Original Research Articles

The Role of Bcl-x(L) Protein in Nucleotide Excision Repair–Facilitated Cell Protection Against Cisplatin-Induced Apoptosis

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Many anticancer drugs target the genomic DNA of cancer cells by generating DNA damage and inducing apoptosis. DNA repair protects cells against DNA damage–induced apoptosis. Although the mechanisms of DNA repair and apoptosis have been extensively studied, the mechanism by which DNA repair prevents DNA damage–induced apoptosis is not fully understood. We studied the role of the antiapoptotic Bcl-x(L) protein in nucleotide excision repair (NER)–facilitated cell protection against cisplatin-induced apoptosis. Using both normal human fibroblasts (NF) and NER-defective xeroderma pigmentosum group A (XPA) and group G (XPG) fibroblasts, we demonstrated that a functional NER is required for cisplatin-induced transcription of the *bcl-x(l)* gene. The results obtained from our Western blots revealed that the cisplatin treatment led to an increase in the level of Bcl-x(L) protein in NF cells, but a decrease in the level of Bcl-x(L) protein in both XPA and XPG cells. The results of our immunofluorescence staining indicated that a functional NER pathway was required for cisplatin-induced translocation of NF- κ B p65 from cytoplasm into nucleus, indicative of NF- κ B activation. Given the important function of NF- κ B in regulating transcription of the *bcl-x(l)* gene and the Bcl-x(L) protein in preventing apoptosis, these results suggest that NER may protect cells against cisplatin-induced apoptosis by activating NF- κ B, which further induces transcription of the *bcl-x(l)* gene, resulting in an accumulation of Bcl-x(L) protein and activation of the cell survival pathway that leads to increased cell survival under cisplatin treatment.

Introduction

DNA DAMAGE HAS THE CAPABILITY of disrupting genomic stability and causing the development of many disease conditions (Friedberg *et al.*, 1995, 2006). DNA damage can also lead to apoptosis of cells. Therefore, the elimination of DNA damage, either by DNA repair or by programmed cell death (apoptosis), is essential for maintaining genetic integrity and preventing disease development. More importantly, many anticancer drugs also target cancer cells by generating DNA damage and promoting apoptosis of cancer cells (Perry, 2001). Therefore, understanding the molecular mechanism by which the DNA repair and apoptosis pathways coordinate to determine the fate of damaged cells has important implications in cancer prevention and treatment. Although great research efforts have been devoted to both DNA repair and apoptosis (Friedberg *et al.*, 1995, 2006; Sancar *et al.*, 2004; Chowdhury *et al.*, 2006; Houtgraaf *et al.*, 2006; Jeggo and Lobrich, 2006), this mechanism remains largely unknown.

DNA repair is a positive response instructing cells to remove DNA damage and to restore disrupted cellular func-

tions. Nucleotide excision repair (NER) is the major DNA repair pathway that repairs bulk DNA damage generated by most environmental factors (e.g., UV radiation) and therapeutic drugs (e.g., cisplatin) (Friedberg *et al.*, 1995, 2006). The NER can be further distinguished into transcription-coupled NER (TC-NER or TCR) and global genome NER (GG-NER or GGR) subpathways. The TCR is initiated by transcription blockage and is used to quickly repair DNA damage in the transcribed sequences (Sarker *et al.*, 2005; Laine and Egly, 2006), whereas the GGR is initiated by DNA damage recognition of an XPC-HR23B complex and is used to repair DNA damage in the entire genome (Wakasugi and Sancar, 1999; Volker *et al.*, 2001; Hey *et al.*, 2002; Janicijevic *et al.*, 2003). The TCR responds to DNA damage very quickly, whereas the GGR takes place at a much slower rate (Hanawalt, 1994, 2002). The signal of DNA damage recognition further recruits other NER components, including RPA, TFIIH, xeroderma pigmentosum group A (XPA) protein, xeroderma pigmentosum group G (XPG) protein, and XPF-ERCC1, to the damaged site (Wakasugi and Sancar, 1999; Volker *et al.*, 2001; Hey *et al.*, 2002; Janicijevic *et al.*, 2003). The damaged bases are excised by the dual incisions made

by XPG and XPF-ERCC1, which creates a ~30 nucleotide single-stranded DNA gap (Evans *et al.*, 1997). The DNA polymerases ($\text{pol } \epsilon$ or δ) fill the gap by resynthesizing DNA using the complementary DNA strand as a template, and DNA ligase seals the gap, completing the NER process (Shivji *et al.*, 1995). The repair of DNA damage generated by DNA crosslinking reagents, such as psoralen and cisplatin, requires the NER and other DNA repair pathways (De Silva *et al.*, 2000; Chen *et al.*, 2003, 2004; Zheng *et al.*, 2003, 2006; Barber *et al.*, 2005; Nojima *et al.*, 2005; Cheng *et al.*, 2006; Liu *et al.*, 2006; Sarkar *et al.*, 2006; Shen *et al.*, 2006). Defects in the NER pathway lead to the increased sensitivity of cells to many DNA-damaging reagents (Satoh and Hanawalt, 1997; de Laat *et al.*, 1998; Matsumura *et al.*, 1998; Koberle *et al.*, 1999; Lalle *et al.*, 2002; Selvakumaran *et al.*, 2003). In addition, defects of NER components have been associated with many types of cancer—especially, skin, lung, and bladder cancer (Chen *et al.*, 2007; Wu *et al.*, 2007).

DNA damage also promotes other cellular responses, including cell cycle arrest and apoptosis (Sancar *et al.*, 2004; Jeggo and Lobrich, 2006). In general, cell cycle arrest is more beneficial to the damaged cells because it will provide the time required for completion of DNA repair and restoration of the disrupted cellular functions. With increased levels of DNA damage, however, the cells will eventually undergo apoptosis to remove the severely damaged cells from the system. Although the mechanism that determines whether the damaged cells will be saved or will undergo apoptosis is unclear, it is believed that the coordination of DNA repair and apoptosis plays a critical role in determining the fate of the damaged cells. In addition, the Bcl-2 family proteins are also known to play an important role in DNA damage-induced cellular responses (Yang *et al.*, 1997; Reed, 2006; Zinkel *et al.*, 2006). Some of the Bcl-2 family proteins, including Bcl-2 and Bcl-x(L), prevent cells against DNA damage-induced apoptosis, whereas other Bcl-2 family proteins, including BAD, BAX, BID, and PUMA, enhance DNA damage-induced apoptosis. The antiapoptotic proteins Bcl-2 and Bcl-x(L) inhibit apoptosis by blocking the release of cytochrome c from the mitochondrial membrane (Kuwana *et al.*, 2002), whereas the proapoptotic proteins BAK and BAX stimulate apoptosis by causing the release of cytochrome c (Wei *et al.*, 2001). PUMA and BAD also function as proapoptotic proteins by inactivating the antiapoptotic Bcl-2 and Bcl-x(L) proteins (Bae *et al.*, 2001; Ming *et al.*, 2006). The release of cytochrome c from the mitochondrial membrane into the cytosol causes sequential activations of a series of caspase cascades, including caspase-8 and caspase-3, resulting in apoptosis of the damaged cells. Several Bcl-2 family genes, including *bax*, *bak*, and *puma*, are transcriptionally regulated by p53 protein (Jeffers *et al.*, 2003; Chipuk *et al.*, 2004; Leu *et al.*, 2004), whereas the antiapoptotic *bcl-x(l)* and *Bcl-2* genes are transcriptionally regulated by NF- κ B (Mori *et al.*, 2001; Huerta-Yepez *et al.*, 2004).

We have studied the role of the antiapoptotic protein Bcl-x(L) in NER-facilitated cell protection against cisplatin-induced apoptosis. The results obtained from our reverse transcription-based quantitative PCR (real-time PCR) studies revealed that the cisplatin treatment caused an increase in transcription of the *bcl-x(l)* gene in the NER-proficient normal human fibroblast (NF) cells but a decrease in transcription of the *bcl-x(l)* gene in the NER-deficient XPA and XPG cells. The

results obtained from our Western blots demonstrated that the cisplatin treatment led to an increase of the Bcl-x(L) protein in NF cells but a decrease of the Bcl-x(L) protein in both XPA and XPG cells. The results of our immunofluorescence staining further demonstrated that a functional NER pathway is required for cisplatin-induced translocation of the NF- κ B from cytoplasm into nucleus, indicative of activation of NF- κ B pathway. Taken together, these results suggest that NER may protect cells against cisplatin-induced apoptosis by activating the NF- κ B, which further induces transcription of the *bcl-x(l)* gene, resulting in an increased accumulation of the antiapoptotic Bcl-x(L) protein and enhanced cell survival under cisplatin treatment.

Materials and Methods

Cell lines and siRNAs

The NF (GM00043), XPA (GM05509), and XPG (GM03021) fibroblasts were obtained from the NIGMS Human Genetic Cell Repository (Corriell Institute for Medical Research, Camden, NJ). All cells were primary fibroblasts and maintained in MEM medium supplemented with 15% FBS, 2 $\times$ essential amino acids, 2 $\times$ nonessential amino acids, and 2 $\times$ vitamins with 2 mM L-glutamine at 37°C with 5% CO₂.

The siRNAs against *xpa* and *xpg* genes have been previously described (Colton *et al.*, 2006) and were synthesized by Ambion (Austin, TX). The siRNA against *xpa* gene (XPA1 siRNA) contained a sequence of 5'GGAGGAGGCUUCAUUUAGtt3', and the siRNA against the *xpg* gene (XPG1 siRNA) contained a sequence of 5'GGGAAGAUCUGGCUGUU Gtt3'. A control siRNA (negative control 2 siRNA) was also purchased from Ambion. Our previous studies have demonstrated the highly specific gene silencing effect of the XPA and XPG siRNAs (Colton *et al.*, 2006). The control siRNA does not bind to any known target gene mRNA sequences.

Cisplatin treatment

The cisplatin was purchased from Sigma (St. Louis, MO). The NF, XPA, and XPG cells were plated onto 100 mm cell culture dishes at a density of 1.2 $\times$ 10⁶ cells/dish and incubated at 37°C overnight. The cisplatin was freshly dissolved into dimethyl sulfoxide (DMSO) and added immediately to the cell culture medium. The cells were cultured in the cisplatin-containing medium for 3 h and then cultured in fresh cell growth medium. The cells were harvested at various time points after the cisplatin treatment and used for further studies.

Real-time quantitative PCR assay

Total RNA was isolated from both untreated and cisplatin-treated cells using an RNeasy mini isolation kit (Qiagen, Valencia, CA). A reverse transcription-based quantitative PCR (real-time PCR) was performed to determine the mRNA level of the *bcl-x(l)* gene from each RNA sample using a master mix for the *bcl-x(l)* gene that contained the forward primer, reverse primer, and 6FAM dye-MGB labeled probe for the *bcl-x(l)* gene (Bcl-xL Hs00169141_m1 from Applied Biosystems, Foster City, CA). The mRNA level of the *bcl-2* gene was also determined for each RNA sample by the real-time PCR using a master mix for the *bcl-2* gene (Bcl-2 Hs00608023_m1 from Applied Biosystems). The mRNA level of *actin* gene was also determined for each RNA sample

using real-time PCR assay. The reverse transcription assay was carried out using 2 µg of total RNA with the protocol suggested by the manufacturer (Applied Biosystems). The PCR procedure was performed using Taq-Man Universal PCR master mix with 100 ng cDNA in a total volume of 20 µL. The PCR assays were completed using the ABI Prism 7500 sequence detection system (Applied Biosystems) with the following conditions: 2 min at 50°C, followed by 20 s at 95°C, and then 40 cycles of 3 s at 95°C and 30 s at 60°C. The real-time PCR data was analyzed using a comparative cycle threshold (C_t) method. Relative quantification was performed to compare gene expression between untreated and cisplatin-treated cells. The *actin* gene was used as an internal control for normalization. Relative expression of the desired target genes was calculated as $2^{-\Delta\Delta C_t}$ where ΔC_t was calculated by subtracting the average normalization gene C_t (*actin*) from the average target gene C_t value in the same cell line. The $\Delta\Delta C_t$ was obtained by the ΔC_t of the treated cells subtracted from the ΔC_t of the untreated cells.

Western blot hybridization and quantification of the Bcl-x(L) protein

Both untreated and cisplatin-treated cells were harvested and lysed in RIPA cell lysis buffer (1×PBS, 1% NP40, 0.5% deoxycholic acid, and 0.1% SDS). The cell lysates (40 µg total protein) were analyzed by SDS-PAGE using 4–20% gradient gel (Bio-Rad, Hercules, CA). The proteins were transferred to a PVDF membrane and hybridized with a Bcl-2 or a Bcl-x(L) antibody (Cell Signaling, Beverly, MA) to detect the respective protein. The same membrane was then soaked in a stripping solution (62.5 mM Tris, pH 6.8, 2% SDS, 0.7% 2-mercaptoethanol) at 50°C for 30 min and then hybridized with an actin antibody (Oncogene, Cambridge, MA) to determine the level of actin in each sample. Quantification of the Western results was performed using a Kodak Image Station 440CF system (Eastman Kodak Inc., Rochester, NY), and the level of Bcl-x(L) protein in each cell lysate was expressed a relative level to that of actin in the same cell lysate. The level of Bcl-x(L) protein in the cisplatin-treated cells was calculated as compared to that of the Bcl-x(L) protein in the untreated cells. The statistical analysis of the Western data was done using GraphPad Prism 4.0 software (GraphPad, San Diego, CA).

siRNA treatment

The cells were collected and resuspended into the Opti-Med I medium at a density of 1.5×10^6 cells/700 µL. The siRNAs were added to the suspended cells at a concentration of 300 nM. The cells were electroporated with a setting of 250 V/950 µF (Bio-Rad). 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 and incubated at 37°C for 24 h. The cells were then treated with various concentrations of cisplatin (0, 10, 20, and 40 µM) for 3 h and incubated in fresh medium for 24 h before the cells were harvested and lysed in RIPA cell lysis buffer. The cell lysates were analyzed by Western blots to determine the protein level of Bcl-x(L) in each lysate. The protein levels of XPA and XPG protein were also determined for the cell lysates on the same membranes to determine the silencing effect of XPA and XPG siRNAs. The protein level of actin was also determined for each cell lysate on the same membrane as a protein

loading control. Quantification of the Bcl-x(L) protein was done as previously described.

Immunofluorescence staining

The NF, XPA, and XPG cells were seeded onto glass coverslips in 100 mm cell culture dishes at a density of 3×10^5 cells/dish and cultured at 37°C overnight to allow for the cells to attach to the coverslips. The individual coverslips were then transferred to 12-well plates that contained 2 mL medium per well. Some of the coverslips were treated with 40 µM cisplatin for 3 h and then placed in fresh medium and cultured at 37°C for 24 h. The cells were fixed to coverslips by incubating in 4% paraformaldehyde/PBS solution for 30 min. The fixed cells were washed three times with 1×PBS and permeabilized with methanol for 5 min on ice. After washing three times with 1×PBS, the coverslips were incubated with 10% FBS/1×PBS for 30 min to block nonspecific immunoglobulin binding. The coverslips were then washed and incubated with NF-κB p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:200 in 10% FBS/1×PBS/0.1% saponin for 1 h at room temperature. After washing three times with 10% FBS/1×PBS, the coverslips were incubated with FITC-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology) in 10% FBS/1×PBS at 1:400 dilution for 1 h at room temperature. After washing three times in 10% FBS/1×PBS solution and a quick wash in water, the cover slips were mounted onto glass slides using UltraCruz mounting medium containing DAPI DNA counterstaining reagent (Santa Cruz Biotechnology). The presence of NF-κB in the stained cells was detected using an Axioplan 2 fluorescence microscope with a 540 nm light source and 40×amplification (Carl Zeiss, München-Hallbergmoos, Germany). The localization of the nucleus of the stained cells was determined using the fluorescence microscope with a 485 nm light source. The digital images were captured using an ApoTome optical sectioning device (Zeiss). The merged image of the NF-κB and nucleus was generated using the Axiovision (Zeiss).

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Statistically significant differences were determined using a one-factor analysis of variance with $p < 0.01$. The quantification of the Bcl-x(L) protein in these studies was obtained from at least three independent experiments.

Results

The requirement of functional NER for cisplatin-induced accumulation of Bcl-x(L) protein

The results of our previous studies revealed the requirement of a functional NER in protecting cells from cisplatin-induced apoptosis (Colton *et al.*, 2006). To elucidate a mechanism by which the NER protects cells against cisplatin-induced apoptosis, we further determined the involvement of Bcl-x(L) and Bcl-2, two important antiapoptotic proteins, in this process.

The NF, XPA, and XPG cells were treated with cisplatin at various concentrations (0, 10, 20, and 40 µM) for 3 h and then cultured in fresh medium for 24 h. The cells were harvested and lysed in RIPA cell lysis buffer. The cell lysates were analyzed by Western blots to determine the level of Bcl-x(L) (Fig. 1A) and Bcl-2 proteins (Fig. 1B). The protein level of actin was

FIG. 1. The cisplatin-induced Bcl-x(L) and Bcl-2 responses in NF, XPA, and XPG cells. The NF, XPA, and XPG cells were treated with cisplatin at various concentrations (0, 10, 20, and 40 μ M) for 3 h and then cultured in fresh medium for 24 h before the cells were harvested and lysed in RIPA cell lysis buffer. **(A)** Cisplatin-induced Bcl-x(L) response in NF, XPA, and XPG cells. **(B)** Cisplatin-induced Bcl-2 response in NF, XPA, and XPG cells. **(C)** Quantification of the cisplatin-induced Bcl-x(L) protein in NF, XPA, and XPG cells. The level of Bcl-x(L) protein in the untreated NF cells was counted as 100%, and the level of Bcl-x(L) protein in the untreated XPA and XPG cells as well as the cisplatin-treated NF, XPA, and XPG cells were calculated as a percentage to that of NF cells. The data represent at least four independent experiments. *Statistical significance in the level of Bcl-x(L) protein between NF and the tested cells at the same cisplatin concentration ($p < 0.01$). NF, normal human fibroblasts; XPA, nucleotide excision repair (NER)-defective xeroderma pigmentosum group A; XPG, NER-defective xeroderma pigmentosum group G.

(A). Cisplatin-induced Bcl-x(L) response in NF, XPA, and XPG cells.

(B). Cisplatin-induced Bcl-2 response in NF, XPA, and XPG cells.

(C). Quantification of the Bcl-x(L) protein in the cisplatin-treated cells.

also determined for each cell lysate on the same membrane as a protein loading control. A low level of Bcl-x(L) protein was detected in the untreated NF cells (Fig. 1A). When the NF cells were treated with cisplatin, however, significant increases in the levels of Bcl-x(L) protein were observed, and the increases were displayed in a concentration-dependent manner (Fig. 1A, C). In contrast, high levels of Bcl-x(L) protein were seen in the untreated XPA and XPG cells (Fig. 1A). After the cisplatin treatment, however, the level of Bcl-x(L) protein significantly decreased in both XPA and XPG cells, and this decrease was also displayed in a concentration-dependent manner for both cell lines (Fig. 1A, C). The protein level of Bcl-2, another important antiapoptotic protein, however, remained unchanged in the NF, XPA, and XPG cells after cisplatin treatment (Fig. 1B). As an internal control, the protein level of actin also remained unchanged in the NF, XPA, and XPG cells after cisplatin treatment.

To further investigate the involvement of Bcl-x(L) protein in cisplatin-induced cellular response, we determined a time-dependent response of the Bcl-x(L) protein in these cells after the cisplatin treatment. The NF, XPA, and XPG cells were treated with 20 μ M cisplatin for 3 h and then cultured in fresh cell growth medium. The cells were harvested at various time points after the treatment (2, 4, 8, 16, 24, and 48 h) and lysed in RIPA cell lysis buffer. As a control, cell lysates were also prepared from the untreated NF, XPA, and XPG cells. The cell lysates were analyzed by Western blots to determine the level of Bcl-x(L) protein at each time point after cisplatin treatment (Fig. 2A and B). When NF cells were treated with

cisplatin, the level of Bcl-x(L) protein started to increase 16 h after the treatment, and this trend continued up to 48 h (Fig. 2). When both XPA and XPG cells were treated with cisplatin at the same condition, however, very different patterns of Bcl-x(L) response were observed: the level of Bcl-x(L) protein initially increased in both XPA and XPG cells (up to 8 h); however, this increase was short-lived, and at the 16-h time point, the level of Bcl-x(L) protein was already below that of the untreated cells; at the 24- and 48-h time points, the levels of Bcl-x(L) protein were significantly lower than that of the untreated cells.

To confirm the observed Bcl-x(L) response of XPA and XPG cells toward cisplatin treatment, which was indeed caused by defects of XPA and XPG proteins in these cells, the *xpa* and *xpg* genes were silenced in NF cells using siRNAs against these genes (Colton *et al.*, 2006) and the cisplatin-induced Bcl-x(L) response was then determined for the NF cells (Fig. 3A and B). As a control, some NF cells were treated with a control siRNA (NC2 siRNA) and the cisplatin-induced Bcl-x(L) response was determined in these cells. No change in the pattern of Bcl-x(L) response was observed when the NF cells were pretreated with the negative control siRNA (Fig. 3A, B). When the NF cells were pretreated with both XPA and XPG siRNAs, however, a reduced level of Bcl-x(L) protein was observed when the NF cells were treated with 40 μ M cisplatin (Fig. 3A, B). The XPA/XPG siRNA-treated NF cells displayed a similar Bcl-x(L) response as that of NF cells at lower concentrations of cisplatin treatment (10 and 20 μ M). One possible explanation is that the XPA/XPG

A. time-dependent Bcl-x(L) response of NF, XPA, and XPG cells following cisplatin treatment.

B. Quantification of Bcl-x(L) protein in the cisplatin-treated NF, XPA, and XPG cells.

lated as 100%, and the level of Bcl-x(L) protein in the untreated XPA and XPG cells as well as the cisplatin-treated NF, XPA, and XPG cells were calculated as a percentage to that of NF cells. The data represent at least four independent experiments. *Statistical significance in the level of Bcl-x(L) protein between NF and the tested cells at the same time point after cisplatin treatment ($p < 0.01$).

A. Cisplatin-induced Bcl-x(L) response in NF cells that were pre-treated with indicated siRNAs.

B. Quantification of the Bcl-x(L) protein in the cisplatin-treated NF and siRNA-pre-treated NF cells.

was calculated as 100%, and the level of Bcl-x(L) in the cisplatin-treated cells was calculated as a percentage to that of the untreated cells. The data represent four independent experiments. *Statistical significance in the level of Bcl-x(L) protein between NF and the siRNA-treated NF cells at the same cisplatin concentration ($p < 0.01$).

FIG. 2. The time-dependent Bcl-x(L) response of NF, XPA, and XPG cells after cisplatin treatment. The NF, XPA, and XPG cells were treated with 20 μM cisplatin for 3 h and then cultured in fresh cell culture medium. The cells were harvested at the indicated time points, lysed in RIPA cell lysis buffer, and analyzed by Western blots to determine the level of Bcl-x(L) protein in each cell lysate. (A) A representative Western blot that shows a time-dependent Bcl-x(L) response of NF, XPA, and XPG cells after cisplatin treatment. (B) Quantification of Bcl-x(L) protein in NF, XPA, and XPG cells at different time points after the cisplatin treatment. The level of Bcl-x(L) protein in the untreated NF cells was calcu-

FIG. 3. The cisplatin-induced Bcl-x(L) response of NF cells in which the *xpa* and *xpg* genes were silenced by siRNAs. The NF cells were either untreated or treated with indicated siRNAs (300 nM each) for 24 h. The cells were then treated with cisplatin at various concentrations (0, 10, 20, and 40 μM) for 3 h and cultured in fresh medium for 24 h before the cells were harvested and cell lysates were prepared from these cells. (A) A representative Western blot that shows the cisplatin-induced Bcl-x(L) response in NF cells, NC2 siRNA-treated NF cells, and XPA/XPG siRNA-treated NF cells. The protein levels of XPA and XPG proteins were also determined from the cell lysates on the same membrane to verify the silencing effects of XPA and XPG siRNAs. (B) Quantification of the cisplatin-induced Bcl-x(L) responses in NF cells, NC2 siRNA-treated NF cells, and XPA/XPG siRNA-treated NF. The results are expressed as mean level $\pm$ SD (from three independent experiments). The protein level of Bcl-x(L) in the untreated cells

siRNA-treated NF cells maintain low levels of NER function, which is sufficient to respond to low levels of DNA damage generated by low concentrations of cisplatin.

Taken together, these results suggest that Bcl-x(L), but not Bcl-2, is involved in the NER-mediated cellular response toward cisplatin treatment. Given the important antiapoptotic role of Bcl-x(L) protein, these results strongly suggest that induced expression of Bcl-x(L) plays an important role in the NER-facilitated cell protection against cisplatin-induced apoptosis.

The requirement of functional NER for cisplatin treatment-induced transcription of bcl-x(l) gene

The results of our Western blots suggest the requirement of the Bcl-x(L) protein in NER-facilitated cell protection against cisplatin-induced apoptosis. To further elucidate the mechanism by which the NER process of the cisplatin DNA damage leads to an accumulation of the Bcl-x(L) protein in NF cells, we determined the cisplatin treatment-induced transcription of the *bcl-x(l)* gene in NF, XPA, and XPG cells.

The NF, XPA, and XPG cells were treated with cisplatin (40 μ M) for 3 h and then cultured in fresh medium. The cells were harvested 8, 12, and 16 h after the cisplatin treatment, and total RNA was isolated. As a control, total RNA was also isolated from the untreated NF, XPA, and XPG cells. A reverse transcription-based quantitative PCR (real-time PCR) was then performed to determine the mRNA levels of *bcl-x(l)* and *bcl-2* genes in each RNA sample. The levels of the *bcl-x(l)* and *bcl-2* mRNAs in the treated cells were calculated as relative levels to that of the untreated cells (Table 1). The cisplatin treatment resulted in an increase in transcription of the *bcl-x(l)* gene in NF cells at each of the tested time points. In XPA and XPG cells, however, the cisplatin treatment led to either reduced or attenuated transcriptions of the *bcl-x(l)* gene at these time points (Table 1). The cisplatin treatment caused decreased transcription of the *bcl-2* gene in both the NF and NER-defective XPA and XPG cells although the degree of decrease for the *bcl-2* mRNA was greater in the XPA and XPG cells than the NF cells (Table 1). It is also worth mentioning that the pattern of the transcriptional response of the *bcl-2* gene (decrease in transcription) was similar between the NF and the NER-defective XPA and XPG cells, indicating that the NER is not involved in the cisplatin treatment-induced transcription downregulation of the *bcl-2* gene. These results suggest that induced transcription of the *bcl-x(l)* gene is a major mechanism for cisplatin-induced accumulation of Bcl-x(L) protein in NF cells.

The requirement of functional NER for cisplatin treatment-induced translocation of NF- κ B

The results of our Western blots and real-time PCR studies demonstrated the involvement of Bcl-x(L) protein in NER-facilitated cell protection against cisplatin-induced apoptosis. Given the important role of NF- κ B in regulating transcription of *bcl-x(l)* gene (Mori *et al.*, 2001; Huerta-Yepez *et al.*, 2004), we further determined the requirement of a functional NER for cisplatin-induced NF- κ B activation.

Under normal cell growth condition, NF- κ B binds with the inhibitory I κ B proteins and is sequestered into the cytoplasm. In response to various stimuli, the I κ B is phosphorylated, which leads to the release of NF- κ B from I κ B and the translocation of NF- κ B from the cytoplasm to the nucleus, resulting in the transcription of responsive genes by NF- κ B. Therefore, activation of NF- κ B can be determined by its translocation from the cytoplasm into the nucleus. We determined the cisplatin treatment-induced NF- κ B translocation in NF, XPA, and XPG cells using immunofluorescence staining with an antibody that recognized the p65 subunit of NF- κ B. The NF, XPA, and XPG cells were treated with 40 μ M cisplatin for 3 h and then cultured in fresh medium for 24 h. The cells were fixed with 4% formaldehyde and stained with the NF- κ B antibody (primary antibody) and a FITC-conjugated secondary antibody. The nuclei were counter-stained by DAPI staining. Detection of NF- κ B and localization of the nucleus in the stained cells were achieved by fluorescence microscopy (Fig. 4A–R). In the untreated NF, XPA, and XPG cells, the NF- κ B was localized mainly in the cytoplasm. After the cisplatin treatment, the NF- κ B started to accumulate in the nuclei of NF cells (Fig. 4D–F). In the cisplatin-treated XPA and XPG cells, however, no accumulation of the NF- κ B in the nuclei occurred, and the NF- κ B remained in the cytoplasm (Fig. 4J–L, P–R). These results indicate that a functional NER is required for the cisplatin-induced translocation of NF- κ B from the cytoplasm into the nucleus. Given the important role of NF- κ B in regulating transcription of the *bcl-x(l)* gene, these results suggest that the activation of NF- κ B plays an important role in cisplatin-induced *bcl-x(l)* gene transcription and NER-facilitated cell protection toward cisplatin treatment.

Discussion

In this work, we studied the role of Bcl-x(L) protein in NER-facilitated cell protection against cisplatin-induced apoptosis and the mechanism by which the NER process leads to induced transcription of the *bcl-x(l)* gene under cisplatin treat-

TABLE 1. CISPLATIN-INDUCED TRANSCRIPTION OF *BCL-X(L)* GENE IN NF, XPA, AND XPG CELLS

	Gene	Fold change		
		NF (GM00043)	XPA (GM05509)	XPG (GM03021)
8 h	<i>bcl-x(l)</i>	1.92 $\pm$ 0.03	–2.02 $\pm$ 0.02	–1.33 $\pm$ 0.02
	<i>bcl-2</i>	–4.48 $\pm$ 0.01	–7.46 $\pm$ 0.10	–8.70 $\pm$ 0.20
12 h	<i>bcl-x(l)</i>	2.65 $\pm$ 0.02	–1.57 $\pm$ 0.01	1.39 $\pm$ 0.01
	<i>bcl-2</i>	–1.49 $\pm$ 0.20	–5.65 $\pm$ 0.02	–2.35 $\pm$ 0.05
16 h	<i>bcl-x(l)</i>	1.84 $\pm$ 0.02	1.32 $\pm$ 0.01	1.01 $\pm$ 0.03
	<i>bcl-2</i>	–1.05 $\pm$ 0.13	–1.31 $\pm$ 0.10	–1.51 $\pm$ 0.01

The mRNA level of the desired target gene was the fold change $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t^{\text{treated}} - \Delta C_t^{\text{untreated}}$ for each cell line. Actin was used to normalize. $\Delta C_t = C_t^{\text{gene}} - C_t^{\text{actin}}$. If $2^{-\Delta\Delta C_t} < 0$, then the fold change is calculated by $-1/2^{-\Delta\Delta C_t}$.

FIG. 4. The cisplatin-induced NF- κ B translocation in NF, XPA, and XPG cells. The NF, XPA, and XPG cells were treated with cisplatin (40 μ M) for 3 h and then cultured in fresh medium for 24 h. The cells were fixed by 4% formaldehyde and then stained with the NF- κ B (p65) antibody and an FITC-conjugated secondary antibody. The nucleus was counterstained with DAPI DNA counterstaining reagent. The NF- κ B signal was detected by an Axioplan 2 fluorescence microscope with a 540 nm light source and 40 $\times$ amplification (Carl Zeiss). The localization of the nucleus in the stained cells was achieved by the fluorescence microscope with a 485 nm light source and 40 $\times$ amplification. The merged image of the NF- κ B and nucleus was generated using the Axiovision (Zeiss).

ment. The results of our Western blots indicate that the cisplatin treatment led to an accumulation of Bcl-x(L) protein in the NF cells but a reduction of the Bcl-x(L) protein in the NER-defective XPA and XPG cells. The results of our real-time PCR studies further revealed that the cisplatin treatment caused an increase in transcription of the *bcl-x(l)* gene in NF cells, but a decrease in transcription of the *bcl-x(l)* gene in both XPA and XPG cells. Given the antiapoptotic role of Bcl-x(L) protein, these results suggest that induced transcription of the *bcl-x(l)* gene is an important mechanism in regard to NER in protecting cells against cisplatin-induced apoptosis.

The mechanism by which NER leads to induced transcription of the *bcl-x(l)* gene is unclear. The results obtained from our immunofluorescence staining reveal that the cisplatin treatment caused translocation of the NF- κ B from the cytoplasm to the nucleus in NF cells; however, this translocation event did not occur in the cisplatin-treated XPA and XPG cells. The results published by others have demonstrated the requirement of NF- κ B translocation for NF- κ B activation and the NF- κ B in regulating transcription of the *bcl-x(l)* gene (Mori *et al.*, 2001; Huerta-Yepes *et al.*, 2004). These results suggest that the NER process may cause an

increase in Bcl-x(L) protein through activation of the NF- κ B, which further induces transcription of the *bcl-x(l)* gene, resulting in an accumulation of the Bcl-x(L) protein and protecting cells against cisplatin-induced apoptosis.

The mechanism by which the NER process causes activation of the NF- κ B is unknown. The results obtained from our recent studies demonstrate the requirement of a functional NER process for cisplatin-induced ATM activation (Colton *et al.*, 2006). The results published by others reveal the requirement of ATM protein in activation of the NF- κ B (Wu *et al.*, 2006; Wuerzberger-Davis *et al.*, 2007). It is possible that the NER process may cause activation of the NF- κ B through activating the ATM protein, which further activates the NF- κ B to lead to an induced transcription of the *bcl-x(l)* gene and increased cell protection toward cisplatin treatment.

The results of our Western blots and real-time PCR studies reveal that the cisplatin treatment causes reduced transcription of the *bcl-x(l)* gene in both XPA and XPG cells, which suggests that the transcription of the *bcl-x(l)* gene was suppressed in the XPA and XPG cells under cisplatin treatment. The mechanism by which the transcription of the *bcl-x(l)* gene is suppressed in the cisplatin-treated XPA and XPG cells is unknown. However, the results obtained from our previous studies have revealed the activation of ATR kinase in the cisplatin-treated XPA and XPG cells (Colton *et al.*, 2006). The work of others also suggests a negative regulation of the NF- κ B by the ATR (Rocha *et al.*, 2005; Rocha and Perkins, 2005; Campbell *et al.*, 2006). It is possible that the inability to repair the cisplatin DNA damage in the NER-defective XPA and XPG cells causes activation of the ATR kinase, which, in turn, negatively regulates the NF- κ B to result in a reduced transcription of the *bcl-x(l)* gene and inhibition of activation of the cell survival pathway, leading to enhanced apoptosis of XPA and XPG cells under cisplatin treatment.

High levels of Bcl-x(L) protein were detected in the untreated XPA and XPG cells. Given the important role of Bcl-x(L) in protecting cells against apoptosis, these results suggest that these cells are more stressed than NF cells. The factors that lead to high levels of Bcl-x(L) protein in these cells are unknown. One possible explanation is that the inability to repair DNA damage results in accumulation of high levels of DNA damage in the genomic DNA; therefore, high levels of Bcl-x(L) protein are required to prevent apoptosis of these cells. Further studies are needed to determine the type and the level of DNA damage in these cells and the mechanism through which the *bcl-x(l)* gene is activated in these cells.

Our real-time PCR studies revealed that the transcription of the *bcl-2* gene decreased in both NF and the NER-defective XPA and XPG cells after cisplatin treatment. Therefore, the NER function is not required for the cisplatin treatment-induced transcription downregulation of the *bcl-2* gene. However, our Western blots indicated that the protein level of the Bcl-2 remained unchanged in these cells after the cisplatin treatment. Hence, the decreased transcription of the *Bcl-2* gene is likely caused by other mechanisms. One possible mechanism is that the cisplatin-induced cell cycle arrest has a global effect in transcription reduction as demonstrated in our previous microarray studies (Wang *et al.*, 2004), which results in a decrease in transcription of the *bcl-2* gene. Another possible mechanism is that the cisplatin treatment may have a prolonged stabilization effect on the Bcl-2 protein (Chanvorachote *et al.*, 2006), which in turn leads to a negative feedback

mechanism toward the transcription of the *bcl-2* gene. Further studies are needed to determine the relationship between the Bcl-2 protein and the downregulation of the transcriptional regulation of the *bcl-2* gene under the cisplatin treatment.

Although the work described here focuses on determining the role of the Bcl-x(L) protein in NER-mediated cell protection against cisplatin-induced apoptosis, it is possible that other proteins are also involved in the NER-facilitated cell protection against cisplatin treatment. For example, the results obtained from our previous studies (Wang *et al.*, 2004) and the works published by others (Mujoo *et al.*, 2003; Di Stefano *et al.*, 2004; Jiang *et al.*, 2004; Yip *et al.*, 2006) have demonstrated the involvement of the p53-signaling pathway in cisplatin-induced cellular responses. Given the important role of p53 in regulating the transcriptions of many important DNA damage-responsive genes, most noticeably the proapoptotic Bcl-2 family genes *bax*, *bad*, and *puma*, it is possible that NER also protects cells against cisplatin-induced apoptosis by downregulating transcriptions of the proapoptotic Bcl-2 family genes through p53, leading to inhibition of the apoptosis pathway and resulting in increased cell survival under cisplatin treatment. In addition, it is known that cisplatin treatment causes the activation of the Akt protein, which can also activate the NF- κ B (Pommier *et al.*, 2004; Beere, 2005; Le Bras *et al.*, 2006). Therefore, it is possible that a complex of the signaling network is involved in cisplatin treatment-induced cellular response, which then determines the fate of the damaged cells.

In conclusion, the results obtained from this work provide an important mechanism by which the NER protects cells against cisplatin-induced apoptosis. It also suggests a possible role of NER in cancer cell resistance to many of the commonly used chemotherapeutic drugs. This knowledge, therefore, has important clinical implications in cancer treatment, as well as anticancer drug design and development.

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Disclosure Statement

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