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Mechanism of T-oligo-induced cell cycle arrest in Mia-Paca pancreatic cancer cells

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Abstract

DNA oligonucleotides with sequence homology to human telomeric DNA (T-oligo) induce cell cycle arrest, followed by apoptosis, senescence, or autophagy in a human cancer cell type-specific manner. T-oligo has potential as a new therapeutic strategy in oncology because of its ability to target certain types of tumor cells while sparing normal ones. In the present study, we demonstrate the T-oligo-induced S-phase cell cycle arrest in four pancreatic cancer cell lines. To further contribute to the mechanistic understanding of T-oligo, we also identify cyclin dependent kinase 2 (cdk2) as a functional mediator in the T-oligo-induced cell cycle arrest of pancreatic cancer cells. Ectopic expression of a constitutively-active cdk2 mutant abrogates T-oligo-induced cell cycle arrest in these tumor cells while knockdown of cdk2 expression alone recapitulates the T-oligo effect. Finally, we demonstrate the dispensability of T-oligo-induced ATM/ATR-mediated DNA damage response-signaling pathways, which have long been considered functional in the T-oligo signaling mechanism.

Keywords

T-oligo; Pancreatic Cancer; Cell Cycle Arrest; Pancreatic Cancer Stem Cells

Introduction

T-oligos are single-stranded DNA oligonucleotides with sequence homology to telomere termini. Exposure to these oligos has produced both cytostatic and cytotoxic effects in multiple cancer cell types, while sparing normal cells (Yaar et al., 2007; Longe et al., 2009), and T-oligos are moving towards clinical testing as tumor-targeted therapeutic agents. A newer generation of these oligos has significant activity against human pancreatic cancer cells and pancreatic cancer stem cells (this report, and Rankin, in preparation). The most widely-accepted mechanistic model for T-oligo action on tumor cells involves the induction of DNA damage-like signaling, characterized by the phosphorylation of the histone variant H2AX, and the ATM and chk2 kinases, followed by a robust cell cycle arrest and cell line-dependent senescence, apoptosis, or autophagy (Yaar et al., 2007; Longe et al., 2009; Eller et al., 2002; Li et al., 2004; Aoki et al., 2007; Puri et al., 2004; Longe et al., 2007). In tandem with activation of this signaling pathway, the tumor suppressor p53 is phosphorylated and stabilized, followed by the transcriptional up-regulation of p21 in p53-proficient cells. Although much work has focused on characterizing the early signaling responses in cells exposed to T-oligo, the molecular mechanism actually mediating T-oligo-

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induced cell cycle arrest is poorly understood. Initial reports focused on the activation of p53 as potentially initiating the arrest. Our recent studies, however, have indicated that cells lacking functional p53 still undergo a robust cell cycle arrest (Longe et al., 2009), indicating that an alternative mechanism must be responsible.

Cell cycle-arrest mechanisms in eukaryotic cells serve a tumor suppression function, halting proliferation in response to specific extrinsic stimuli, including growth factor withdrawal and DNA damage (Norbury et al., 1992). Cell cycle progression is regulated by balancing the activation and inhibition of cyclin-dependent kinase (cdk) activity, through dephosphorylation events and the binding of cyclin-dependent kinase inhibitors (CDKIs), respectively. During the G₁ to S phase transition specifically, the primary regulatory kinase, cyclin-dependent kinase 2 (cdk2), is partially activated following the disruption of its association with the CDKIs p21^{cip1/waf-1} or p27^{KIP-1} (Cuadrado et al., 2009; Morgan et al., 1997; Harper et al., 1993; Montagnoli et al., 1999). Following additional activating dephosphorylation, cdk2 then hyperphosphorylates and inactivates the Rb tumor suppressor protein, allowing induction of E2F transcription factor-dependent S phase-specific gene induction (Chellappan et al., 1991; Dyson, 1998).

A recently-developed deoxyguanosine-rich 16-mer T-oligo analogue bearing four GGTT tandem repeats demonstrated significantly increased effectiveness over previous T-oligo sequences in inducing cytostasis and cytotoxicity in several cancer cell types (unpublished data, and Sarkar and Faller, 2011; Sharifatabrizi and Eller, 2007). The aim of this study was (1) to elucidate functionally-relevant cell cycle mediators in T-oligo-induced cell cycle arrest in pancreatic cancer cells, and (2) to determine the functional importance of T-oligo-induced activation of DNA damage-responses in pancreatic cancer cells.

We show here that human pancreatic tumor cells lacking a functional p53 undergo cell cycle arrest in response to T-oligo exposure, that inhibition of cdk2 by T-oligo mediates the T-oligo-induced arrest, and that ATM-mediated DNA damage-signaling induced by T-oligo exposure is not required for the arrest.

Materials and Methods

Chemicals and reagents

Deoxyguanosine-rich oligonucleotides (5'GGTTGGTTGGTTGGTT3'), and control oligonucleotides (5'AAACCTACACCTAACCC3') were purchased from The Midland Certified Reagent Co. (Midland, TX, USA). Antibody against phosphorylated ataxia-telangiectasia-mutated (ATM) (Ser1981) protein was purchased from Novus Biologicals (Littleton, CO, USA). Antibodies against total and phosphorylated check point kinase 2 (chk-2) (Thr68), total and phosphorylated cyclin dependent kinase 2 (cdk2) (Thr14,Tyr15), total cyclin-dependent kinase 4 (cdk4), and total β -tubulin proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against total ATM and total ataxia-telangiectasia-mutated related (ATR) proteins were purchased from GeneTex Inc. (Irvine, CA, USA) while antibodies against total and phosphorylated histone H2AX (Ser139) protein were purchased from Cell Signaling (Danvers, MA, USA). Propidium Iodide, RNase A, and antibody against total β -actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human-specific siRNA smartpools against ATM, ATR, and cdk2, as well as transfection reagent (Dharmafect), were purchased from Dharmacon RNAi Technologies (Lafayette, CO, USA). Chemicals used in cell culture, including penicillin/streptomycin and L-glutamine were purchased from GIBCO BRL (Carlsbad, CA, USA). FITC BrdU flow kit was purchased from BD Biosciences (San Jose, CA, USA). 7-aminoactinomycin D (7-AAD) was purchased as a component within the aforementioned FITC BrdU flow kit. Western Lightning chemiluminescence reagent was purchased from

Perkin Elmer (Waltham, MA, USA). The expression vector for constitutively-active cdk2 was kindly provided by D.J. Tindall (Mayo Clinic College of Medicine, Rochester, MN, USA).

Cell Culture

The human pancreatic cancer cell lines Mia-PaCa 2, Panc-1 and AsPC-1, were obtained from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) purchased from Sigma-Aldrich (St. Louis, MO, USA) and supplemented with 2 mM L-glutamine and 100 units/ml penicillin, both purchased from GIBCO BRL (Carlsbad, CA, USA), and 10% fetal bovine serum purchased from Hyclone (Waltham, MA, USA). Human pancreatic cancer stem cells were obtained from Celprogen (San Pedro, CA, USA) and were cultured in Pancreatic Cancer Stem Cell Growth Medium (manufacturer's proprietary formulation) and on Pancreatic Cancer Stem Cell Extracellular Matrix-coated cell culture plates; both of which were purchased from Celprogen. As indicated in Celprogen's published product data sheet, the pancreatic cancer stem cells were clonally isolated from human pancreatic cancer tissue and were tested positive for CA19-9, carcinoembryonic antigen (CEA), galactosyl transferase II, glutamic acid decarboxylase (GAD), α -1-antitrypsin, mucin, keratin, CK7, and CD133. All cells were cultured at 37°C with 5% CO₂.

Cell Proliferation Assay

Cells were seeded onto 6-well plates at a density of 1600 cells/cm² on day 0. On day 2, triplicate cultures were exposed to T-oligo or control oligo at concentrations up to 20 μ M for the indicated times and collected by trypsinization. A 10 μ l aliquot of cell suspension from each sample was combined in a 1:1 ratio with trypan blue purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue-negative cells were then counted manually, using disposable hemacytometers purchased from Fisher Scientific (Pittsburgh, PA, USA).

Cell cycle analysis

Cells were seeded onto 6-well plates at a density of 1600 cells/cm² on day 0. On day 2, triplicate cultures were exposed to T-oligo or control oligo at concentrations up to 20 μ M for the indicated times, trypsinized, fixed in 35% (vol/vol) ethanol/DMEM, washed once with phosphate buffered saline (PBS), incubated for 30 min at 37°C in phosphate-buffered saline containing 25 μ g propidium iodide/ml and 25 μ M RNASE A, and analyzed for DNA content on a fluorescence-activated cell sorter purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Distribution of cells containing DNA characteristic of G₀, G₁, S, and G₂/M cell cycle phases was determined using CellQuest Software purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

5'-bromo-2'-deoxyuridine Incorporation Analysis

Cells were seeded onto 6-well plates at a density of 1600 cells/cm² on day 0. On day 2, triplicate cultures were exposed to T-oligo or control oligo at a concentration of 20 μ M for 24 h and analyzed for ability to incorporate 5'-bromo-2'-deoxyuridine (BrdU) using a FITC BrdU flow kit and manufacturer's protocol. Briefly, cells were incubated for 45 min with 1 mM BrdU, fixed, permeabilized, and stained with α -BrdU antibody before being analyzed by flow cytometry for quantification of BrdU-positive cells.

Immunoblot Analysis

Cells were treated with T-oligo or control oligo (20 μ M) as described and then lysed in RIPA immunoblotting lysis buffer (25 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% SDS; 1.0% sodium deoxycholate; 1.0% Triton X-100). Samples were separated by electrophoresis

on an 8, 10, 12, or 15 percent poly-acrylamide gel, transferred to nitrocellulose membrane, and analyzed by immunoblot for the following targets: α -total or -phosphorylated ATM (Ser1981), -total or -phosphorylated chk-2 (Thr68), -total or -phosphorylated cdk2 (Thr14, Tyr15), -total cdk4, -total ATR, -total or -phosphorylated H2AX (Ser139), -total β -actin, or total β -tubulin.

Transfection with siRNA

Cells were transfected with Dharmafect transfection reagent containing 250 nM cdk2 siRNA, or 150 nM ATM or ATR siRNA, or 100 nM each of ATM and ATR siRNAs, or 150 nM negative siRNA control for 96 h in DMEM supplemented with 10 percent fetal bovine serum and 2 mM L-glutamine, but no antibiotics, according to the manufacturer's instructions. Cells were lysed in RIPA buffer, and samples analyzed by immunoblot to detect total cdk2, ATM or ATR proteins, to confirm protein knockdown.

Statistical Analyses

All experiments were repeated a minimum of three times, and representative results are shown. Statistical significance was assessed with Student t-test or ANOVA, as applicable.

Results

Recent work has demonstrated the effectiveness of a 16-mer deoxyguanosine-rich T-oligo containing four tandem repeats of GGTT in inducing cytostatic/cytotoxic effects in cancer cell lines (unpublished data and Sarker and Faller, 2011; Sharifabrizi and Eller, 2007). To determine whether T-oligo would induce cytostatic effects in human pancreatic cancer cells, Mia-PaCa 2 cells were treated with T-oligo and assessed for the ability of the T-oligo to inhibit cell proliferation, using trypan blue exclusion as a proxy for viable cells. Compared with cells exposed to vehicle or to a control oligo of equal length but containing an unrelated sequence, exposure of Mia-PaCa 2 cells to T-oligo (at 20 μ M) reduced cell numbers by $74 \pm 5\%$ within 48 h (Fig. 1).

To determine whether the T-oligo-induced inhibition of cell proliferation was caused by cell cycle arrest, Mia-PaCa 2, Panc-1, AsPC-1, or human pancreatic cancer stem cells were exposed to vehicle, or exposed to 20 μ M of T-oligo for 6, 12, or 24 h or 20 μ M control oligo, and analyzed for DNA content using propidium iodide staining and flow cytometric analysis. Within 12 h, Mia-PaCa 2 cells exposed to T-oligo at 20 μ M demonstrated a statistically-significant increase in "S phase" levels of DNA, compared to control cultures (approximately 38%) ($p=0.0001$, $n=3$) (Fig. 2a). Similarly, within only 12 h of exposure to T-oligo at 10 μ M, $60 \pm 1\%$ of pancreatic cancer stem cells exhibited "S phase" DNA levels, compared with $36 \pm 1\%$ of cells exposed to vehicle alone ($p=0.0001$, $n=3$) (Fig. 2b). Panc-1 and AsPC-1 pancreatic cancer cells demonstrated comparable accumulations of cells with "S phase" levels of DNA, within 48 h (Fig. 2c).

To determine whether the T-oligo-induced accumulation of cells with S phase levels of DNA content was due to a cell cycle arrest, as defined by the inhibition of DNA synthesis, Mia-PaCa 2 cells were exposed either to vehicle or to T-oligo or to control oligo for 24 h and then assayed for their ability to incorporate 5'-bromo-2'-deoxyuridine (BrdU). Only $7 \pm 4\%$ of cells treated with T-oligo were positive for BrdU, compared with $46 \pm 1\%$ and $47 \pm 3\%$ of cells exposed to vehicle or control oligo, respectively (Fig. 3).

These findings that exposure to T-oligo induces an S phase cell cycle arrest (as evidenced by an increase in the percentage of Mia-PaCa 2 cells with S phase levels of DNA content and a reduction in the percentage of cells incorporating BrdU), together with the established role of cdk2 in mediating the G₁/S phase cell cycle transition in mammalian cells, prompted

analysis of cdk2 and other cell cycle regulating proteins for T-oligo-induced post-translational and total protein modulations that might regulate their activity. Immunoblot analysis of total protein extracts from Mia-PaCa 2 cells exposed to vehicle or T-oligo or control oligo for 12 h revealed modulation of post-translationally-modified proteins known to regulate G₁ to S phase cell cycle transition (Fig. 4). The dual phosphorylation of cdk2 at Thr14 and Tyr15, a modification which inhibits cdk2 kinase activity, increased robustly through 48 h of T-oligo exposure, but cdk2 was not modified in cells exposed to the control oligo. Total cdk2 protein levels also increased, reaching maximal levels at 48 h of T-oligo treatment. In contrast to the T-oligo-induced time-dependent modulation in cdk2 post-translational modifications and total protein levels, no change was observed in total levels of cdk4, a cyclin-dependent kinase whose function regulates G₁ phase progression. In each case, β -actin served as a loading control.

To formally test whether inhibition of cdk2 activity is necessary for T-oligo-induced cell cycle arrest, Mia-PaCa 2 cells were stably-transfected with a vector expressing a V5 epitope-labeled, constitutively-active cdk2 which is incapable of inhibition by phosphorylation on Thr14 and Tyr15 due to the selective mutation of these amino acids to alanine and phenylalanine respectively. Immunoblot analysis demonstrated efficient expression of the mutated cdk2 in transfected cells (Fig. 5a). Transfected cells were exposed to vehicle or to T-oligo for 24 or 48 h and analyzed for DNA content using propidium iodide staining and flow cytometry. Cells expressing constitutively-active cdk2 proteins demonstrated a $38 \pm 8\%$ to $34 \pm 4\%$ reduction in the percentage of cells containing S phase levels of DNA compared with mock transfected Mia-PaCa 2 cells exposed to T-oligo for the same duration (Fig. 5b).

If cdk2 is indeed functionally critical in mediating T-oligo-induced cell cycle arrest, its selective depletion in Mia-PaCa 2 cells independently of T-oligo exposure should recapitulate a T-oligo-induced cell cycle arrest. Mia-PaCa 2 cells were either left untransfected, or transfected with cdk2-specific siRNA, or non-targeting siRNA control, or vehicle for 96 h. Cells treated with cdk2-specific siRNA demonstrated a decrease in cdk2 protein of 71.7%, compared with levels of cdk2 protein in cells under any of the control conditions (Fig. 6a). Depletion of total cdk2 protein levels in Mia-PaCa 2 cells using specific siRNA effectively modulated the cell cycle profiles, producing a 4-fold increase in the percentage of cells with S phase levels of DNA when compared with untransfected cells (Fig. 6b). These findings demonstrate that selective depletion of cdk2 alone in these cells is sufficient to arrest G₁/S phase cell cycle transition, recapitulating the arrest observed in response to T-oligo exposure.

Despite the wealth of published descriptive data obtained from multiple cancer cell lines demonstrating upregulation of DNA damage responses, characterized by phosphorylation of ATM, chk2, and histone H2AX following T-oligo treatment (Yaar et al., 2007; Longe et al., 2009; Puri et al., 2004; Eller et al., 2006), any functional role for these specific DNA damage-response proteins, including ATM, ATR, and chk2, in the cellular response to T-oligos, has largely been untested. Exposure to T-oligo induces a DNA damage-response signaling pattern in Mia-PaCa 2 cells similar to that reported in other cancer cell types (Fig. 7). Specifically, phosphorylation of ATM (Ser1981), chk2 (Thr68), and histone H2AX (Ser139) increased within 12–24 h of T-oligo exposure. In contrast, cells exposed to control oligo demonstrated no increase in phosphorylation of these proteins when compared to vehicle-treated controls. Total ATM, ATR, chk2, and histone H2AX protein levels were not changed by any of these experimental conditions.

A potential role for ATM was explored first, because it is among the earliest of DNA damage-sensing proteins to become activated following DNA double-strand breaks (Lim et

al., 2000; Canman et al., 1998; Burma et al., 2001). To test for a functional role of the ATM DNA damage-response signaling pathway in the cellular response to T-oligo treatment, ATM-specific siRNA-mediated knockdown was employed. Should ATM serve a functional role in T-oligo-induced DNA damage signaling and cell cycle arrest, its selective depletion would abrogate these responses following T-oligo exposure. Mia-PaCa 2 cells were either left untransfected, or transfected with ATM-specific siRNA, or a non-targeted siRNA control, or exposed to vehicle only, for 96 h. Cells transfected with ATM-specific, or negative control siRNA demonstrated cell cycle profiles, as determined by propidium iodide staining and flow cytometric analysis for DNA content, similar to profiles of untransfected cells. Following exposure to T-oligo for 24 h, no difference in cell cycle profile was observed between cells left untransfected and those transfected with ATM-specific siRNA or negative-control siRNA (data not shown).

Because selective depletion of ATM did not abrogate T-oligo-induced cell cycle arrest in Mia-PaCa 2 cells, a functional role for ATR was investigated. ATR is known to phosphorylate and activate several cell cycle regulatory proteins, including chk2, previously thought to specifically lie downstream of ATM (Jazayeri et al., 2006; Cuadrado et al., 2006). ATR total protein was depleted in Mia-PaCa 2 cells in order to test whether it played a functional role in cellular responses to T-oligo treatment. Efficient knockdown of ATR protein was verified by immunoblot. Knockdown of ATR did not alter the S phase arrest observed after exposure to T-oligo (data not shown).

Cellular responses to internal or external stimuli often rely on parallel and converging signaling pathways in order to increase the probability of a biological outcome. In the context of DNA damage, several redundant pathways have the capability of converging on critical effector proteins which then stimulate the cell to either initiate DNA repair mechanisms, or in the case of irreparable DNA damage, arrest the cell cycle and initiate apoptosis (Cuadrado et al., 2006; Kastan and Bartek, 2004). To test the hypothesis that ATM and ATR function may be redundant with each other in the context of cellular responses to T-oligo treatment, both proteins were simultaneously depleted in Mia-PaCa 2 cells using specific siRNAs followed by treatment with T-oligo. Knockdown of both ATM and ATR proteins (by 87.6% and 90.5%, respectively) was verified by immunoblot (Fig. 8a). Knockdown of both ATM and ATR concurrently did not affect the ability of the T-oligo to induce S phase arrest (Fig. 8b).

Discussion

Pancreatic cancer is among the least survivable of all diagnosed cancers, with conventional therapeutic approaches being largely ineffectual in prolonging life following diagnosis, necessitating the development of novel therapeutics (Regine et al., 2008; Jemal et al., 2009; Bilimoria et al., 2007). In this report, we demonstrated the strong cytostatic action of a recently-developed deoxyguanosine-rich T-oligo analogue, bearing four GGTT tandem repeats, in pancreatic cancer cells. The lack of toxicity of these T-oligos to normal cells *in vitro*, and in *in vivo* studies, has been previously demonstrated by ourselves and others (Yaar et al., 2007; Longe et al., 2009). The currently-accepted model for mechanism of action of T-oligos is *via* the up-regulation of DNA damage-response signaling pathways involving the phosphorylation of ATM, chk2, p95/NBS1, and histone H2AX, followed by cell cycle arrest and initiation of apoptotic or senescence programs (Yaar et al., 2007; Longe et al., 2009; Puri et al., 2004; Eller et al., 2006). The purpose of this study was two-fold: first, to elucidate functionally-relevant cell cycle mediators in T-oligo-induced cell cycle arrest, and second, to determine the functional importance of the T-oligo-induced activation of DNA damage-signaling in pancreatic cancer cells.

T-oligo produces substantial cytostatic effects on Mia-PaCa 2 pancreatic cancer cells and human pancreatic cancer stem cells within 12 h of treatment, as evidenced by a marked decrease in proliferation and a robust modulation of the cell cycle profiles of T-oligo-treated cells, with an increase in the percentage of cells exhibiting DNA content consistent with S phase. This effect was also seen in Panc-1 and AsPC-1 pancreatic cancer cells, albeit at a later time point. Additionally, BrdU incorporation analysis demonstrated that T-oligo exposure arrests cycling pancreatic cancer cells within 24 h, producing a nearly complete abrogation of BrdU incorporation. Furthermore, T-oligo exposure induced profound cell cycle arrest in pancreatic cancer stem cells within 12 h. Discrepancy observed between the percentage of Mia-Paca 2 cells in S phase as gauged by propidium iodide staining (26 percent) versus the percentage observed according to BrdU labeling (46 percent) can be explained by the relative clarity of distinct cell populations detected by the two assays; namely that BrdU incorporation allows for more precise distinction between cell populations based on whether they are actively undergoing DNA replication versus the less specific and broad measurement of total DNA content as assessed by propidium iodide staining.

Despite the wealth of descriptive data reporting the T-oligo-induced up-regulation of DNA damage-response signaling, few studies have tested the functional importance of DNA damage-response proteins in T-oligo-induced cell cycle arrest. Studies to date have been limited to exploring the involvement of the WRN, ATM, and p95/Nbs1 proteins. Specifically, in osteosarcoma cells depleted of WRN protein *via* transfection with WRN-specific siRNA, phosphorylation of ATM and H2AX on Ser1981 and Ser139, respectively, were reduced following T-oligo treatment compared to controls transfected with a scrambled siRNA and exposed to T-oligo (Eller et al., 2006). Cells from a patient with Nijmegen breakage syndrome (NBS), when exposed to T-oligo, exhibited altered cell cycle profiles compared to control fibroblast cells. Finally, cells derived from patients with Ataxia-Telangiectasia, when exposed to T-oligo, exhibited reduced levels of phosphorylated p95/Nbs1 (Eller et al., 2003).

A number of DNA damage-activated signaling pathways, because of their activation, or because of an increase in their levels after exposure to T-oligos, have been hypothesized to mediate the cell cycle arrest induced by T-oligos. These include ATM/chk2 (Yaar et al., 2007; Longe et al., 2009) and p53/p21 (Longe et al., 2009; Eller et al., 2002; Li et al., 2003). The p53 axis is frequently non-functional in human tumors. We have previously reported that p53-deficient tumor cell lines remain responsive to the cytostatic and subsequent cytotoxic actions of T-oligos. The current report verifies and extends these findings. Mia-PaCa 2 cells lack a functional p53 protein, and p21^{cip1/waf-1} is not inducible in these cells by either T-oligo or classic DNA-damaging chemotherapeutic agents, yet these cells are sensitive to T-oligo mediated cell cycle arrest and subsequent cytotoxic effects.

In contrast to p53, ATM and chk2 are indeed activated in these cells in response to T-oligo exposure, consistent with findings in other tumor cell types (Yaar et al., 2007; Longe et al., 2009). Interestingly, the kinetics of this activation (maximum at 48 h) appear to lag behind the S phase arrest (apparent at 12–24 hr). Nonetheless, evidence for a functional and necessary role of the ATM/chk2 axis in the cytostatic action of T-oligos was explored, and rigorously ruled out, in our studies. Refuting prior speculation, ablation of the ATM protein did not abrogate T-oligo-induced cell cycle arrest. One potential explanation for this outcome could be the presence of a redundant signaling pathway, paralleling that initiated by ATM activation. ATR is a protein kinase known to initiate DNA damage signaling in response to pyrimidine dimers, stalled replication forks, and DNA double-strand breaks, whereas ATM functions primarily as a mediator of DNA damage signaling following DNA double-strand breaks (Osborn et al., 2002). It was conceivable that in the absence of ATM protein, ATR may serve as the mediator of a T-oligo-induced DNA damage signaling.

Interestingly, ATR depletion similarly had no effect on T-oligo-induced cell cycle arrest. Furthermore, ablation of both ATM and ATR proteins simultaneously had no effect on T-oligo-induced cell cycle arrest. These findings demonstrate that the DNA damage-sensing functions of ATM and ATR are not required for the T-oligo-induced cell cycle arrest of cells.

In contrast to our rigorous demonstration that ATM, ATR, p53 and p21^{cip1/waf-1} play no functional roles in the tumor cell response to T-oligos, we found that cdk2 is required to mediate the cytostatic actions of the T-oligo. T-oligo exposure induced an increase in cdk2 phosphorylation at Thr14 and Tyr15, two post-translational modifications that block the ATP-binding pocket of cdk2 and, consequently, its kinase activity, suggesting its potential involvement.

To explore a potential functional role for cdk2 in T-oligo-induced cell cycle arrest, a constitutively-active cdk2 mutant protein was stably over-expressed in these cells. The marked reduction in the percentage of cells with S phase levels of DNA following T-oligo treatment in those cells expressing active cdk2 demonstrated that reconstitution of cdk2 activity could abrogate the arrest, consistent with a functional role for cdk2 inhibition in T-oligo-induced cell cycle arrest. Thus, cdk2 repression by T-oligo is necessary for the arrest induced by T-oligo.

To determine whether inhibition of cdk2 activity is sufficient alone to recapitulate the arrest of the G₁/S phase transition observed in tumor cells after exposure to T-oligos, cdk2-was knocked-down with specific siRNA. Cells transfected with cdk2-specific siRNA demonstrated a nearly four-fold increase in the percentage of cells with S phase DNA content, comparable to, but not quite equaling, the effects of T-oligo. (This attenuation might be due to lack of absolute depletion of cdk2 by siRNA, or to the significant length of time required for siRNA to deplete protein levels [96 h], compared to the comparatively rapid actions of T-oligo on inactivating cdk2 [within 12 h].)

In this report, we demonstrate the ability of a deoxyguanosine-rich T-oligo analog to initiate robust cytostasis in pancreatic cancer cells and cancer stem cells. Additionally, we functionally validate a central role for cdk2 inhibition in T-oligo-induced cell cycle arrest. Furthermore, our findings that ATM and ATR function, and p53/p21^{cip1/waf-1} function, are not required for the actions of T-oligo have potential importance. The ability of T-oligo to function independently of DNA-damage signaling pathways, components of which are frequently mutant or inactive in tumor cells, may widen the potential application of T-oligos as cancer therapeutics.

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Figure 1.

T-oligo exposure inhibits the cell growth of Mia-PaCa 2 cells. Triplicate cultures of Mia-PaCa 2 cells were treated with T-oligo (20 μ M), control oligo (20 μ M), or vehicle for 24 or 48 h. Viable cell number was determined by enumeration of trypan blue-negative cells. Error bars represent SEM. Statistical significance was determined by one-way ANOVA.

Figure 2.

T-oligo exposure modulates cell cycle profiles. (A) For each condition, triplicate cultures of Mia-PaCa 2 cells were exposed to vehicle or to 20 μM T-oligo for 6, 12, or 24 h, harvested, stained with propidium iodide, and analyzed by flow cytometry. Insets: Average and standard deviations of percentage of cells with DNA content consistent with G₁, S, or G₂/M cell cycle phases. (B) Triplicate cultures of human pancreatic cancer stem cells were treated with vehicle or with T-oligo (10 μM) for 12 h and analyzed for DNA content in an identical fashion to the Mia-PaCa 2 cells in earlier panels. (Insets) Average and standard deviations of percentage of cells bearing DNA content consistent with G₁, S, or G₂/M cell cycle phases. (C) For each condition, triplicate cultures of Panc-1 or AsPC-1 pancreatic cancer cells were exposed to vehicle or to 20 μM T-oligo for 48 h, harvested, stained with propidium iodide, and analyzed by flow cytometry. Insets: Average and standard deviations of percentage of cells with DNA content consistent with G₁, S, or G₂/M cell cycle phases.

Figure 3.

Exposure to T-oligo inhibits DNA synthesis. Mia-PaCa 2 cells were exposed to vehicle or T-oligo or control oligo (both at 20 μ M) for 24 h, followed by incubation for 45 min in complete media supplemented with BrdU (1 mM). Cells were fixed, permeabilized, and stained with α -BrdU antibody before being analyzed by flow cytometry for quantification of BrdU-positive cells. Insets: Average and standard deviations of percentage of cells incorporating BrdU.

Figure 4.

T-oligo exposure modulates cell cycle proteins regulating the G_1/S phase transition. Total protein extracted from Mia-PaCa-2 cells exposed to vehicle (Veh) or T-oligo for 12, 24, or 48 h or control oligo (Co) (each at 20 μ M) for 48 h and immunoblotted for phosphorylated cdk2 (Thr14, Tyr15), total cdk2, or total cdk4. Each blot was stripped and re-probed for β -actin as a control for equal loading.

Figure 5.

Abrogation of T-oligo-induced cell cycle arrest in cells stably-expressing constitutively-active cdk2. (A) Immunoblot for the V5 epitope in Mia-PaCa 2 cells mock- or stably-transfected with an expression plasmid encoding a V5-tagged, constitutively-active cdk2 gene. (B) Triplicate cultures of Mia-PaCa 2 cells stably-expressing mutant, constitutively-active cdk2 were exposed to vehicle or T-oligo (20 μ M) for 24 or 48 h, stained with propidium iodide, and analyzed by flow cytometry. Insets: Average and standard deviations of percentage of cells containing S phase levels of DNA.

Figure 6.

Specific siRNA-mediated depletion of cdk2 protein recapitulates T-oligo-induced cell cycle arrest. (A) Immunoblot of total cdk2 protein 96 h after Mia-PaCa 2 cells were either left untreated, or transfected with cdk2-specific siRNA or with non-targeting (neg. siRNA) (both at 250 nM), or exposed to vehicle. (B) Triplicate cultures of Mia-PaCa 2 cells were left untreated or transfected with cdk2-specific or non-targeting control siRNA for 96 h, harvested, stained with propidium iodide, and analyzed by flow cytometry. Insets: Average and standard deviations of percentage of cells containing levels of DNA consistent with G₁, S, and G₂/M cell cycle phases. Error bars represent SEM. Statistical significance was determined by one way ANOVA.

Figure 7.

Exposure to T-oligo induces modulation of DNA-damage response signaling proteins. Total protein was extracted from Mia-PaCa 2 cells exposed to either vehicle (Veh) or T-oligo or control oligo (CO) (both at 20 μ M) for 12, 24, or 48 h and analyzed by immunoblot for modulation of total and phosphorylated ATM (Ser1981), total and phosphorylated chk2 (Thr60), total and phosphorylated H2AX (Ser139) and total ATR levels. Total β -tubulin or β -actin levels serve as loading controls.

Figure 8.

RNA interference-mediated simultaneous depletion of ATM and ATR proteins does not abrogate T-oligo-induced cell cycle arrest. **(A)** Immunoblot for total ATM and ATR proteins 96 h after Mia-PaCa 2 cells were either left untreated, transfected simultaneously with ATM- and ATR- specific siRNA (ATM/ATR) or with non-targeting (negative control) siRNA (Neg.siRNA or Neg) (all at 125 nM), or exposed to vehicle. **(B)** Duplicate cultures of Mia-PaCa 2 cells were either left untreated, transfected with 125 nM each of ATM- and ATR-specific or non-targeting (Neg. siRNA), or vehicle for 96 h, then either treated with vehicle or with T-oligo (20 μ M) for 24 h, harvested and stained with propidium iodide, and analyzed by flow cytometry. Insets: Average and standard deviations of percentage of cells bearing levels of DNA consistent with G₁, S, and G₂/M cell cycle phases.