Telomere-Homologous G-Rich Oligonucleotides Sensitize Human Ovarian Cancer Cells to TRAIL-Induced Growth Inhibition and Apoptosis

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G-rich T-oligos (GT-oligos; oligonucleotides with homology to telomeres) elicit a DNA damage response in cells and induce cytotoxic effects in certain tumor cell lines. We have previously shown that GT-oligo inhibits growth, arrests cell cycle, and induces apoptosis in ovarian, pancreatic, and prostate cancer cells. However, not all ovarian cancer cell lines are susceptible to GT-oligo exposure. GT-oligo was found to induce transcript expression of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors DR-4 and DR-5, which are generally silenced in ovarian cancer cells, rendering them insensitive to TRAIL. Exposure of TRAIL- and GT-oligo-resistant cell lines to GT-oligo rendered them sensitive to the cytotoxic effects of TRAIL, producing more than additive inhibition of growth. An intracellular inhibitor of the extrinsic apoptotic pathway, FLICE-like Inhibitory Protein-Short (FLIP_s), was down-regulated and Jun kinase (JNK) was activated by exposure to GT-oligo. JNK inhibition partially reversed the growth inhibition caused by the combination of GT-oligo and TRAIL indicating partial involvement of the Jun kinase pathway in the resulting cytotoxic effect. Both caspase-8 and caspases 3/7 were activated by exposure to GT-oligo plus TRAIL, consistent with activation of the extrinsic apoptotic pathway. These results demonstrate a novel way of sensitizing resistant ovarian cancer cells to TRAIL-mediated cytotoxicity.

Introduction

CONVENTIONAL TREATMENT OF ovarian epithelial cancer includes surgery and systemic chemotherapy. Although 70% of ovarian cancer patients with advanced disease initially respond to platinum- or taxane-based chemotherapy, the majority experience recurrence and overall survival rate is very poor (Liu and Matulonis, 2010). Current chemotherapy is non-specific and relatively toxic, producing unwanted side effects. Thus, more effective and more targeted therapies are urgently needed.

It has been previously shown that oligonucleotides homologous with the repeated sequence of telomeres (T-oligos) appear to exert selectively cytotoxic effects on malignant cells compared with their normal, non-transformed counterparts *in vitro* and *in vivo*. Treatment of melanoma or human breast cancer xenografts, or murine lymphomas, by systemic injection of various types of T-oligos resulted in reduced tumorigenicity and metastatic potential (Puri et al., 2004; Yaar et al., 2007; Longe et al., 2009). We have recently shown that a GC-rich variant of T-oligo, designated "GT-oligo," is more efficient than the parental T-oligo at inhibiting growth and

inducing apoptosis in selected ovarian cancer cell lines (Sarkar and Faller, 2011), although certain other ovarian cancer cell lines showed variable resistance to GT-oligo, with human ovarian carcinoma cell line SKOV-3 cells being the most resistant. We observed that SKOV-3 cells could be sensitized to GT-oligo by exposure to histone deacetylase (HDAC) inhibitors (Sarkar and Faller, 2011). This result raised the possibility that T-oligos may be more broadly useful for the treatment of epithelial cancers when used in combination with other anti-cancer therapeutics, rather than as single agents.

We initiated the current study to explore the mechanism of GT-oligo-induced apoptosis in oligo-sensitive ovarian cancer cells. We observed that GT-oligo, in addition to inhibiting cell cycle progression and activating caspase-9 (Sarkar and Faller, 2011), also induced expression of the tumor necrosis factor (TNF) family of death receptors (TNFRs). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is the ligand for the TNFRs, and TRAIL and TRAIL-related agonists are under clinical investigation for many forms of malignancies, particularly leukemias (Wiley et al., 1995; Pitti et al., 1996). TRAIL-based approaches, however, are not generally effective against solid tumors, including ovarian cancers. Ovarian

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cancer cells in particular lack expression of most of the TNF death receptors and thus are not responsive to TRAIL treatment. In this study, we report that exposure to GT-oligo renders ovarian cancer cell lines susceptible to TRAIL, and that the combination of GT-oligo and TRAIL inhibited growth of ovarian cancer cell lines that were resistant to GT-oligo or TRAIL alone.

Materials and Methods

Reagents

The GT-oligo sequence was 50-p-GGTTGGTTGGTTGGTT-30. GT-oligo was obtained from Midland Certified Reagent. The oligonucleotides were purified from the trityl group by gel filtration chromatography. Purity was >98% for all oligonucleotides. The caspase-9 and caspase-3 activity measurement kit was from Promega. The apoptosis gene array plate was from SA Biosciences. Complementary DNA (cDNA) preparation kit was from Qiagen (SA Biosciences). SYBR green real-time quantitative polymerase chain reaction (qPCR) mix was from Applied Biosciences. Primers were from Invitrogen. Fluorescein isothiocyanate (FITC)-conjugated death receptor (DR)-4 and DR-5 antibodies were from Novus Biologicals. Other reagents were purchased from Sigma.

Cell culture

The human ovarian cancer cell lines PA1, CAOV-3, and SKOV-3 were obtained from ATCC. CAOV-3 and SKOV-3 cells were grown in RPMI 1640 media (Invitrogen) containing 5 mL penicillin/streptomycin and 10% heat-inactivated fetal bovine serum. PA1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells at 70%–80% confluence were exposed to either vehicle (water or dimethyl sulfoxide) or various concentrations of reagents. Cells were incubated for different times before processing, as described in the figure legends.

Apoptosis gene array analysis

PA1 ovarian cancer cells were treated with GT-oligo at the indicated concentrations for 24 hours. Total RNA was isolated from both treated and untreated cells and cDNA were prepared. Quantitative PCR (qPCR) was performed after adding prepared cDNA to the gene array plates according to manufacturer's protocol. Data from treated and untreated cells were uploaded to the provider of the array website, which calculated the up- or down-regulation of apoptosis related genes.

Cell survival assays

Viable cell enumeration was performed using a trypan blue exclusion assay in 6-well plates. Cells were trypsinized and 20 mL cell suspensions were mixed with 20 mL trypan blue, and viable cells (dye-excluding cells) were enumerated. The percentages of viable cell counts were plotted against time of incubation. An increase in viable cell counts over time provides a measure of growth.

Flow cytometric assays

For cell cycle analyses, cells were treated with different agents for the indicated times and stained with propidium

iodide (PI) as described elsewhere and then analyzed for DNA content by flow cytometry. Briefly, cells were washed with phosphate-buffered saline (PBS), fixed in the medium containing 35% ethanol for 5 minutes at room temperature, and stained for 30 minutes in the dark with PI 25 mg/mL and RNase 50 mg/mL in phosphate-buffered saline, before flow cytometric analysis. For cell surface protein expression analysis, cells were treated with GT-oligo at 10 μ M for 72 hours, harvested, and resuspended in ice-cold PBS, stained with FITC-conjugated DR-4 or DR-5 antibodies at 5 μ g/mL for 30 minutes on ice, washed twice with cold PBS, and resuspended in 1 mL PBS before flow cytometric analysis.

Caspase-3/7 and caspase-9 assays

Cells were treated with GT-oligo for 72 hours and lysed, and protein was quantitated. One microgram of protein in each sample was incubated with the synthetic substrate in the assay buffer in a volume of 200 μ L for 1 hour. The substrate containing assay buffer served as a blank control. Emitted light was quantitated in a luminometer. The results were presented in arbitrary units and were the average of 3 independent assays (Sarkar and Faller, 2011).

qPCR

Total RNA was prepared from cells by Trizol reagent (Invitrogen) according to manufacturer's protocol. Real-time qPCR analysis was performed with the cDNA prepared for quantitation of mRNA transcripts. The results were normalized with the expression level of β -actin transcript in each sample. The PCR was performed in triplicate for each sample. The means are presented with the standard deviations. Sequences of primers: DR-6, forward primer 5'-CCA CAG CTC AGC CAG AAC AG-3', reverse primer 5'-CGG TGG CAC GGT CAA CAT-3'; DR-4, forward primer 5'-CTGAGCAA CGCAGACTCGCTGTCCAC3', reverse primer 5'-TCCAAGG ACACGGCAGAGCCTGTGCCAT3'; DR-5, forward primer 5'-CCTCATGGACAATGAGATAAAGGGTGGCT3', reverse primer 5'-CCAAATCTCAAAGTACGCACAAACGG3'; FLIP_L, forward primer 5'-AATTCAAGGCTCAGAAGCGA3', reverse primer 5'-GGCAGAACTCTGCTGTTC3'; FLIP_S, forward primer 5'-GGCCGAGGCAAGATAAGCAAGG3', reverse primer 5'-GCGCGGTACCTCACATGGAACAATTT CCAAG3'. Annealing temperatures of all primers were 60°C. All other conditions were as previously described (Sarkar and Faller, 2011).

Statistical methods

For statistical analysis a standard Student's *t*-test was performed.

Results

Gene expression profiling after exposure to GT-oligo

The 16-mer "GT-oligo" (5'-p-GGTTGGTTGGTTGGTT-3') has recently been shown to exert anti-proliferative and cytotoxic activity against ovarian cancer cell lines (Sarkar and Faller, 2011). Exposure to GT-oligo inhibits cell growth and induces apoptosis of ovarian cancer cells to different extents, depending upon the particular cell line (Sarkar and Faller, 2011). To begin to elucidate the mechanisms underlying the

differential sensitivities of ovarian cancer cells to GT-oligo, we examined the changes in the gene expression patterns of regulators of apoptosis after exposure to GT-oligo. The GT-oligo-sensitive ovarian cancer cell PA1 was exposed to GT-oligo at 20 μ M for 24 hours. Analysis of cDNA generated from total RNA was performed using a gene array plate for apoptosis-related genes. Significant up-regulation of 2 apoptosis-related genes, tumor necrosis factor receptor superfamily member 21 (*TNFRSF21*) and *CD-70*, was observed (Table 1). *TNFRSF21* is an orphan receptor, also called DR-6, and a member of the family of TNFR receptors/death receptors, which also includes DR-4 and DR-5. These latter receptors can initiate the extrinsic pathway of apoptosis after binding of ligand. TRAIL is an established ligand for DR-4 and DR-5 (Camidge, 2008; Hotte et al., 2008). *CD-70* is involved the development of hematopoietic and lymphoid cells, can promote apoptosis of lymphocytes after binding to CD-27, and its overexpression by tumor cells may promoter evasion of immune surveillance. [The apparent down-regulation of the X-linked inhibitor of apoptosis protein (XIAP) inhibitor of apoptosis in the array studies was not confirmed in subsequent independent qPCR assays. GT-oligo treatment produced little reduction in the *XIAP* transcript levels, with a nonsignificant *P* value of 0.3727.]

Expression of death receptors in human ovarian cancer cell lines

To validate the results from the array analysis, we examined the transcript expression of orphan death receptor DR-6 in SKOV-3 cells after exposure to GT-oligo at 10 μ M for 48 hours. DR-6 transcript expression was enhanced by GT-oligo exposure (Fig. 1A). To determine the extent of DR induction by GT-oligo, we further investigated the expression profile of the other two death receptors, DR-4 and DR-5, in the GT-oligo-resistant SKOV-3 and CAOV-3 ovarian cancer cell lines after exposure to GT-oligo (10 or 20 μ M) for 48 hours. Both *DR-4* and *DR-5* (also called *TR1* and *TR2*) transcript expression increased in a dose-dependent manner in both SKOV-3

and CAOV-3 ovarian cancer cell lines by exposure to GT-oligo (Fig. 1B, C, D, E). To determine whether changes in transcript expression corresponded with protein expression of death receptors, we analyzed the cell surface expression of DR-4 and DR-5 in SKOV-3 cells after exposing them to GT-oligo for 48 hours. Flow cytometric analysis after staining with anti-DR-4 and DR-5 antibodies revealed a consistent increase in cell surface expression of DR-4, and to a lesser extent, DR-5 (Fig. 2A). Analysis of CAOV-3 cells revealed that DR-5 surface expression was increased by exposure to GT-oligo (Fig. 2 B).

Effects of GT-Oligo and TRAIL on the growth of human ovarian cancer cell lines

Exposure to GT-oligo produces variable anti-proliferative effects against different lines of ovarian cancer cells (Sarkar and Faller, 2011). While PA1 cells are sensitive and CAOV-3 and OVCAR-3 cells are moderately resistant, SKOV-3 cells are very resistant to GT-oligo as a single agent. Furthermore, all of these lines are resistant to TRAIL (Viganti et al., 2002; Park et al., 2009), as has been previously reported for ovarian cancer cells and proposed to be secondary to low or inadequate expression of death receptors. The re-expression of TNFR receptors by exposure to GT-oligo raised the possibility that GT-oligo may sensitize these cells to TRAIL.

SKOV-3 or CAOV-3 ovarian cancer cells were exposed to GT-oligo and TRAIL, alone or in combination. The results confirmed the resistance of the cell lines to GT-oligo and TRAIL as single agents, as they only produced a modest effect (GT-oligo) or no effect (TRAIL) on cell number in SKOV-3 or CAOV-3 lines (Fig. 3A, B). The combination, however, produced significant more-than-additive decreases in cell number of the SKOV-3 cell line ($P=0.0001$ compared with control, $P=0.006$ compared with GT-oligo alone) (Fig. 3A). Parallel studies in CAOV-3 cells showed similar effects of the combination of GT-oligo and TRAIL ($P=0.0001$ compared with control, $P=0.0039$ compared with GT-oligo alone) (Fig. 3B). SKOV-3 ovarian cancer cells were almost completely resistant to TRAIL even at very high (40 ng/mL) concentrations (Fig. 3C).

TABLE 1. APOPTOSIS GENE EXPRESSION ANALYSIS AFTER TREATING PA1 OVARIAN CANCER CELLS WITH G-RICH T-OLIGOS

Genes up-regulated	Fold change	Genes up-regulated	Fold change	Genes down-regulated	Fold change
<i>ABL1</i>	6.83	<i>TNFRSF10B</i>	1.11	<i>BCL10</i>	-1.02
<i>AKT1</i>	1.73	<i>TNFRSF1A</i>	1.3	<i>BCL2A1</i>	-1.27
<i>APAF1</i>	1.19	<i>TNFRSF21</i>	84.99	<i>BCLAF1</i>	-1.02
<i>BAD</i>	1.1	<i>CD27</i>	1.33	<i>BIK</i>	-1.02
<i>BAG1</i>	1.25	<i>TNFRSF9</i>	1.24	<i>XIAP</i>	-429.59
<i>BAG3</i>	1.14	<i>TNFSF10</i>	2.02	<i>CASP1</i>	-2.1
<i>BAG4</i>	1.07	<i>CD70</i>	3.25	<i>CASP4</i>	-1.38
<i>BAK1</i>	1.36	<i>TNFSF8</i>	1.18	<i>CASP8</i>	-1.08
<i>BAX</i>	1.12	<i>TRADD</i>	1.05	<i>FADD</i>	-2.47
<i>BCL2</i>	1	<i>TRAF2</i>	1.12	<i>FAS</i>	-67.73
<i>BCL2L11</i>	1.39	<i>TRAF3</i>	1.09	<i>LTA</i>	-1.29
<i>BCL2L2</i>	1.48	<i>TRAF4</i>	1.6	<i>PYCARD</i>	-2.22
<i>BFAR</i>	1.91			<i>TNFRSF25</i>	-38.96
<i>BID</i>	1.29			<i>TP53BP2</i>	-1.21

PA1 ovarian cancer cells were exposed to G-rich T-oligos at 20 μ M for 24 hours. Total RNA was isolated from both oligo-treated and vehicle-treated cells and complementary DNA (cDNA) was prepared. Quantitative PCR was performed after adding prepared cDNA to the gene array plates. Relative transcript expression levels are shown.

FIG. 1. Effect of GT-oligo exposure on death receptor expression: SKOV-3 ovarian cancer cells (**A, B, C**) and CAOV-3 ovarian cancer cells (**D, E**) were exposed to GT-oligo at 10 μ M or 20 μ M concentrations, for 48 hours. Real-time quantitative polymerase chain reaction was performed and the results are expressed as relative transcript expression compared with the untreated control cells. The results were normalized to β -actin transcript expression levels in each sample. (**A**) Death receptor (DR)-6 transcripts in SKOV-3 cells; (**B**) DR-4 transcripts in SKOV-3 cells; (**C**) DR-5 transcripts in SKOV-3 cells; (**D**) DR-4 transcripts in CAOV-3 cells; (**E**): DR-5 transcripts in CAOV-3 cells. Panel A: * $P=0.008$ compared with control. Panel B: * $P=0.0648$ compared with control; ** $P=0.003$ compared with control. Panel C: * $P=0.104$ compared with control; ** $P=0.0104$ compared with control. Panel D: * $P=0.001$ compared with control; ** $P=0.001$ compared with control. Panel E: * $P=0.001$ compared with control; ** $P=0.0001$ compared with control.

Effects of GT-oligo and TRAIL on apoptosis

Our previous study showed that exposure to GT-oligo induces the activity of caspase-9 and caspase-3 in the oligo-sensitive ovarian cancer cell line PA1, and that it induces

apoptosis (Sarkar and Faller, 2011). Caspase-9 activation is regulated by mitochondrial permeability and v-akt murine thymoma viral oncogene homolog 3 (AKT) inhibition, and comprises in part the intrinsic pathway of apoptosis. In contrast, TRAIL-induced apoptosis is mediated by death

FIG. 2. Ovarian cancer cells were exposed GT-oligo at 10 μ M for 72 hours, harvested, fixed, and stained with FITC-conjugated DR-4 or DR-5 antibodies. After flow cytometric analysis, the histograms of DR-4 and DR-5 from GT-oligo-treated cells and vehicle-treated cells were superimposed. (**A**) Results from SKOV-3. (**B**) Results from CAOV-3 cells.

FIG. 3. Effects of GT-oligo and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on the growth of SKOV-3 and CAOV-3 ovarian cancer cells: SKOV-3 (**A**) and CAOV-3 (**B**) cells were treated for 96 hours with G-rich T-oligo (GT-oligo) at 10 μ M, TRAIL at 20 ng/mL, or with the combination of GT-oligo (10 μ M) and TRAIL (20 ng/mL). Live cells were enumerated. The number of % live cells in vehicle-treated cultures was normalized to 100%. (**C**) A dose-response experiment with SKOV-3 ovarian cancer cells was performed by exposing the cells to TRAIL at 20 or 40 ng/mL for 96 hours. Results were expressed as percentage of live cells at that time compared with control, as described in **A** and **B**. Panel A: * $P=0.0001$ compared with control; ** $P=0.006$ compared with GT-oligo alone. Panel B: * $P=0.0001$ compared with control; ** $P=0.0039$ compared with GT-oligo alone.

receptors and the extrinsic pathway utilizing caspase-8. To determine whether the extrinsic pathway is also operative during exposure to the combination of GT-oligo and TRAIL, we investigated the activation of caspase-8 in SKOV-3 cells. Activation of caspase-8 was significantly greater in response to the combination of agents, compared with the individual agents (Fig. 4A). Caspase 3/7 activation is a final common effector of both the extrinsic and intrinsic pathways of apoptosis. Caspase 3/7 activation was also more pronounced for the combination of agents (Fig. 4B). The induction of caspase 8 and caspase 3/7 activity induced by the combination of TRAIL plus GT-oligo compared to the control or the single agents alone was significant ($P=0.01$).

To further characterize the involvement of the extrinsic pathway in this process, the expression of FLIP_L and FLIP_S proteins was analyzed. FLIP proteins are inhibitors of the extrinsic pathway of apoptosis (Chawla-Sarkar et al., 2004). Down-regulation of FLIP_S can enhance TRAIL-mediated apoptosis, and conversely, up-regulation of FLIP_S inhibits death receptor-mediated apoptosis. GT-oligo exposure down-regulated FLIP_S in SKOV-3 cells (Fig. 4C). The decrease in FLIP_S transcript level was highly significant ($P=0.0001$).

The activation of TNF receptors, the activation of caspase-8, the down regulation of FLIP_S, and the more-than-additive effects of GT-oligo plus TRAIL on the growth inhibition of resistant ovarian cancer cells collectively suggested that the cytotoxic effect is mediated at least partially by downstream effectors of the death receptor pathway. JNK is one of the downstream apoptotic effectors of the TNF death receptors. Immunoblot analysis of lysates prepared from untreated and GT-oligo-treated SKOV-3 cells showed that exposure to GT-oligo resulted in phosphorylation (activation) of JNK (Fig.

4D), which was inhibited by JNK inhibitor SP60025. To determine a functional role for JNK activation in the actions of the agents, the effects of JNK inhibition on the cytotoxicity induced by the combination of TRAIL and GT-oligo was studied. The effect of the combination of GT-oligo plus TRAIL compared to the control was significant ($P=0.0019$). Pretreatment of cells with a JNK inhibitor SP60025 partially reversed the cytotoxic effects of the combination of GT-oligo plus TRAIL ($P=0.026$) (Fig. 4E), suggesting a significant contribution from JNK-mediated apoptotic signaling.

Discussion

We have previously demonstrated the susceptibility of multiple human ovarian cancer cell lines to 2 distinct T-oligo sequences (T-oligo and GT-oligo) and have shown the enhanced activity of these T-oligos in combination with a new class of targeted tumor agents, the histone deacetylase (HDAC) inhibitors. We and others have also shown the safety and activity of T-oligos in animal models (Puri et al., 2004; Yaar et al., 2007; Longe et al., 2009). These T-oligos may therefore hold potential as therapeutics, either as single agents or in combination regimens, for the treatment of ovarian cancer.

Importantly, our molecular characterization of the mechanisms underlying this cytotoxic activity demonstrated that the actions of these T-oligos on eliciting a DNA-damage-like response is not dependent upon complete telomere homology, nor is the cytotoxic activity mediated through telomere-protective mechanisms. We proposed that they form G-quadruplexes that mimic DNA damage, initiating signaling characterized by the phosphorylation of the histone variant histone 2AX (H2AX), and the ataxia telangiectasia mutated

FIG. 4. Effects of GT-oligo and TRAIL on caspase-8 and caspases 3/7 and expression levels of FLIP_S, and mitigation of effects on growth by inhibition of JNK. SKOV-3 cells were exposed to vehicle, GT-oligo (10 μ M), TRAIL (20ng/mL), or to the combination of GT-oligo (10 μ M) and TRAIL (20ng/mL) for 96 hours. Cells were harvested and lysed and caspase 8 (**A**) and caspase 3/7 (**B**) assays were performed on the lysates. The induction of caspase 8 and caspase 3/7 activity induced by the combination of TRAIL plus GT-oligo compared with the control or the single agents alone was significant (panel A: * $P=0.004$; panel B: * $P=0.006$). (**C**) SKOV-3 ovarian cancer cells were exposed to GT-oligo at 10 μ M for 48 hours. Results are expressed as relative transcript expression levels compared to the untreated control cells. The results were normalized to β -actin transcript expression levels in each sample. The decrease in FLIP_S transcript level compared to control was highly significant (* $P=0.0001$). (**D**) SKOV-3 cells were exposed for 96 hours to either vehicle or GT-oligo (10 μ M), protein lysates were prepared and equal amount of proteins were resolved in SDS-PAGE, transferred to membranes, and blotted against anti-phospho JNK antibody, stripped, and blotted against anti-JNK antibody. The position of bands corresponding to phospho-JNK (pJNK) and total JNK are indicated. (**E**) SKOV-3 cells were exposed for 96 hours to vehicle, GT-oligo (10 μ M), TRAIL (20 ng/mL), SP60025, the combination of GT-oligo + TRAIL, or the combination of GT-oligo + TRAIL + SP60025. Cells were harvested and enumerated using trypan blue dye exclusion as marker of live cells. Y-axis indicates the percentage of live cells in each treatment group after normalizing to the vehicle-treated control cells, which were assigned a value of 100%. The effect of the combination of GT-oligo plus TRAIL compared with the control (* $P=0.0019$) and compared with the addition of SP60025 (** $P=0.026$) were significant.

(ATM) and checkpoint-kinase 2 (Chk2) kinases, followed by a robust cell cycle arrest and cell line-dependent senescence, apoptosis or autophagy (Rankin et al., 2011). Importantly, we demonstrated that the tumor protein 53 (p53), tumor protein 73 (p73), and ATM status of ovarian carcinoma cells are not critical determinants of responsiveness to T-oligos. This finding is particularly relevant in a potential therapeutic for ovarian cancers, which are often deficient in p53 (Sarkar and Faller, 2011).

One potential limitation to therapeutic application of T-oligos, however, was the variable range of sensitivity or response to T-oligos among different human ovarian cancer lines, with some cells showing high sensitivity (PA1 cells), some lower sensitivity (CAOV-3 cells), while still others (SKOV-3 cells) were completely resistant at concentrations of

T- or GT-oligo, which could be realistically achieved *in vivo*. While this is not unexpected for a targeted therapeutic agent, a broader range of activity would certainly be desirable. Combination of GT-oligo with HDAC inhibitors (HDACi), as mentioned above, did confer sensitivity in a number of the lines resistant to T-oligo as a single agent, but we sought another way of increasing the proportion of ovarian cancers which might be treatable with this novel, targeted therapeutic modality.

In analyzing genes that were differentially regulated in the ovarian cancer cell lines in response to GT-oligos, we found that the genes which were up/down-regulated to the greatest extent were predominantly related to the extrinsic pathway of apoptosis, and more specifically, were genes whose products are related to TRAIL death-receptor signaling. The expression

of TRAIL death receptors DR-4, DR-5 (and also DR-6/TNFRSF-21/TNF-super family receptor 21) transcripts were dramatically increased by exposure of ovarian cancer cells to GT-oligo. We observed coincidental increases in the expression the DR-4 receptor on the surface of SKOV-3 cells, and to a lesser extent on CAOV-3 cells, after exposure to GT-oligo. (A discrepancy between transcript levels and surface protein expression for death receptors is not uncommon. In many types of cancer cells, the lack of death receptor expression on the cell surface [and consequent TRAIL resistance] is the result of differential vesicular transport processes (Zhang et al., 2008).

Since TRAIL was first described more than a decade ago, it has been the focus of extensive cancer-related research owing to its selective induction of apoptosis in malignant cells (Wiley et al., 1995; Pitti et al., 1996). TRAIL kills a variety of tumor cells, but importantly, has no or minimal effects on normal cells because TRAIL death receptors are expressed essentially only in transformed cells. Great excitement for the potential use of TRAIL for ovarian cancers was initially generated, as TRAIL or TRAIL agonists produced significant cytotoxicity in certain ovarian cancer cell lines and xenografts (Zhao et al., 1995; Estes et al., 2007; Duiker et al., 2009). TRAIL activation of the extrinsic apoptotic pathway occurs independently of p53 status, an important issue in ovarian cancer therapy, as many tumors possess p53 mutations, rendering them resistant to chemotherapy (Igney and Krammer, 2002). TRAIL resistance in ovarian malignancies is frequently due to lack of, or loss of, expression of the death receptor DR-4 (and DR-5), in some cases due to hypermethylation (Tomek et al., 2004; Horak et al., 2005; Bae et al., 2008). Hypermethylation of the *DR-4* and *DR-5* promoters correlates with TRAIL resistance in both ovarian cancer cell lines and primary tumors (Bae et al., 2008). However, we found minimal or no methylation of CpG residues in the upstream promoter regions of *DR-4* in either CAOV-3 or SKOV-3 ovarian cancer cells, and methylation levels did not change after exposure to GT-oligo (data not shown). This result indicates that alteration in promoter methylation does not play a role in the re-expression of DR-4 mediated by GT-oligo. Other cases of TRAIL resistance are due to increases in *FLIP* (Hietakangas et al., 2003; Chawla-Sarkar et al., 2004; Cummins et al., 2004; Lane et al., 2007; Bae et al., 2008).

The SKOV-3 and CAOV-3 ovarian carcinoma cells are resistant to GT-oligos (Sarkar and Faller, 2011) and to TRAIL. They express low levels of the TRAIL death receptors DR-4 and DR-5, which is likely the cause of TRAIL resistance (Tomek et al., 2004; Horak et al., 2005). Exposure to GT-oligo sensitized TRAIL-resistant (and T-oligo-resistant) human ovarian carcinoma cells to TRAIL, coincident with the induction of these death receptors. Concentrations of TRAIL, which alone had no effect on SKOV-3 cells, generated a significant cytotoxic effect in the presence of non-cytotoxic concentrations of GT-oligo. This sensitization occurred in the absence of a functional p53 in these cells. [Cytotoxic drugs have sometimes produced some tumor sensitization to TRAIL, but this effect is p53-dependent, and ovarian carcinomas, including SKOV-3 cells, frequently lack functional p53 (Igney and Krammer, 2002)]. One of the downstream effectors of extrinsic apoptosis pathway is activation of JNK. JNK was activated by GT-oligo treatment, as indicated by its phosphorylation. An inhibitor of JNK partially reversed the growth inhibition produced by the combination of GT-oligo

and TRAIL, indicating that the JNK pathway is partially involved in mediating the apoptosis induced by the combination. The partial reversal of growth inhibition indicates that the intrinsic pathway also plays a role in the observed growth inhibition and GT-oligo is involved in this process in addition to its role in inducing DR-4 and DR-5 expression.

Caspase-8 was activated by the combination of GT-oligo and TRAIL in SKOV-3 cells, supporting the mechanism that TRAIL-mediated cell death operates through the extrinsic pathway. The common terminal apoptotic caspases 3/7 were activated by the combination. Resistance to TRAIL in ovarian cancers can also be conferred by over-expression of the FLIP_S and XIAP proteins (Lane et al., 2007). Exposure to GT-oligo markedly downregulated the transcript expression of FLIP_S. The relative contribution of this activity on FLIP compared with the induction of death receptor expression in sensitizing the tumor cells to TRAIL is the subject of future studies.

Ovarian cancer remains a leading cause of death among women worldwide, and current treatment regimens for advanced disease are inadequate. Continued preclinical development of targeted combination therapeutic approaches, particularly those using novel biological agents, has the potential to significantly impact the care of ovarian cancer patients.

Acknowledgments

Supported by research grants from the Department of Defense: W81XWH-07-1-0577 (OC060348) and W81XWH-06-1-0408; the National Cancer Institute (CA133654); and the Karin Grunebaum Cancer Research Foundation (DVF).

The experiments were planned by SS and DVF. The experiments were carried out by SS. Data was analyzed by SS and DVF. The manuscript was written by SS and DVF.

Author Disclosure Statement

No competing financial interests exist.

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Received for publication October 11, 2012; accepted after revision November 8, 2012.