were grown to 70% confluence (48 h), washed with PBS, and lysed in ice-cold hypotonic lysis buffer [50 mM Tris-HCl (pH 7.5), 5 mM EGTA (pH 8.5), 150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM sodium molybdate]. Lysates were centrifuged for 6 min at 13,000 x g. Protein concentrations of supernatants were determined using the Bio-Rad Protein Assay Kit (Richmond, CA). Cell lysates (30 μg) were resolved on 10% or 12% SDS-PAGE and then transferred electrophoretically overnight onto Immobilon-P membranes (Millipore, Bedford, MA). After transfer, membranes were blocked with 1% blocking agent in Tris-buffered saline [20 mM Tris-HCl and 137 mM NaCl (pH 7.5)] before probing overnight at 4°C with the appropriate primary antibody: anti-Raf-1 (R19120; Transduction Laboratories, Lexington, KY), anti-phosphoMEK (NEB 9121), anti-phosphoERK (NEB 9101), or anti-actin (CP01; Oncogene Research Products, Cambridge MA). Immunoreactive bands were detected using enhanced chemiluminescent reagents (1520709; Roche, Basel, Switzerland) and Hyperfilm ECL film (Amersham, Buckinghamshire, United Kingdom). In experiments using transforming growth factor α (TGF-α), cells were transferred to double charcoal stripped 5% FCS for 24 h before addition of growth factor. TGF-α was added for 15 min in double charcoal stripped 5% FCS, and lysates were collected immediately after.

**Cell Cycle Analysis.** Flow cytometric DNA analysis of treated cells was carried out using methodology described by Levack et al. (15). After trypsinization, cells were resuspended in 100 μl of citrate buffer and stored at −20°C before flow cytometric DNA analysis using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

**PARP Cleavage Assay.** SKOV-3, PE01, and OVCAR5 cells were assayed for late-stage apoptosis using an anti-poly-(ADP-ribose) polymerase (PARP) antibody (AF-800-NA; R&D Systems, Minneapolis, MN). Media first were decanted from flasks and transferred to 50-ml universal containers to be pooled with cell extracts. Cells were washed with PBS and trypsinized, washing the flask using collected media from the respective flasks. The pooled cells and media were centrifuged at 1500 x g for 5 min and resuspended in RPMI 1640 (4 ml), and a 200-μl aliquot was counted. Cells (0.8 x 10^6) then were taken from each tube and transferred to fresh universal containers before again centrifuging and resuspending in lysis buffer (200 μl). After breaking up the pellet, all of the samples were sonicated for 30 s on ice and stored at −20°C until analyzed. Samples were heated at 65°C for 10 min before loading on 7.5% SDS-PAGE gels for Western blot detection.

**Antisense Oligonucleotide Fluorescence Uptake Studies.** The uptake of ASO into SKOV-3 and OVCAR5 cell lines was assessed using fluorescent (6-carboxyfluorescein) FAM-labeled ISIS 5132 (synthesized by ICRF, Clare Hall Laboratories, South Mimms, United Kingdom). Cells were plated in Petri dishes and exposed to FAM-5132 as described previously before being harvested immediately after oligonucleotide incubation and resuspended in PBS (0.5 ml). Fluorescence (FL1) then was measured in 20,000 cells using a Becton Dickinson FACSCalibur flow cytometer. Visualization of oligonucleotide incorporation also was carried out by fluorescent microscopy using a Cox stereomicroscope equipped with a fluorescence arc lamp. Duplicate images were captured using phase contrast and RGF2 filtration and then layered together using Adobe Photoshop software (San Jose, CA).

**RNA Extraction and Measurement.** RNA was extracted from the panel of 15 cell lines using commercially available TriReagent as per the described protocol (T-9429; Sigma, St. Louis, MO). Treatment with DNase 1 (10 units/ml, 776785; Roche) in the presence of RNase inhibitor (40 units/ml, 799017; Roche) was followed by re-extraction using a standard phenol-chloroform extraction protocol. RNA was reverse transcribed into single-stranded cDNA using the first-strand cDNA synthesis kit for reverse transcription-PCR (143 188; Roche) as described in the protocol provided. Two ml of cDNA were analyzed by real-time PCR using a Rotorgene 2000 (Corbett Research, Sydney, Australia). Reactions included Excite 2× Master Mix (Ex001; Biogene, Kimbolton, United Kingdom), SYBR green dye at a final concentration of 1:20,000 (1765; Biogene), and forward and reverse primers at 0.4 mM. The following primer pairs were used:

- for Raf-1, GGAGAGACATGGGATTTGAG and GCTGTGAAAGGAGGACGTG
- for A-Raf, ATGTTCGTCTCTGCCCTGAT and GATGAGGAGAGGCTG
- for B-Raf, CCTCCGGAGAGGCTG and AGTTCTGCAGGTAGCTCTCCAGAAT; and
for β-actin, CTACGTCCCTGGACTTCGAC and GATGGAGCCGCCGATCCACACGG.

Standard curves were obtained by performing reactions with predetermined amounts of target template DNA for each primer pair. Contamination of RNA by genomic DNA was excluded by performing reactions on RNA that had not been reverse transcribed.

**Raf Immunocomplex Kinase Assays.** A two-step assay was used to detect Raf kinase activity (16, 17). Cells were treated and lysed as described previously. Protein lysates (300 μg) were incubated overnight at 4°C with antibodies (1 μg) to A-Raf (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), B-Raf (F-7; Santa Cruz Biotechnology), or Raf-1 (R19120; Transduction Laboratories), together with protein-A/G plus Sepharose (15 μl). Immunoprecipitates then were centrifuged, and the pellet was washed three times in ice-cold wash buffer [20 mM Tris HCl (pH 7.4), 150 mM NaCl, and 1% Triton-X100], followed by two additional washes in ice-cold dilution buffer 1 [50 mM Tris HCl (pH 7.5), 75 mM NaCl, 5 mM EGTA, and 5 mM MgCl₂]. Sepharose pellets were resuspended in 30 μl dilution buffer 2.

**Fig. 1** Effect of Raf-1 antisense oligonucleotides ISIS 5132 and ISIS 13650 and the mismatch oligonucleotide ISIS 16971 on cell growth in a panel of ovarian cancer cell lines. A and B, ovarian cancer cell lines were treated with (A) ISIS 5132 (200 nM) or ISIS 13650 (200 nM) or (B) ISIS 16971 (200 nM) for 3 h and then counted at 72 h on a cell counter. Cell counts at time 0 (commencement of treatment) were deducted from the 72-h cell count. The value shown is the percentage growth compared with the value obtained from untreated cells grown for 72 h (100%). The data shown represent the mean (±SE) of three independent experiments. C, Raf-1 protein expression in ovarian cancer cell lines. Expression of Raf-1 protein in ovarian cancer cell lines was detected by Western blot analysis. Cells were lysed, and samples were electrophoresed as described in “Materials and Methods.” Blots were probed with anti-Raf-1 antibody. The result from one set of lysates from the panel of cell lines is shown, and this is representative of data obtained from four separate sets of lysates. Actin values for these samples also are shown to indicate loading. D, Raf-1 kinase activity in a panel of ovarian cancer cell lines. Lysates were immunoprecipitated with anti-Raf-1, and subsequent immunoprecipitates were incubated with mitogen-activated protein/extracellular signal-related kinase kinase and extracellular signal-related kinase to phosphorylate myelin basic protein in the presence of [γ-32P]-ATP.
buffer 2 (1 mM DTT and 1 mM NaVO₃ made up in dilution buffer 1) and transferred to a 96-well round-bottomed microtiter plate. Reaction mixture A (0.1 μg MEK, 1 μg ERK, 25 mM MgCl₂, and 0.25 mM ATP made up in 10 μl dilution buffer 2) was added to immunoprecipitates to allow MEK and subsequent ERK phosphorylation. Reaction mixture B [11.5 μg myelin basic protein (MBP) substrate, 2 mM MgCl₂, 0.25 mM ATP, and 3²P-γ-ATP (3000 Ci/mmol; 0.2 μl) made up in 25 μl dilution buffer 2] was added to reaction mixture A for an additional 20 min and incubated to allow MBP phosphorylation. The reaction was stopped by adding SDS-PAGE sample buffer (25 μl), and the samples were run on a 12% SDS-PAGE gel before exposing to Hyperfilm ECL (Amersham) overnight at −70°C.

For experiments exploring the effect of TGF-α on Raf kinase activity, the same conditions as for Western analysis were used, except that a 5-min time point was selected because preliminary experiments had shown increases in kinase activities to be maximal at this time point.

**RESULTS**

Raf-1 ASOs Inhibit the Growth of Ovarian Cancer Cell Lines and Markedly Reduce Raf-1 Protein Expression.

The ability of Raf-1 ASOs to inhibit cellular growth was demonstrated with ISIS 5132 (first-generation oligonucleotide) and ISIS 13650 (second-generation oligonucleotide) in a panel of 15 ovarian cancer cell lines (Fig. 1A). All of the cell lines were growth inhibited by both oligonucleotides but to differing degrees. Whereas cell lines such as SKOV-3 and OAW42 were growth inhibited by ≥90%, cell lines such as OVCAR5 and CAOV3 were inhibited by only 10–40%. The control mismatch ODN ISIS 16971 produced minimal effects on growth (<20% inhibition) in this series of cell lines; examples are shown in Fig. 1B. In cells grown under similar culture conditions, the extent of growth inhibition was not simply associated with either Raf-1 protein expression (Fig. 1C) or with Raf-1 kinase activity (Fig. 1D).
Treatment with either ISIS 5132 or ISIS 13650 produced marked inhibition (≥75%) of Raf-1 protein in all of the cell lines (Fig. 2A), whereas treatment with a mismatch oligonucleotide had a minimal effect (<15% inhibition; Fig. 2B). This effect was concentration dependent and accompanied by a concomitant decrease in the number of cells in G0-G1 phases. Treatment with mismatch ISIS 16971 had no effect on the cell cycle distribution at concentrations <200 nM (data not shown). Only minimal changes in cell cycle distribution were demonstrated in PE01 cells, and no effects were observed in OVCAR5 cells (Fig. 2A and B).

PARP cleavage (indicative of late-stage apoptosis) was observed in SKOV-3 cells after treatment with ISIS 13650 (200 nM). This ASO also produced a low level of cleavage in PE01 cells but none in OVCAR5 cells (Fig. 3C). These data indicate that Raf-1 ASO growth inhibition in SKOV-3 cells is associated with arrest in S and G2-M phases and with increased apoptosis.

**Raf-1 ASO Uptake Is Similar in Growth-Sensitive and Growth-Resistant Cell Lines.** To investigate whether the diversity in growth response might result in part from differential

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Fig. 2 Anti-Raf-1 ODNs can cause alterations in the cell cycle distribution and induce late-stage apoptosis. A and B, SKOV-3, PE01, and OVCAR5 cells were treated with ISIS 5132 (A) or ISIS 13650 (B; 50–200 nM), and cell cycle analysis was performed by flow cytometry. Forty-eight h after treatment with either oligonucleotide, cells were trypsinized and stained for total DNA content using propidium iodide. Cell cycle distribution was undertaken using a FACS Calibur flow cytometer, and results were analyzed using Modfit software. The relative proportions of cells in the G0-G1, S, and G2-M phases of the cell cycle are shown. Mean percentage values of triplicate groups are shown. C, effect of ISIS 13650 on poly(ADP-ribose) polymerase (PARP) cleavage. Cells were treated for 3 h with 200 nM ISIS 13650, and PARP cleavage was assessed 48 h after initiation of treatment by Western blot analysis as described in “Materials and Methods.” Apoptosis was indicated by cleavage of full-length PARP (M, 116,000) to the M, 85,000 fragment. Data shown are representative of two independent experiments.

Fig. 3 Anti-Raf-1 ODNs can cause alterations in the cell cycle distribution and induce late-stage apoptosis. A and B, SKOV-3, PE01, and OVCAR5 cells were treated with ISIS 5132 (A) or ISIS 13650 (B; 50–200 nM), and cell cycle analysis was performed by flow cytometry. Forty-eight h after treatment with either oligonucleotide, cells were trypsinized and stained for total DNA content using propidium iodide. Cell cycle distribution was undertaken using a FACS Caliber flow cytometer, and results were analyzed using Modfit software. The relative proportions of cells in the G0-G1, S, and G2-M phases of the cell cycle are shown. Mean percentage values of triplicate groups are shown. C, effect of ISIS 13650 on poly(ADP-ribose) polymerase (PARP) cleavage. Cells were treated for 3 h with 200 nM ISIS 13650, and PARP cleavage was assessed 48 h after initiation of treatment by Western blot analysis as described in “Materials and Methods.” Apoptosis was indicated by cleavage of full-length PARP (M, 116,000) to the M, 85,000 fragment.

Treatment with either ISIS 5132 or ISIS 13650 produced marked inhibition (≥75%) of Raf-1 protein in all of the cell lines (Fig. 2A), whereas treatment with a mismatch oligonucleotide had a minimal effect (<5% inhibition; Fig. 2B). The Raf-1 protein knockout with ISIS 5132 and ISIS 13650 was concentration dependent; examples are shown in Fig. 2C.

**Raf-1 ASOs Affect Cell Cycle Distribution and Induce Apoptosis.** Three cell lines were selected for more detailed studies: SKOV-3 (the most sensitive cell line), OVCAR5 (the least sensitive line), and PE01 (intermediate in its sensitivity). Following ASO treatment, SKOV-3 cells treated with ISIS 5132 and ISIS 13650 showed an increase in the proportion of cells in the G2-M and S phases of the cell cycle (Fig. 3, A and B). This effect was concentration dependent and accompanied by a concomitant decrease in the number of cells in G0-G1 phases. Treatment with mismatch ISIS 16971 had no effect on the cell cycle distribution at concentrations <200 nM (data not shown). Only minimal changes in cell cycle distribution were demonstrated in PE01 cells, and no effects were observed in OVCAR5 cells (Fig. 3, A and B).

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Fig. 4 Uptake of FAM-labeled ISIS 5132 in ovarian cancer cells. A, SKOV-3 and OVCAR5 cells were treated with FAM-labeled ISIS 5132 (50 nM and 100 nM) for 1–3 h and analyzed by flow cytometry. Mean fluorescence was assessed in triplicate groups of cells, and the mean (±SD) percentage of cells exhibiting positive staining is shown. B, incorporation of FAM-labeled ISIS 5132 in SKOV-3 cells. FAM-labeled ISIS 5132 is shown as red and clearly localizes to the cell nucleus.
uptake of oligonucleotide, FAM-labeled ISIS 5132 was used to monitor oligonucleotide entry into the SKOV-3 (most-sensitive) and OVCAR5 (most-resistant) cell lines. After a 1-h exposure to FAM-5132, 92% of SKOV-3 cells demonstrated uptake, increasing to 99% after 3 h (Fig. 4A). Although incorporation into OVCAR5 cells was slower, ~80% of cells were positive at 3 h (Fig. 4A). Fluorescence microscopy demonstrated that oligonucleotide was localized in the nucleus of SKOV-3 and OVCAR5 cell lines (Fig. 4B).

Raf-1 ASO Growth Response Is Associated with the Proportion of Raf-1 mRNA Expression. The mRNA expression levels of A-Raf, B-Raf, and Raf-1 were quantified in the series of cell lines. Expression levels varied, but all of the cell lines possessed measurable levels of mRNA for the three Raf isoforms, albeit in differing ratios (Fig. 5, A–C). No simple correlation was found between Raf-1, A-Raf, or B-Raf mRNA levels and growth response to ASO. However, when the proportion of Raf-1 mRNA relative to the total Raf mRNA content...
(Raf-1 + A-Raf + B-Raf) was considered, an association between growth response to ISIS 5132 was observed. A comparison using five of the seven most sensitive cell lines (PE06 and PE016 were not investigated) revealed that these lines had a higher median proportion of Raf-1 than the eight least sensitive cell lines (Fig. 5D; \( P = 0.019 \), Mann-Whitney nonparametric \( U \) test). This would be consistent with the view that all of the three forms of Raf are signaling, and the Raf-1 ASO knockout of Raf-1 mRNA (and thereby protein) will be most effective in those cell lines where Raf-1 is the dominantly expressed form.

**Raf-1 ASO Knockout Reduces ERK Phosphorylation in SKOV-3 but not in OVCAR5 Cells.** To demonstrate that the Raf/MEK/ERK pathway was functional and intact in these cell lines, TGF-\( \alpha \) was added to the SKOV-3 and OVCAR5 cell lines, and the effects on Raf-1 and MEK and ERK phosphorylation were assessed by Western blot analysis (Fig. 6A). TGF-\( \alpha \) activation of this pathway stimulated a Raf-1 band shift (consistent with phosphorylation) and increased phosphorylation of MEK and ERK in both cell lines.

Whereas ISIS 5132 and ISIS 13650 reduced Raf-1 expression in SKOV-3 and OVCAR5 cell lines, they reduced phosphorylation of ERK in SKOV-3 but not in OVCAR5 cells (Fig. 6B). This may reflect preferential dependency on Raf-1 (as opposed to other Raf isoforms) in SKOV-3 cells compared with OVCAR5 cells. This possibility was investigated by using a TGF-\( \alpha \) stimulus and immunoprecipitating A-Raf, B-Raf, and Raf-1 separately in the presence of MEK and ERK to phosphorylate MBP. Under these conditions, SKOV-3 cells demonstrated preferential activation of Raf-1, whereas OVCAR5 cells predominantly used B-Raf (Fig. 6C).

**Induction of Raf-1 Kinase Activity Is Associated with Growth Inhibition after Raf-1 Antisense Treatment.** To assess whether greater use of Raf-1 was indicative of cell lines that were more sensitive to Raf-1 ASO, the effect of TGF-\( \alpha \) stimulation on Raf-1 kinase was correlated with the degree of growth inhibition following ASO treatment. The four cell lines demonstrating the greatest growth inhibition (SKOV-3, PE06, PE016, and OAW42) showed a more than threefold increase in Raf-1 kinase activity after TGF-\( \alpha \) treatment. Across the panel, growth inhibition was significantly greater in cell lines that demonstrated a twofold or greater increase in Raf-1 kinase activity after TGF-\( \alpha \) stimulation compared with those that showed a lesser effect (\( P = 0.04 \), Mann-Whitney nonparametric \( U \) test; Fig. 7).

**DISCUSSION**

In this study, we have sought to characterize features that determine growth sensitivity to anti-Raf-1 ASOs in ovarian cancer. We are not aware of any previous study that has asso-
associated growth response to antisense targeting in a predictive manner. The use of a series of cell line models has demonstrated that growth response was not uniform because certain cell lines were growth inhibited completely, whereas others showed relatively minor (<40%) inhibition. Several features may explain this diversity. First, uptake of oligonucleotide may vary across the panel of cell lines, and if uptake is poor, insufficient ODN may be available to interact with target Raf-1 mRNA. Alternatively, the growth response may be only partially dependent on (or completely independent of) the Raf-1 pathway, such that its blockade may have little or no effect on growth response.

Comparison of SKOV-3 cells (which are growth inhibited by Raf-1 ASO) and OVCAR5 cells (which are relatively insensitive) suggested that although there may be some differences in the rate of oligonucleotide uptake between the two lines, not only did the majority of cells show incorporation after 3 h but also oligonucleotide accumulated in the nucleus. Although there was some minor difference between the two cell lines in the number of cells demonstrating incorporation, Raf-1 protein expression was markedly depleted (>75%) in both these lines and in all of the cell lines examined, suggesting that the targeting of Raf-1 mRNA and subsequent reduction of Raf-1 protein synthesis were not limiting. Inhibition of Raf-1 expression was marked with most cell lines, demonstrating >80% reduction of expression. Growth inhibition was associated with enhanced apoptosis and blockade of cells in the S and G2-M phases of the cell cycle.

Because uptake was not the key determinant for growth response, it seemed likely that it was the dependence of the cells on the Raf-1 pathway for growth that was limiting. Simple association with the expression of total Raf-1 protein or the level of Raf-1 kinase did not explain the varying growth response. In a parallel study, we have demonstrated that all of these cell lines express not only Raf-1 but also A-Raf and B-Raf proteins, and all three may be involved in signaling as indicated by their increased Raf kinase activities on growth factor addition.3 Although the use of Western blot analysis did not allow for quantitative comparisons, it was feasible to quantify relative amounts of mRNA for the three Raf isoforms. Although growth response to anti-Raf-1 ASO did not correlate directly with the expression of Raf-1 mRNA, when the expression of Raf-1 as a proportion of total Raf was considered, a significant association was noted. This would be consistent with the possibility that all three isoforms of Raf contribute to signaling; therefore, inhibition of Raf-1 production would have greatest impact in those cell lines in which it was the predominantly expressed form. In SKOV-3 and OVCAR5 cells, TGF-α stimulated the Ras/Raf/MEK/ERK cascade, resulting in phosphorylation of MEK and ERK. In addition, TGF-α produced a band shift in Raf-1, which is consistent with a change in phosphorylation status, although given the multiplicity of phosphorylation sites in Raf-1, some of which are associated with activation but others with inhibition, this does not necessarily imply activation (18, 19). Whereas ISIS 13650 could reduce Raf-1 expression in SKOV-3 and OVCAR5 cell lines, phospho-ERK was reduced only in

Fig. 7 Transforming growth factor α (TGF-α) stimulation of Raf-1 kinase activity and its association with ISIS 5132 growth response. A, TGF-α stimulation of Raf-1 kinase in ovarian cancer cell lines. Cell lines were stimulated with TGF-α (1 nm) for 5 min, and lysates were collected. Lysates were immunoprecipitated with anti-Raf-1 antibody, and subsequent immunoprecipitates were used in a two-step kinase assay to phosphorylate mitogen-activated protein/extracellular signal-related kinase (MEK) and extracellular signal-related kinase (ERK) and then myelin basic protein (MBP) in the presence of 32P-γ-ATP. The values shown are fold-increases in the Raf-1 kinase activity in the presence of TGF-α compared with that in its absence. B, association between TGF-α stimulation of Raf-1 kinase activity and growth response to ISIS 5132. Cell lines demonstrating a twofold or greater increase in Raf-1 kinase activity after TGF-α treatment had significantly higher growth inhibitions than cell lines with less than twofold activation (P = 0.04, Mann-Whitney nonparametric U test).

3 Unpublished observations.
SKOV-3 cells. One explanation would be that Raf-1 was the dominant activator of MEK and ERK in SKOV-3 cells but not in OVCAR-5 cells. This possibility was explored by immuno-precipitating individual isoforms of Raf in these cell lines where activation by TGF-α indicated that although Raf-1 activity markedly increased in SKOV-3 cells relative to A-Raf or B-Raf activation, it was B-Raf activity that was the most markedly increased in OVCAR-5 cells. These results suggest that Raf-1 signaling in OVCAR5 cells is not critical for growth. Additional investigation of Raf-1 kinase activity following TGF-α activation across the panel of cell lines indicated that those cell lines with a greater than twofold increase in Raf-1 kinase activation after addition of TGF-α demonstrated a significantly greater growth inhibition by anti-Raf-1 ASO treatment. This again is consistent with the view that the impact of the ASO on growth will be greatest in cell lines where Raf-1 is used most.

In summary, Raf-1 ASO treatment not only markedly reduces Raf-1 protein expression but also results in cellular growth inhibition. These effects vary from cell line to cell line, a phenomenon that is likely to be paralleled in patients undergoing clinical trials. Such differences are not simply the result of incomplete oligonucleotide transfer and subsequent protein reduction. Determination of A-Raf, B-Raf, and Raf-1 mRNA levels revealed that the proportion of Raf-1 mRNA relative to the total of all three helps predict cell lines responsive to a Raf-1 ASO approach. Furthermore, the degree of Raf-1 kinase activity following growth factor stimulation also was informative. These characteristics could be measured in primary ovarian cancers and might provide a useful insight as to which patients are likely to respond to treatment in a clinical setting. These observations suggest a subgroup worth targeting in future clinical studies.

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