The Involvement of Ataxia-telangiectasia Mutated Protein Activation in Nucleotide Excision Repair-facilitated Cell Survival with Cisplatin Treatment*

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DNA damage can lead to either DNA repair with cell survival or to apoptotic cell death. Although the biochemical processes underlying DNA repair and apoptosis have been extensively studied, the mechanisms by which cells determine whether the damage will be repaired or the apoptotic pathway will be activated is largely unknown. We have studied the role of nucleotide excision repair (NER) in cisplatin DNA damage-induced apoptosis (2–7), the molecular mechanism that determines whether the damage will be repaired or that the damaged cells will undergo apoptosis is largely unknown. The lack of such knowledge has significantly limited our understandings of cancer cell drug resistance and hindered our abilities in the design and development of new drugs for effective cancer treatment.

Nucleotide excision repair (NER) is the major DNA repair pathway utilized in the repair of bulky DNA damage generated by most environmental insults and therapeutic drugs (1, 8, 9). The NER process is initiated by DNA damage recognition and the binding of the XPC-HR23B complex to damaged DNA (10–14), which further recruits other NER components including XPA, TFIIH, XPG, and XPF/ERCC1 to the damaged site (12, 13, 15, 16). The XPG protein makes a 3' incision, which is followed by a 5' incision made by the XPF/ERCC1, resulting in a single-stranded gap of 27–32 nucleotides (17). The DNA polymerases (pol ε or pol δ) fill the gap and the DNA ligase seals the gap to complete the DNA repair process. Interestingly, defects in most of the NER proteins, including XPA, XPB, XPD, XPF, and XPG, lead to elevated sensitivities of the cells to many DNA damaging reagents. However, defects of XPC and XPE proteins do not cause increased sensitivity of the cells to DNA damaging treatment (18). Therefore, studying the DNA damage-mediated signaling process in these NER-defective cells will provide important insights into the mechanism of DNA repair in preventing DNA damage-induced apoptosis.

DNA damage also promotes cell cycle checkpoint regulation. Both ATM and ATR proteins play important roles in DNA damage-induced cell cycle checkpoint regulation (19–21). The ATM protein is activated by DNA double strand breaks (22–25) and the ATR protein is activated by arrested replication forks (26–29). Once the ATM and/or ATR protein is activated, the kinase activity of the ATM and ATR proteins further phosphorylates a series of downstream protein targets including P53 (22, 24), BRCA1 (30), NBS1 (31), and CHK1/CHK2 (32–35). This causes cell cycle arrest at both the G1 and G2/M checkpoints.

Many anticancer drugs are targeted to the genomic DNA of cancer cells to generate DNA damage and block DNA replication and/or gene transcription, resulting in cell cycle arrest and apoptotic cell death (apoptosis). However, cancer cells can avoid this DNA damage-induced cell death through several mechanisms. DNA repair is one of the most important mechanisms that prevent DNA damage-induced cell death (1). Although many studies have been done regarding DNA repair and apoptosis (2–7), the molecular mechanism that determines whether the damage will be repaired or that the damaged cells will undergo apoptosis is largely unknown. The lack of such knowledge has significantly limited our understandings of cancer cell drug resistance and hindered our abilities in the design and development of new drugs for effective cancer treatment.

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2 The abbreviations used are: NER, nucleotide excision repair; NF, normal fibroblasts; Q-PCR, quantitative PCR; XPA, xeroderma pigmentosum group A; XPG, xeroderma pigmentosum group G; IP, immunoprecipitation; siRNA, small interfering RNA; AMC, 7-amino-4-methylcoumarin; ICL, intra- and interstrand cross-links.
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points, which allow DNA repair to take place and result in cell survival after DNA damaging treatment. Therefore, the activation of ATM/ATR proteins is essential for cell survival to various DNA damaging treatments.

We have studied the mechanisms by which the NER process prevents cisplatin treatment-induced apoptosis. Using both normal human fibroblasts (NF) and NER-defective xeroderma pigmentosum group A (XPA) and group G (XPG) cells, we have demonstrated that the NER defects caused an increased caspase-3 activation after cisplatin treatment. The results obtained from our Western blots reveal that cisplatin treatment results in enhanced CHK1 phosphorylation and p21 induction in both XPA and XPG cells. In contrast, cisplatin treatment-induced ATM phosphorylation at Ser-1891 is attenuated in XPA and XPG cells. The results obtained from our immunoprecipitation (IP) experiments indicate that the ATM protein interacts with the TFIH basal transcription factor, and that this interaction also requires the presence of the XPG protein. In addition, the IP results further reveal the requirement of a functional XPC protein for the association of the ATM protein to genomic DNA. These results suggest that the NER process may prevent cisplatin treatment-induced apoptosis by activating the ATM pathway, which in turn, enhances cell cycle arrest to allow for DNA repair to take place. The DNA damage recognition and binding of XPC protein helps recruit the ATM protein to the DNA template. These results provide an important insight into the mechanism by which the NER pathway coordinates with other cellular processes including cell cycle arrest and apoptosis in determining the fate of damaged cells.

MATERIALS AND METHODS

Cell Lines and siRNAs—The NF (GM00043), XPA (GM05509), XPC (GM16684), XPG (GM03021), and ataxiatelangiectasia mutated (ATM) (GM02052) cells were obtained from the NIGMS Human Genetic Cell Repository (Corriel Institute for Medical Research, Camden, NJ). All the cells are primary fibroblasts and were maintained in minimal essential medium supplemented with 15% fetal bovine serum, 2 mM glutamine, nonessential amino acids, and vitamins at 37 °C with 5% CO2.

The siRNAs against XPA and XPG genes were synthesized by Ambion Inc. (Austin, TX) and purified by high performance liquid chromatography. The siRNA against the XPA gene (XPA1 siRNA) contained a sequence of 5′-GGAGGAGCCUCUUUUG-3′ and the siRNA against the XPG gene (XPG1 siRNA) contained a sequence of 5′-GGAGAUGCUUGCCGUUG-3′. A control siRNA (Negative Control 2 siRNA) was also purchased from Ambion Inc. The control siRNA did not bind to any known target gene mRNA sequences.

Cisplatin Treatment—Cisplatin was purchased from Sigma and prepared fresh before the experiments. Cisplatin was dissolved into dimethyl sulfoxide reagent and added to the cell growth medium at a ratio of 1:2000 to desired final concentrations (0–40 μM). The 10 μM cisplatin is relevant to the dosage used in most of anticancer treatments. The cells were cultured at 37 °C for 3 h. The medium was replaced with fresh cell growth medium and the cells were cultured for various lengths of times before harvesting. For the caspase-3 assay, the cells were harvested 40 h after cisplatin treatment. For the Western blotting assay, the cells were harvested 16 h after the treatment.

Caspase-3 Assay—The cells were lysed in insect cell lysis buffer (BD Biosciences). The protein concentrations of the cell lysates were determined by a BCA assay (Sigma). Caspase-3 assay was performed in a 96-well plate using fluorogenic Ac-DEVD-AMC as a substrate (BD Biosciences). All samples and controls were run in triplicate. Caspase-3 activity was determined by a spectrophotometer (Molecular Devices) for detecting free AMC released from the substrate during a 15-min incubation period at 37 °C with an excitation wavelength of 380 nm and an emission wavelength of 430–460 nm. Caspase-3 activity (nanomole of AMC/min/mg of protein) was determined for each cell lysate.

siRNA Treatment—The NF cells were harvested and resuspended into Opti-Med I medium (Invitrogen Inc.) at a density of 1.5 × 10^6 cells/700 μl. The siRNA was added into the medium at 300 nM and the mixture was electropolitated with a setting of 250 V/950 μF. The cells were incubated at room temperature for 30 min and then plated onto 100-mm cell culture dishes at a density of 5 × 10^5 cells/dish. The cells were cultured at 37 °C for 24 h. Some cells were collected and lysed in RIPA cell lysis buffer for determining the silencing effect of the siRNAs using a Western blot assay. The rest of the cells were treated with cisplatin (0, 10, 20, and 40 μM) for 3 h and then cultured in fresh medium for various lengths of time before the cells were harvested for the desired assays.

Western Blot Assay—The cell lysates were prepared from the cisplatin-treated cells 16 h after the treatment. Western blotting was performed as described previously (36). The p-ATM (Ser-1891) (10H11.E12), CHK1, CHK2, p-CHK1 (Ser-317), pCHK1 (Ser-345), and pCHK2 (Thr-68) antibodies were purchased from Cell Signaling Inc. (Beverly, MA). The ATM (2C1), ATR (N-19), DNA-PK (H-163), CDK7 (N-19), p21 (C-19), p62 (Q-19), and p89 (S-19) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The XPC antibody (mouse monoclonal antibody) was purchased from Abcam Inc. (Cambridgeshire, United Kingdom). The β-actin antibody (Ab-1) was purchased from Oncogene Research Products (San Diego, CA). A mouse monoclonal antibody against human ATM protein (8F4) was generated in the laboratory of Dr. Y. A. Wang as described elsewhere (37).

Flow Cytometry Assay—The cells were seeded onto 100-mm dishes at a density of 3 × 10^5 cells/dish and incubated at 37 °C overnight. Some of the dishes were treated with 40 or 10 μM cisplatin for 3 h and then replaced with fresh cell growth medium. The cells were incubated at 37 °C and harvested at various lengths of time (day 0, day 1, day 2, and day 3). The cells were washed twice in 1× phosphate-buffered saline and fixed in 70% ethanol. For the flow cytometry assay, the cells were washed in 1× phosphate-buffered saline three times and then digested with RNase A (10 μg/ml) at 37 °C for 30 min. The cells were stained with 5 μg/ml propidium iodide at room temperature for 10 min and analyzed by FACS Calibur (BD Bioscience). The cell cycle profile was determined with ModFit LT software.

IP Assay—The IP assay was performed using a protocol designed for the chromatin immunoprecipitation assay (Upstate Biotechnology, Lake Placid, NY) with some modifications.
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RESULTS

The NER Defects Led to an Increased Caspase-3 Activation after Cisplatin Treatment—Although it is known that NER-defective cells are hypersensitive to many DNA damaging reagents, it is important to determine whether these elevated sensitivities are caused by DNA damage-induced apoptosis or other cellular responses such as necrosis. Therefore, we first determined the cisplatin treatment-induced activation of caspase-3, an important effector caspase indicative of irreversible apoptosis, in the NER-defective XPA and XPG cells (Fig. 1A). As a control, cisplatin treatment-induced caspase-3 activation was also determined in human NF cells (Fig. 1A). The cisplatin treatment-induced caspase-3 activation was also determined in human NF cells (Fig. 1A). The cisplatin treatment-induced caspase-3 activation was also determined in human NF cells (Fig. 1A).

In brief, the cultured cells (40–50% confluence) were treated with cisplatin (40 μM) for 3 h and then cultured in fresh cell growth medium for 3 h. The cells were fixed in 1% formaldehyde at 37 °C for 10 min and then washed with ice-cold 1× phosphate-buffered saline three times. The cells were resuspended into SDS lysis buffer (1 × 10^6 cells/200 μl) and sonicated in the same condition. The cell lysates were centrifuged at 4 °C for 10 min and the supernatants were collected. For the IP assay, the cell lysate (200 μl) was diluted at a ratio of 1:10 in the chromatin immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). A desired primary antibody (2 μg) was then added into the cell lysate and the mixture was incubated at 4 °C overnight. The mixture was incubated with Protein A/G Plus-conjugated agarose beads (40 μl) (Santa Cruz Biotechnologies) at 4 °C for 2 h. The beads were collected and washed three times in a washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl). Half of the beads were analyzed by Western blot for the detection of the desired proteins, including ATM, XPC, CDK7, and p89. The rest of the beads were resuspended into 100 μl of elution buffer (0.1 M NaCO3, 1% SDS, 200 mM NaCl) and incubated at 65 °C for 4 h to reverse the protein-DNA cross-links. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The amount of DNA carried by the beads was determined by a quantitative PCR (Q-PCR) assay for the detection of both β-actin and p53 gene sequences using primers that bind to either the β-actin or p53 gene coding region sequences. The primers used in the Q-PCR assay are the p53 forward primer (5′-CTGCACAGCAGCTCTACACC-3′), the p53 reverse primer (5′-CGTGCAAGTCAGAGACTCTTGCTG-3′), the β-actin forward primer (5′-GTACGTTGCTATCCAGGCTTG-3′), and the β-actin reverse primer (5′-CATGAGGTAGTCAGTCAGGTC-3′). The threshold cycle (Ct) obtained from the Q-PCR assay was used to determine the relative amount of DNA pulled down by the beads from individual cell lysates. We have chosen the β-actin and p53 genes as our targets for the Q-PCR assay because of their different responses to the DNA damaging treatment: the level of β-actin protein is not affected by DNA damaging treatment, whereas the level of p53 is increased following most DNA damaging treatments.

Statistic Analysis—Results are expressed as the mean ± S.D. Statistically significant differences were determined using a one-factor analysis of variance with p < 0.01.
respectively. As a negative control, cisplatin treatment did not cause a significant increase of caspase-3 activity in XPC cells even at 40 μM (Fig. 1A).

It was necessary to determine whether the increased caspase-3 activity of XPA and XPG cells following cisplatin treatment was indeed caused by defects of the XPA and XPG proteins, but not the other possible defects carried by these cells. Therefore, we studied further cisplatin treatment-induced caspase-3 activation in NF cells in which the XPA and XPG genes were individually silenced with specific siRNAs (Fig. 1B). As expected, silencing the XPA and XPG genes led to greater caspase-3 activations in NF cells following cisplatin treatment than in those of NF cells in which the XPA and XPG functions remained (Fig. 1C). Interestingly, when both XPA and XPG genes were silenced in the same experiment, more increased caspase-3 activities were detected in NF cells following cisplatin treatment. As a negative control, when the NF cells were treated with a control siRNA (negative control 2 siRNA) at the same concentration, cisplatin treatment-induced caspase-3 activity remained similar to that of the NF cells. These results suggest that a functional NER pathway is required for cell survival from cisplatin treatment and that defects of XPA and/or XPG proteins result in an increased apoptotic cell death of the cells following cisplatin treatment.

The NER Defects Resulted in Altered Cell Cycle Arrest following Cisplatin Treatment—The results obtained from our caspase-3 activation assay suggested an important role of the NER process in preventing cisplatin treatment-induced apoptosis. To determine whether the pattern of cisplatin treatment-induced cell cycle arrest was also affected by the NER defects, we conducted a flow cytometry assay for cisplatin-treated NF, XPA, and XPG cells (Fig. 2). Indeed, the pattern of cisplatin treatment-induced cell cycle arrest was affected by NER defects. In NF cells, 40 μM cisplatin treatment caused a more transient accumulation of cells at the G2/M checkpoint of the cell cycle. In both XPA and XPG cells, however, the cisplatin treatment-induced G2/M arrest was diminished. Instead, a large proportion of the cells were accumulated at the G1 phase. The sub-G1 cell population, indicative of cell death, also had accumulated over time in cisplatin-treated XPA and XPG cells (Fig. 2). To determine whether cisplatin concentrations have some effect on the cell cycle profile of the cells, we also performed a flow cytometry assay using cells treated with 10 μM cisplatin (supplemental data Fig. 1). The results obtained from this study revealed similar cell cycle profiles for the cells treated with 10 or 40 μM cisplatin. The proportion of the sub-G1 population in 10 μM cisplatin-treated XPA and XPG cells, however, was less than those treated with 40 μM cisplatin. These results
suggest that the NER defects disrupt the normal cellular process and cause altered cell cycle regulation, resulting in increased cell death following cisplatin treatment.

The NER Defects Caused an Enhanced CHK1 Phosphorylation and p21 Induction after Cisplatin Treatment—To determine whether the increased G1 population in cisplatin-treated XPA and XPG cells was caused by an altered cell cycle checkpoint regulation or continued cell growth, we determined the cisplatin treatment-induced phosphorylation of CHK1 (Ser-317 and Ser-345) and the induction of p21CIP1 in both NF and the NER-defective XPA and XPG cells. Phosphorylation of CHK1 at Ser-317 and Ser-345 is an important indication of CHK1 activation that leads to cell cycle arrest at the G1 phase (34, 38, 39). Similarly, induction of p21CIP1 is important for maintaining cells in the G1 phase (40, 41). The levels of the phosphorylated CHK1 protein (both Ser-317 and Ser-345) were significantly affected by the NER defects following cisplatin treatment (Fig. 3, A and B). Cisplatin treatment caused a modest increase in CHK1 phosphorylation in the NF cells (Fig. 3A, lanes 1–4). In XPA and XPG cells, the magnitude of increase in CHK1 phosphorylation in response to cisplatin treatment was greater, and the maximum increases occurred at lower cisplatin concentrations (Fig. 3A). The level of the phosphorylated CHK2 (Thr-68), another important checkpoint kinase, however, was not significantly affected by the NER defects following cisplatin treatment (data not shown).

To confirm that the enhanced CHK1 phosphorylation observed in the cisplatin-treated XPA and XPG cells were indeed caused by defects of the XPA and XPG proteins of the cells, we further determined cisplatin treatment-induced CHK1 phosphorylation in NF cells in which the XPA and XPG genes were silenced by siRNAs (Fig. 3C). Silencing of individual XPA and XPG genes resulted in enhanced CHK1 phosphorylation of NF cells following cisplatin treatment. When both XPA and XPG genes were silenced in the same experiment, cisplatin treatment-induced phosphorylation of CHK1 in these cells was even stronger than in those of the NF cells for which the XPA or XPG genes were individually silenced by the siRNAs.

The NER defects also caused an enhanced p21CIP1 response following cisplatin treatment (Fig. 4). In NF cells, cisplatin treatment caused a modest increase in p21CIP1 protein. In the XPA and XPG cells, however, cisplatin treatment resulted in greater increases in the level of p21CIP1 protein. These results suggest that the altered cell cycle distributions of both XPA and XPG cells following cisplatin treatment were indeed caused by an altered cell cycle arrest of the cells.

The NER Defects Caused Attenuated ATM Phosphorylation after Cisplatin Treatment—The results obtained from both the caspase-3 activation and flow cytometry experiments suggest that a functional NER pathway is required for cell survival to cisplatin treatment. The results obtained from our previous studies also demonstrated the involvement of the p53 signaling pathway in the cisplatin treatment-induced signaling process (36). To elucidate a mechanism by which the NER process leads to increased cell survival and p53 activation following cisplatin treatment, we further determined the involvement of ATM/ ATR activation in this process.

It is known that phosphorylation of the ATM protein at Ser-1981 plays an important role in ATM activation (22–25). Therefore, we first determined cisplatin treatment-induced ATM phosphorylation at Ser-1981 in both NF and NER-defective XPA and XPG cells (Fig. 5A). Cisplatin treatment caused a dose-dependent increase in the level of phosphorylated ATM protein (Ser-1981) in NF cells (Fig. 5A, lanes 1–4). When the XPA and XPG cells were treated with cisplatin at similar concentrations, however, the levels of phosphorylated ATM protein were attenuated (Fig. 5A, lanes 5–8 and 9–12 versus lanes 1–4). The level of total ATM protein, however, was not affected by cisplatin treatment in these cells (Fig. 5A). As controls, the levels of DNA-PK and β-actin were not affected by cisplatin treatment (Fig. 5A). We also determined the protein level of
ATR protein in these cells. No significant change was seen in the ATR protein level between the NF and NER-defective cells following cisplatin treatment (Fig. 5B). Taken together, these results suggest that the NER process is required for ATM phosphorylation, implying an important role of ATM activation in NER-facilitated cell survival following cisplatin treatment.

The ATM Protein Interacted with TFIIH Basal Transcription Factor and XPG Protein —The results obtained from our Western blot experiments suggest the involvement of ATM phosphorylation in the cellular response to cisplatin treatment. To elucidate a mechanism by which the ATM protein is linked with the NER process, we further determined the NER components that interacted with the ATM protein using an IP assay. An ATM antibody was used to pull down the ATM protein and the NER components that were pulled down with the ATM protein in the IP assay were identified by Western blot (Fig. 6A). The results obtained from our Western blot revealed that the ATM protein was pulled down from all cell lysates (Fig. 6A, top panel). CDK7 and p89, both of which are components of the TFIIH basal transcription factor, were pulled down from the cell lysates of NF, XPA, and XPC cells but not the XPG cells (Fig. 6A, lanes 1, 2, and 5–8 versus lanes 3 and 4). The XPC protein was pulled down from the cell lysates of NF and XPA cells but not XPG or XPC cells (Fig. 6A, lanes 1, 2, 5, and 6 versus lanes 3, 4, 7, and 8). As a negative control, none of these proteins were pulled down from the NF cell lysates by the Protein G-conjugated agarose beads alone without the ATM antibody (Fig. 6A, lanes 9 and 10 versus lanes 1–8).

We also determined the NER components required for recruitment of the ATM protein to the genomic DNA by detection of the genomic DNA pulled down with the ATM protein in the IP experiments. A Q-PCR assay was used for the study (Table 1) (42). The results obtained from the Q-PCR assay indicated that high levels of genomic DNA were pulled down from the NF and XPA cells, but not the XPC cells as evidenced by the amounts of p53 and actin gene DNA detected from the beads in the IP experiment (Table 1). Low levels of p53 and actin DNA were also pulled down from the XPG cell lysates in the IP experiment (Table 1).

To confirm the ATM IP results, we further performed a reciprocal IP experiment using antibodies that recognized either the p62 of TFIIH or the XPC protein (Fig. 6B, 8, and C). When the TFIIH p62 antibody was used as the primary antibody for the IP experiment, both ATM and XPC proteins were pulled down from the NF, XPG, and XPA cells (Fig. 6B). The amounts of ATM protein pulled down from the XPG cell lysates, however, were less than those from other cell lysates (Fig. 6B). Similarly, when the XPC antibody was used as the input antibody in the IP experiment, both TFIIH (CDK7) and ATM proteins were pulled down from the NF, XPA, and XPG cells (Fig. 6C). As controls, no ATM protein was pulled down from the ATM cells or the NF cells that contained no XPC gene DNA detected from the beads (Fig. 6C, lanes 7–10 versus lanes 1–6). These results confirmed the ATM IP results and suggested that the ATM protein interacted with the TFIIH and the XPC protein.

**FIGURE 5.** Cisplatin treatment-mediated ATM and ATR responses in NF, XPA, and XPG cells. The cells were treated with cisplatin at the indicated concentrations for 3 h and then cultured in fresh cell growth medium for 16 h before the cells were harvested and cell lysates were prepared. A, the cisplatin treatment-induced phosphorylation of the ATM protein at Ser-1981. The protein levels of ATM, phosphorylated ATM (Ser-1981), DNA-PK, and β-actin were determined in the cell lysates. B, cisplatin treatment-induced ATR expression. The protein levels of ATR and β-actin were determined in the cell lysates.

**FIGURE 6.** Identification of the NER components that interact with the ATM protein using an IP assay. Both untreated and cisplatin-treated cells were fixed in formaldehyde and sonicated to shear genomic DNA to lengths of 200–1000 bp. An IP assay was then performed to pull down the desired target proteins and the proteins that were associated with the target proteins. As controls, some of the reactions contained only the cell lysates and beads but without the primary antibody (lanes 9 and 10 in A, lane 9 in B, and lane 9 in C, this figure) and some of the reactions contained the normal IgG instead of the primary antibody (lane 10 in C). The NF cell lysate was used as the positive control (lane 10 in B and lane 11 in C).
decreased ATM protein pulled down from the XPG cell lysates also suggested the requirement of XPG protein for the interaction of ATM protein with the TFIIH.

All these results suggest that the ATM protein interacts with the TFIIH basal transcription factor and that the presence of XPG protein is required for this interaction. These results also suggest that the function of XPC protein is essential for the association of the ATM protein with genomic DNA.

DISCUSSION

We have studied the role of NER in cisplatin treatment-induced apoptotic cell death and the involvement of ATM activation in this process. Using both NF and NER-defective XPA and XPG cells, we demonstrated that the NER defects led to an altered cell cycle arrest and an increased apoptotic cell death after cisplatin treatment. The results obtained from our Western blotting indicated that cisplatin treatment resulted in enhanced CHK1 phosphorylation and p21 induction in both XPA and XPG cells. We further demonstrated that the ATM protein was phosphorylated in cisplatin-treated NF cells, whereas this cisplatin treatment-induced ATM phosphorylation was attenuated in XPA and XPG cells. The results obtained from our IP experiment indicated that the ATM protein interacted with the TFIIH basal transcription factor and the XPG protein might stabilize this interaction. In addition, the Q-PCR results also suggested the requirement of a functional XPC protein for the association of ATM protein with genomic DNA. Taken together, these results suggest that the NER process may prevent cisplatin treatment-induced apoptotic cell death by phosphorylating the ATM protein and activating the ATM pathway, resulting in enhanced cell cycle arrest for DNA repair and cell survival after the treatment. These results further suggest that the XPC protein plays an important role in recruiting the ATM protein to genomic DNA and in linking the NER pathway with other cellular processes such as cell cycle regulation and apoptosis. Therefore, the results obtained from these studies provide important insights into the mechanism of cisplatin treatment-mediated cellular responses including DNA repair, cell cycle arrest, and apoptosis and suggest that the interplay of the NER and ATM pathways helps determine the fate of the damaged cells.

The results obtained from the caspase-3 activation experiments demonstrated that the NER defects resulted in increased caspase-3 activation following cisplatin treatment. The results obtained from our Western blot experiments revealed that cisplatin treatment caused phosphorylation of the ATM protein at Ser-1981 in NF cells, whereas the level of this ATM phosphorylation was attenuated in the NER-defective XPA and XPG cells after treatment. Because of the important role of ATM phosphorylation in the activation of the ATM pathway, these results suggest that the ATM pathway also plays an important role in NER-facilitated cell survival from cisplatin treatment. However, the ATM protein is normally activated by double strand breaks generated by DNA damaging treatment such as ionizing radiation and x-ray (25, 43). In comparison, the major DNA damage generated by cisplatin is bulky DNA damage such as intra- and interstrand cross-links (ICLs) (44 – 46). The mechanism that is responsible for ATM activation following cisplatin treatment is unknown. However, attenuated ATM phosphorylation in both XPA and XPG cells following cisplatin treatment suggests that the NER process is essential for this ATM phosphorylation. Because the NER process can generate many different types of DNA repair intermediates such as strand breaks, gaps, and single-stranded regions (8, 9, 21), it is possible that some of these repair intermediates (e.g. strand breaks) cause an activation of the ATM protein (21). In addition, the unique DNA damage generated by cisplatin, ICLs, may also contribute to this ATM activation. Although the mechanism of repairing ICLs is not fully understood, it is known that NER is involved in this repair process (47 – 55). It is possible that the ICL-mediated NER process generates the DNA repair intermediates required for ATM activation, whereas the defects of XPA or XPG proteins prevent the NER process and the generation of these DNA repair intermediates. In the absence of these repair intermediates, the ATM protein remains inactive and cisplatin treatment causes an altered cellular process and an increased apoptotic cell death. Further studies need to determine the DNA repair intermediates required for the ATM activation.

The results obtained from our IP studies suggest that the ATM protein interacts with the TFIIH basal transcription factor and this interaction also requires the presence of XPG protein. The results obtained from the Q-PCR experiments further demonstrate the requirement of XPC protein for the association of the ATM protein with genomic DNA. These results may provide important insights into a mechanism by which the ATM pathway is linked with the NER pathway. It is known that TFIIH binds with the XPC/HR23B complex to form an XPC-TFIIH complex during the NER process (56 – 60). Binding of XPC protein at the damaged DNA is essential for recruiting other NER components and initiating the NER process (12, 13, 61, 62). The results obtained from this study further reveal that the ATM protein interacts with the TFIIH basal transcription factor. It is possible that the ATM protein is linked to the NER process through these interactions and that XPC-TFIIH complex formation helps bring the ATM protein to the genomic DNA. The NER process-generated DNA repair intermediates then cause phosphorylation of the ATM protein, which leads to the activation of the ATM pathway and results in cell cycle arrest for DNA repair and cell survival after cisplatin treatment.

### TABLE 1

Determination of the level of DNA pulled down with the ATM protein in the IP assay

| Gene     | NF | NF + cis-Pt | XPA | XPA + cis-Pt | XPG | XPG + cis-Pt | XPC | XPC + cis-Pt | Primers alone |
|----------|----|-------------|-----|--------------|-----|--------------|-----|--------------|---------------|
| p53      | 1.0| 23 ± 0.5    | 0.1 ± 0.01 | 5.8 ± 0.8 | <0.01 | ND*          | ND  | ND           | ND            |
| β-Actin  | 1.0| 1.6 ± 0.1   | 0.3 ± 0.1  | 8.6 ± 0.3 | 0.01 | ND*          | ND  | ND           | ND            |

*ND, not detected.*
Further studies are needed to determine the roles of these interactions in DNA damage-induced cellular responses including DNA repair, cell cycle arrest, and apoptosis.

Many studies have demonstrated the requirement of the Mre11-Rad50-Nbs1 (MRN) protein complex in recruiting ATM proteins to double strand break damage sites (63–67). The results obtained from this study revealed the recruitment of the ATM protein to genomic DNA via the NER components. These results suggest that the ATM protein may play an important role in a broad range of DNA damage-induced cell cycle checkpoint regulations. Therefore, the results obtained from this study will have important clinical implications in cancer treatment for both radiation therapy and chemotherapy.

The results obtained from our flow cytometry studies revealed that cisplatin treatment causes a G1/M arrest in NF cells, whereas the NER defects reduce this arrest and result in an increased G1 arrest in both XPA and XPG cells. This result is consistent with the Western blot data that demonstrated the requirement of the NER pathway in cisplatin treatment-induced phosphorylation of the ATM protein. Because the activation of the ATM protein can cause both G1 and G2/M arrests, it is likely that the NER process is required for the G1 progression that results in the accumulation of damaged cells at the G2/M phase, whereas the NER defects of both XPA and XPG cells fail this G1 progression, resulting in an increased accumulation of damaged cells at the G1 phase and increased apoptotic cell death over time. In addition, the results obtained from our Western blot experiments also revealed an increased CHK1 phosphorylation in both XPA and XPG cells following cisplatin treatment. Because the CHK1 protein is preferentially phosphorylated by the ATR protein (68–71), these results suggest a possible role of ATR activation in this altered cell cycle arrest. The results obtained from this study revealed the recruitment of ATM proteins to double strand break damage sites (63–67). The MRN protein complex in recruiting DNA repair, cell cycle arrest, and apoptosis.

Further studies are needed to determine the relationship of ATM and ATR activation in DNA damage-induced cellular responses including DNA repair, cell cycle arrest, and apoptosis.

Although we focused our work on studying the role of the NER process and ATM activation in cell survival to cisplatin treatment, it is possible that many other cellular processes are also affected by the NER defects following cisplatin treatment, which may contribute to the increased sensitivity of the NER-defective cells with treatment. Further studies need to identify these pathways and determine their roles in cisplatin treatment-induced cellular responses.

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