Single-stranded DNA Induces Ataxia Telangiectasia Mutant (ATM)/p53-dependent DNA Damage and Apoptotic Signals

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Single-stranded DNA has been speculated to be the initial signal in the DNA damage signaling pathway. We showed that introduction of single-stranded DNA with diverse sequences into mammalian cells induced DNA damage as well as apoptosis signals. Like DNA damaging agents, single-stranded DNA up-regulated p53 and activated the nuclear kinase ataxia telangiectasia mutant (ATM) as evidenced by phosphorylation of histone 2AX, an endogenous ATM substrate. Single-stranded DNA also triggered apoptosis as evidenced by the formation of caspase-dependent chromosomal DNA strand breaks, cytochrome c release, and increase in reactive oxygen species production. Moreover, single-stranded DNA-induced apoptosis was reduced significantly in p53 null cells and in cells treated with ATM small interfering RNA. These results suggest that single-stranded DNA may act upstream of ATM/p53 in DNA damage signaling.

DNA damage plays an important role in neuron degeneration, carcinogenesis, aging, as well as cell killing by chemotherapeutic agents (1–3). At the cellular level, DNA damage induces complicated cellular responses including checkpoint activation and apoptotic cell death (4, 5). Ataxia telangiectasia mutant (ATM)1 and p53 are two key regulatory molecules in DNA damage signaling and apoptosis. The tumor suppressor p53 is known to mediate G1 cell cycle arrest and apoptosis (6). DNA damage signaling and apoptosis. The tumor suppressor p53-dependent DNA damage as well as apoptosis signals. Like DNA damaging agents, single-stranded DNA up-regulated p53 and activated the nuclear kinase ataxia telangiectasia mutant (ATM) as evidenced by phosphorylation of histone 2AX, an endogenous ATM substrate. Single-stranded DNA also triggered apoptosis as evidenced by the formation of caspase-dependent chromosomal DNA strand breaks, cytochrome c release, and increase in reactive oxygen species production. Moreover, single-stranded DNA-induced apoptosis was reduced significantly in p53 null cells and in cells treated with ATM small interfering RNA. These results suggest that single-stranded DNA may act upstream of ATM/p53 in DNA damage signaling.

The nuclear kinase, ATM, and its related kinase ATR are critically involved in integrating the initial DNA damage signals to cell cycle checkpoints and apoptosis (1, 2). Activation of ATM/ATR by DNA damage has been widely demonstrated (1, 11). For example, ionizing radiation and etoposide have been shown to induce ATM-dependent apoptosis (12), while camptothecin and UV have been shown to induce ATR-dependent apoptosis (13, 14). Oxidative stress-induced neuron degeneration has also been shown to depend on ATM (15). It is generally believed that ATM mediates a signal from DNA double-strand breaks, whereas ATR mediates a signal from replication fork arrest (1, 16).

Activation of the ATM kinase, which is evidenced by phosphorylation of the ATM target proteins, has been demonstrated in cells treated with DNA damage agents, especially those that produce DNA double-strand breaks (17, 18). ATM phosphorylates a large number of proteins in response to DNA damage, e.g. Ser-139 of histone 2AX (H2AX) (19), Thr-68 of Chk2 (20), and Ser-15 of p53 (21–23). While ATM kinase is considered to be one of the earliest regulatory molecules in DNA damage signaling, the initial DNA damage signal that triggers ATM activation remains unclear.

Single-stranded DNA, which activates the RecA protease activity toward LexA, is known to be the initial signal for activating the SOS repair response in bacteria (24). Single-stranded DNA has also been speculated to act as the initial signal for DNA damage responses in eukaryotic cells (16). In the current study, we have investigated the effect of single-stranded oligonucleotides on DNA damage signaling and apoptosis. We showed that transfection with synthetic oligodeoxynucleotides (oligos) as short as 5-mer up-regulated p53 and activated ATM. Moreover, single-stranded oligos induced ATM/p53-dependent apoptosis. Our results thus suggest that single-stranded DNA may act as a signal upstream of ATM/p53 in DNA damage and apoptosis signaling.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture cells used in the current study include BALB/c 3T3, NIH 3T3, p53 (wild type), and p53−/− mouse embryo fibroblasts (MEF) (obtained from Dr. Baoji Li, Institute of Molecular and Cell Biology, The National University of Singapore, Singapore), HCT116 and HCT-116 p53−/− (obtained from Dr. Bert Vogelstein, Johns Hopkins Medical School, Baltimore, MD), and HeLa cells. BALB/c 3T3 (p53 wild type) and BALB/c 3T3(10)1 (p53 mutated) were obtained from Dr. Arnold Levine, The Rockefeller University, New York. Antibodies against a synthetic peptide consisting of the last nine amino acids (KATQASQEY) of H2AX with phospho-Ser-139 were obtained from Dr. D. Chen (Lawrence Berkeley National Laboratory). Antibodies against histone 2A, cytochrome c, p53, ATM, and actin were purchased from Santa Cruz Biotechnology, Inc. The caspase 3 inhibitor (Z-DEVD-FMK) was purchased from Calbiochem. Oligos were synthesized by Integrated DNA Technologies, Inc. CaspACE (FITC-VAD-FMK), Lipofectin, and Oligo-foctamine were purchased from Invitrogen. FuGENE was purchased from Roche Diagnostics Corporation.

Cell Culture and Transfection of Oligodeoxynucleotides—BALB/c 3T3

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cells were cultured to semiconfluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine serum at 37°C in an environment containing 5% CO2. Oligos (200 nM or as indicated) or oligos complexed with 10 μg Cellfectin (Invitrogen) were mixed with 2 ml of serum-free DMEM according to the supplier's protocol and then added to cells. Cells were harvested 4 h after transfection. The following single-stranded oligodeoxynucleotides were used: NT36 (5'-AAG AGG TGG TGG AGG AGG TGG TGG AGG AGG TGG AGG-3'), NT27 (5'-TTG AAT TCC TAG TTT CCC AGA TAC AGT-3'), NT12 (5'-TCG GTA CTCGG-3'), NT18 (5'-TTA GGG TTA GGG TTA GGG-3'), THIONT5 (a phosphorothioate oligo of the sequence 5'-CTTGA-3'), NT8F (5'-GCACAGTGGC-3'), and NT8R (5'-CGCTGAATGC-3').

Alkaline Single Cell Gel Electrophoresis (SCGE) Assay—Genomic DNA strand breaks were analyzed by alkaline SCGE assay. Representative nuclei with intact DNA (no comet) and broken strands (comet) are shown. The THIONT5 oligo (1 μM) was used, and the alkaline SCGE assays were performed after 1, 2, and 3 h of transfection. D, single-stranded, but not double-stranded, oligos induce nuclear DNA strand breaks. NT8F, NT8R, and annealed NT8F:NT8R duplex oligos (1 μM each) were transfected into BALB/c 3T3 cells, and nuclear DNA strand breaks were monitored by alkaline SCGE assay. Each experiment was repeated three times showing similar results.

FIG. 1. Oligos induce nuclear DNA strand breaks in BALB/c 3T3 cells. BALB/c 3T3 cells were cultured to semiconfluency. Each oligo was complexed with 10 μg of Cellfectin and then transfected into BALB/c 3T3 cells as described under "Experimental Procedures." Nuclear DNA strand breaks were monitored after 4 h using an alkaline SCGE assay. A, induction of nuclear DNA strand breaks by different oligos. Different oligos (NT36, NT27, THIONT5, NT18, or NT12, 200 nM each) were transfected into BALB/c 3T3 cells, and nuclear DNA strand breaks were determined by alkaline SCGE assay. B, dose-dependent increase of oligo-induced nuclear DNA strand breaks. The NT36 oligo was used in this experiment. The concentration of the NT36 oligo for each reaction is indicated at the bottom of the figure in nM. C, time-dependent increase of oligo-induced nuclear DNA strand breaks. The THIONT5 oligo (1 μM) was used, and the alkaline SCGE assays were performed after 1, 2, and 3 h of transfection. D, single-stranded, but not double-stranded, oligos induce nuclear DNA strand breaks. NT8F, NT8R, and annealed NT8F:NT8R duplex oligos (1 μM each) were transfected into BALB/c 3T3 cells, and nuclear DNA strand breaks were monitored by alkaline SCGE assay. Each experiment was repeated three times showing similar results.

FIG. 2. Oligos induce caspase-dependent DNA strand breaks and cell killing in BALB/c 3T3 cells. A, oligo-induced caspase 3-dependent DNA strand breaks. The caspase 3 inhibitor, Z-DEVD-FMK (50 μM), was added together with each oligo:Cellfectin complex to BALB/c 3T3 cells for 4 h. Nuclear DNA strand breaks were monitored by alkaline SCGE assay. B, oligos kill BALB/c 3T3 cells. A 200 nM concentration of each oligo was used to transfect BALB/c 3T3 cells. Cell survival was determined by MTT assay as described under "Experimental Procedures."
Preparation of Cytoplasmic Fraction—BALB/c 3T3 cells were harvested and lysed in a modified buffer containing 100 mM Tris, pH 7.4, 10 mM MgCl$_2$, 10 mM CaCl$_2$, 1 mM dithiothreitol, 100 μM EGTA, 0.5% Nonidet P-40, and 10 μg/ml each of leupeptin, pepstatin, and aprotinin for 10 min at 4°C. Supernatants were mixed with the Lamelli SDS sample buffer and analyzed by Western blotting.

Measurement of Reactive Oxygen Species (ROS) Production—ROS production was measured using flow cytometry as described previously (27). Briefly, BALB/c 3T3 cells were grown to semiconfluence and transfected with the oligo:Cellfectin (10 μg) complex as described above. After 4 h, 5-(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes) was added to the medium to a final concentration of 20 μM. Cultures were then incubated again for 40 min at 37°C to allow accumulation of DCFH. Cells were washed with PBS, trypsinnized, and suspended in ice-cold PBS. Cellular fluorescence from a sample of 15,000 cells was analyzed using a Coulter EPICS Profile II flow cytometer (Coulter Electronics, Miami, FL). Fluorescence, excited at 488 nm, was detected using a 525 ± 20 band pass filter. Cellular debris was excluded from analysis by gating based on forward angle light scatter. Histograms were analyzed using EPICS Workstation Software (version 4).

Apoptotic Chromatin Condensation and Caspase Activation Assays—After transfection, cells were harvested and washed twice with 1× PBS buffer. Hoechst 33342 (0.5 μg in PBS) and CaspACE (10 μg in PBS) were then added to the cells. Cells were then incubated for 20 min at 37°C. Both chromatin condensation and caspase activation were examined by fluorescence microscopy.

RESULTS

Single-stranded DNA Induces Apoptotic Nuclease-mediated Chromosomal DNA Cleavage—To test whether single-stranded
DNA could induce apoptosis, apoptotic nuclease-mediated DNA cleavage in the chromosomal DNA was monitored by an alkaline SCGE assay. Different single-stranded oligos were complexed with Cellfectin and used to transfect BALB/c 3T3 cells. Transfected cells were then analyzed for apoptotic nuclease-mediated DNA strand breaks in genomic DNA using the alkaline SCGE assay. As shown in Fig. 1A, oligos with different sequences and sizes (from 5 to 36 nucleotides) were found to induce genomic DNA strand breaks. The deoxyribonucleoside triphosphates (dNTPs), on the other hand, were unable to induce genomic DNA strand breaks above the background level (Fig. 1A). We have also tested different transfection agents, including Oligofectamine, Cellfectin, Lipofectamin, and FuGENE, all of which are effective in mediating the effect of oligos. These results suggest that single-stranded oligos can indeed activate a nuclease (see below for the involvement of an apoptotic nuclease) in treated cells.

The oligo-induced chromosomal DNA cleavage showed dose dependence from 0 to 200 nM (Fig. 1B). A maximum of 60% of cells was shown to exhibit DNA cleavage (Fig. 1A). Above 200 nM, the oligo was unable to increase the level of DNA cleavage. The oligo-induced chromosomal DNA cleavage were found to be time-dependent (Fig. 1C). DNA strand breaks were detectable within 1 h of treatment and found to increase with time (Fig. 1C). The oligo-induced DNA strand breaks were shown to depend on the single-strandedness of the oligo. As shown in Fig. 1D, the double-stranded oligo was much less effective than the single-stranded oligo in inducing DNA strand breaks. However, at higher concentrations of double-stranded oligo, DNA cleavage was also detectable (data not shown).

We have studied a possible involvement of apoptotic nuclease in oligo-induced cleavage in the genomic DNA. Treatment of BALB/c 3T3 cells with the caspase 3 inhibitor Z-DEVD-FMK was found to inhibit oligo-induced cleavage in the genomic DNA (Fig. 2A), suggesting that a caspase-activated apoptotic nuclease(s) is involved in oligo-induced chromosomal DNA cleavage.

The fact that oligos can induce an apoptotic nuclease in treated cells has prompted us to examine whether oligos can induce (apoptotic) cell death. As shown in Fig. 2B, oligos indeed induced cell death in BALB/c 3T3 cells as evidenced by the MTT assay.

**Oligos Induce Other Apoptotic Cell Death Markers**—To test whether oligo-induced cell death is due to apoptosis, we have measured the expression of various apoptotic markers in oligo-transfected cells. As shown in Fig. 3A, transfection of BALB/c 3T3 cells with different oligos caused release of cytochrome c from the mitochondria into the cytoplasm (Fig. 3A). Transfection of oligos also caused increased production of ROS as evidenced by increased fluorescence of oxidized DCFH (Fig. 3B). In addition, the NT8R oligo was shown to induce both chromat in condensation (Fig. 3C, upper panel) and caspase activation (Fig. 3C, lower panel) in BALB/c 3T3 cells. About equal numbers of cells (50%) were shown to undergo chromatin condensation and caspase activation in about equal numbers of cells (80%). In the aggregate, these results strongly suggest that oligos can induce apoptotic cell death. Thus, it seems likely that oligo-induced DNA strand breaks, which are blocked by a caspase 3 inhibitor (Fig. 2A), are due to activation of an apoptotic nuclease(s).

**Oligos Induce Up-regulation of p53 and Activate ATM Kinase**—Oligos could mimic a DNA damage signal(s) to trigger apoptosis. One of the most important cellular responses to DNA damage is up-regulation of p53 (29, 30). We have thus tested whether oligos up-regulate p53 in BALB/c 3T3 cells. As shown in Fig. 4A, both oligos, NT36 and NT27, up-regulated p53 in BALB/c 3T3 cells.

Activation of the nuclear kinase ATM is another important cellular response to many DNA damaging agents (18, 31). H2AX is a target protein of the ATM kinase and is known to be phosphorylated at Ser-139 by ATM (19). Using an antibody specific for the phosphorylated peptide epitope of H2AX, we have measured the amount of phosphorylated H2AX in BALB/c 3T3 cells treated with different oligos. As shown in Fig. 4B, NT36 and NT27 oligos stimulated phosphorylation of H2AX in BALB/c 3T3 cells. The increase in the amount of phosphorylated H2AX was not due to an overall elevation of histone H2A (Fig. 4B, lower panel). Phosphorylation of H2AX at Ser-139 suggests that the ATM kinase is activated by oligos. Our results thus suggest that oligos can mimic a DNA damage signal(s) to both up-regulate p53 and activate ATM kinase.

**Single-stranded DNA-induced Apoptosis (SIA) Is p53- and ATM-dependent**—DNA damage is known to induce ATM/p53-dependent apoptosis (32). As shown in Fig. 5A, SIA as monitored by the alkaline SCGE assay was found to be significantly reduced (about 80% reduction) in p53−/− MEF as compared with wild type MEF (Fig. 5A). Similar results were obtained using two other isogenic pairs of p53 cell lines, HCT116/
HCT116p53 −/− and BALB/c 3T3 (p53 wild type)/BALB/c 3T3(10)1 (p53 mutated) (Fig. 5, B and C). These results indicate that oligos act upstream of p53 in SIA.

To study the role of ATM in SIA, ATM siRNA was employed to knock down the expression of ATM in BALB/c 3T3 cells (33, 34). As shown in Fig. 6A, ATM siRNA significantly reduced (about 60%) SIA in BALB/c 3T3 cells as monitored by the alkaline SCGE assay. As a control, the corresponding ATM sense strand RNA did not affect SIA (Fig. 6A). Western blot analysis indicated that the ATM protein level was reduced more than 3-fold in ATM siRNA-treated, but not the corresponding ATM sense-strand RNA-treated, BALB/c 3T3 cells (Fig. 6A). We have also monitored the effect of ATM siRNA on cytochrome c release from mitochondria in BALB/c 3T3 cells treated with different oligos. As shown in Fig. 6B, oligo-induced release of cytochrome c from the mitochondria was greatly reduced in BALB/c 3T3 cells treated with ATM siRNA. These results suggest that oligos act upstream of ATM in SIA. Taken together, our results suggest that oligos may mimic a DNA damage signal(s) to induce ATM/p53-dependent apoptosis.

DISCUSSION

DNA damage is known to trigger a myriad of cellular responses including cell cycle arrest and apoptosis (35, 36). While many key regulatory proteins (e.g. ATM and p53) have been identified, the immediate signal following DNA damage remains unclear (37). A number of studies have demonstrated that introduction of fragmented or single-stranded DNA into cells can influence the cell cycle and induce cell death, suggesting a potential role of DNA ends or single-stranded DNA in DNA damage signaling. For example, transfection with randomly fragmented herring sperm DNA induces apoptosis (38). Nuclear injection of linearized plasmid DNA, circular DNA with a large gap, or single-stranded circular phagemid induces a p53-dependent G1 arrest (39). Transfection of a plasmid containing the telomeric sequence was found to activate p53 (40). Surprisingly, a CpG sequence has previously been reported to be required for induction of apoptosis in T lymphocyte cell lines (41).

Our studies have shown that single-stranded oligos are more effective than duplex forms in inducing apoptosis. In our system, all oligos tested induce apoptosis. In addition to the oligos shown in Fig. 1, we have tested over a dozen other oligos, all of which induce apoptosis. We did not observe a CpG sequence requirement. However, some oligos appear to be more effective than others in inducing apoptosis. Oligos containing the human telomeric G-tail sequence or a stretch of Gs are much more effective in inducing apoptosis.2 Indeed, a recent report has shown that poly(G) motif-containing oligos can induce apoptosis in prostate cancer cells (42). The molecular basis for the observed sequence specificity is unclear and is currently under investigation.

Oligos have been used extensively in antisense research (43). SIA could potentially be explained by an antisense effect. However, two observations argue against the antisense explanation for apoptosis induction. First, we have shown that oligos as short as a 5-mer can induce apoptosis. It is unlikely that a 5-mer antisense oligo can form a stable RNA/DNA hybrid (44). Second, all sequences used in this study induce apoptosis.

Fig. 5. Oligo-induced nuclear DNA strand breaks is p53-dependent. Three independent pairs of cell lines with wild type and mutant p53 were used in this study. Cells were transfected with different oligos (as indicated). A, MEF p53 −/− and two independent MEF (p53 −/−) from p53 −/− mice. Cellfectin was abbreviated as cellF in this figure. B, human colorectal cancer cell lines HCT116 p53 −/− and p53 −/−. C, BALB/c 3T3 (p53 wild type) and BALB/c 3T3(10)1 (p53 mutated). The experiments were repeated three times showing similar results.

a H. Qi, T.-K. Li, A. Nur-E-Kamal, and L. F. Liu, unpublished results.
These sequences are not designed to be antisense oligos. It seems highly unlikely that all these sequences act as antisense oligos.

The oligos could potentially activate apoptosis by acting on cytoplasmic or mitochondrial apoptosis modulators. However, our studies have suggested that oligos act in the nucleus. First, oligos activate the nuclear kinase ATM as evidenced by phosphorylation of the ATM target protein H2AX in the nucleus. Second, like many DNA damaging agents, oligos up-regulate p53. Third, like DNA damage-induced apoptosis, SIA was found to be ATM- and p53-dependent. The similarity between SIA and DNA damage-induced apoptosis suggests that oligos may mimic a DNA damage signal in the nucleus to activate ATM/p53. Activated ATM/p53 signals are transduced to the mitochondria leading to the activation of caspase pathway and apoptotic cell death.

The molecular mechanism by which oligos activate ATM is unclear. One possibility is that oligos directly activate ATM kinase, which then triggers DNA damage responses. Consistent with this hypothesis, single-stranded DNA and linear DNA have been shown to activate replication protein A (RPA) phosphorylation by the ATM immunocomplex (45). However, it is unclear whether the role of single-stranded DNA is for direct ATM kinase activation or for binding to the ATM substrate RPA. Alternatively, oligos may act by titrating single-stranded DNA binding proteins (e.g. RPA) (46, 47). Such a titration may cause deprotection of single-stranded regions in the genome (e.g. replication forks), which in turn could trigger DNA damage responses. Regardless of the mechanism, our results suggest that the single-stranded DNA must act upstream of ATM/p53 in DNA damage signaling. As shown in Fig. 7, a schematic model for the role of single-stranded DNA in DNA damage and apoptotic signaling is presented. In this model, DNA damage is first converted into single-stranded DNA by a nuclease. The single-stranded DNA then activates ATM/p53-dependent DNA damage and apoptotic signaling. A double-strand break in the chromosomal DNA or a telomere end may not activate ATM/p53-dependent DNA damage and apoptotic signaling unless the end is invaded by exonuclease/helicase or de-protected (e.g. inactivation of telomere protecting proteins) to produce single-stranded DNA. This is in contrast to the DNA-PK signaling pathway in which the ends of a DNA double-strand break may be sufficient to signal (48). Similarly, DNA adducts (e.g. UV and carcinogenic DNA adducts) may not signal through this pathway unless they are processed by nuclease/helicase to expose single-stranded DNA.

**Fig. 6. SIA is ATM-dependent.** A, ATM siRNA was used to knock down ATM kinase in BALB/c 3T3 cells as described under “Experimental Procedures.” The level of ATM in BALB/c 3T3 cells was determined by immunoblotting using ATM antibodies. Equal loading of protein was determined by immunoblotting using actin antibodies (Santa Cruz Biotechnology). B, treatment of ATM siRNA inhibits oligo-induced nuclear DNA strand breaks. Cells were transfected with either ATM sense RNA or ATM siRNA (145 ng). 24 h later, cells were again transfected with Cellfectin complexed with dNTPs (siRNA), NT36, or THIONT oligos. 4 h after transfection, cells were harvested and nuclear DNA strand breaks were monitored by alkaline SCGE assay. The ATM protein level in ATM siRNA-treated cells was determined by immunoblotting with anti-ATM antibodies (A). C, ATM siRNA abolishes oligo-induced cytochrome c release from mitochondria. BALB/c 3T3 cells were treated with ATM siRNA to knock down ATM expression as described in A. 24 h later, cells were transfected with different oligos (NT36 and NT27). 6 h after oligo transfection, cells were fixed and immunostained using cytochrome c antibodies followed by treatment with the FITC-conjugated anti-rabbit IgG secondary antibodies. Oligo-induced cytochrome c release from mitochondria was then examined using a fluorescent microscope. The release of cytochrome c (green fluorescence) is evidenced by transition from punctuated staining in the mitochondria (the micrograph labeled siRNA/NT36) to the smooth distribution of cytochrome c in the cytoplasm and punctuated distribution in the nucleus (the micrograph labeled sense RNA/NT36). The red fluorescence is due to the DNA stain (propidium iodide). The number of cells exhibiting cytochrome c release was plotted for different treatment conditions.

**Fig. 7. A proposed model for the role of single-stranded DNA in DNA damage/apoptotic signaling.** DNA damage in the form of DNA adducts (A) and a double-strand break (B) is first processed by nuclease/helicase to produce single-stranded DNA (e.g. a single-stranded gap or single-stranded oligos). The single-stranded DNA then activates ATM/p53-dependent DNA damage/apoptotic signaling.
Transfection of oligos to induce DNA damage/apoptosis signals might provide a very useful system to study the molecular mechanism of apoptotic signal transduction pathways. The potency of oligos in inducing apoptosis also suggests their potential use as anticancer agents. Clearly, more studies are necessary to establish the molecular mechanism of SIA and the potential application of oligos in cancer therapy. It should be noted that oligos have already been successfully used to treat tumors in animals (49). However, the antitumor activity of the oligos has been attributed to the antisense effect (50). It remains to be determined whether SIA may play a role in the antitumor activity of oligos.

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