

Implications of Apurinic/Apyrimidinic Endonuclease in Reactive Oxygen Signaling Response after Cisplatin Treatment of Dorsal Root Ganglion Neurons

Yanlin Jiang,¹ Chunlu Guo,³ Michael R. Vasko,³ and Mark R. Kelley^{1,2,3}

¹Department of Pediatrics (Section of Hematology/Oncology), Herman B. Wells Center for Pediatric Research, Departments of ²Biochemistry and Molecular Biology and ³Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana

Abstract

Peripheral neuropathy is one of the major side effects of the anticancer drug cisplatin. Although previous work suggests that this neuropathy correlates with formation of DNA adducts in sensory neurons, growing evidence suggests that cisplatin also increases the generation of reactive oxygen species (ROS), which could cause DNA damage. Apurinic/aprimidinic endonuclease/redox factor-1 (Ape1/Ref-1) is a multifunctional protein involved in DNA base excision repair of oxidative DNA damage and in redox regulation of a number of transcription factors. Therefore, we asked whether altering Ape1 functions would influence cisplatin-induced neurotoxicity. Sensory neurons in culture were exposed to cisplatin for 24 hours and several end points of toxicity were measured, including production of ROS, cell death, apoptosis, and release of the immunoreactive calcitonin gene-related peptide (iCGRP). Reducing expression of Ape1 in neuronal cultures using small interfering RNA (siRNA) enhances cisplatin-induced cell killing, apoptosis, ROS generation, and cisplatin-induced reduction in iCGRP release. Overexpressing wild-type Ape1 attenuates all the toxic effects of cisplatin in cells containing normal endogenous levels of Ape1 and in cells with reduced Ape1 levels after Ape1siRNA treatment. Overexpressing the redox deficient/repair competent C65-Ape1 provides partial rescue, whereas the repair-deficient Ape1 (N226A + R177A) does not protect neurons from cisplatin toxicity. We also observe an increase in phosphorylation of p53 after a decrease in Ape1 levels in sensory neuronal cultures. These results strongly support the notion that Ape1 is a potential translational target such that protecting Ape1 levels and particularly its DNA repair function could reduce peripheral neuropathy in patients undergoing cisplatin treatment. [Cancer Res 2008;68(15):6425–34]

Introduction

Cisplatin is one of the most commonly used cytotoxic agents in the treatment of a variety of solid malignant tumors, including testicular, bladder, lung, esophagus, stomach, and ovarian cancers, as well as sarcomas and lymphomas (1). One of the major limitations of the use of cisplatin, however, is peripheral neuropathy (2, 3) which seems to be caused mainly by the action of the drug on

dorsal root ganglion (DRG) neurons (4). Cisplatin therapy and cytotoxicity has been shown to involve the formation of intrastrand and interstrand adducts in DNA (5), and this is thought to be one of the major mechanisms for cisplatin-induced damage in sensory neurons (3, 6). Indeed, mice deficient in nucleotide excision repair (NER), the pathway once thought to be the major repair response to cisplatin, show an increase in symptoms of neuropathy after cisplatin treatment compared with mice with NER intact (7). However, there is growing evidence suggesting that cisplatin toxicity is closely associated with increased generation of reactive oxygen species (ROS; refs. 8–10).

Apurinic/aprimidinic endonuclease/redox factor (Ape1/Ref-1; which will be called Ape1) is a ubiquitous multifunctional protein involved in the DNA base excision repair (BER) pathway of oxidative DNA damage that can occur from either endogenous or exogenous agents generating ROS (11–13). In addition, Ape1 functions as a major reducing-oxidizing (redox) factor augmenting the binding activity of a number of transcription factors to DNA including, but not limited to, nuclear factor- κ B, p53, activator protein 1, and cAMP-responsive element binding protein (13). In neurons, these transcription factors play important roles in neuronal survival, stress response, and various disorders (14–16).

Previous studies by our laboratory showed that Ape1 protects sensory neuronal cells from oxidative DNA damage, secondary to exposure to H₂O₂ (17). Other groups have shown that overexpression of Ape1 protected melanoma cells from cisplatin-induced or H₂O₂-induced apoptosis (18), and we have shown that Ape1 overexpression protected germ cell tumor cells from radiation and bleomycin treatment (19). Thus, there is precedent in mitotic cancer cell lines that Ape1 plays a role in cisplatin-induced cell death. The question remains, however, whether Ape1 could be neuroprotective against cisplatin-induced toxicity in postmitotic sensory neurons and thus have the potential to reduce peripheral neuropathy. In this article, we show that cisplatin produces significant amounts of ROS in sensory neuronal cultures and that reducing Ape1 expression in these cultures results in an increase in cisplatin-induced neurotoxicity and an increase in the activity of the p53 signaling pathway. Conversely, overexpressing wild-type (WT) and redox-deficient Ape1 in neuronal cultures attenuates cisplatin-induced toxicity whereas a repair-deficient Ape1 does not. These findings have potential translational applications such that modifying or protecting the function of Ape1 in neuronal cells may have chemoprotective effects for patients undergoing chemotherapy with platinating agents.

Materials and Methods

Cell culture. Adult dorsal root ganglion neuronal cell cultures were prepared, as previously described (20, 21). Briefly, adult male (150–175 g)

Requests for reprints: Mark R. Kelley, Department of Pediatrics, Herman B. Wells Center for Pediatric Research, 1044 West Walnut, R4-302C, Indianapolis, IN 46202. Phone: 317-274-2755; Fax: 317-278-9298; E-mail: mkelley@iupui.edu.

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Sprague-Dawley rats (Harlan) were euthanized by CO₂ asphyxiation. DRGs were collected from the spinal column, incubated in collagenase, and mechanically dissociated. For studies measuring cell viability, ROS, apoptosis, and signaling, ~60,000 cells were plated into each well of poly-D-lysine and laminin-coated six-well culture plates. For studies measuring calcitonin gene-related peptide (CGRP) release, ~15,000 cells were plated in 12-well culture plates. Cells were maintained in F-12 media (Invitrogen) supplemented with 10% horse serum, 2 mmol/L glutamine, 100 µg/mL normocin, 50 µg/mL penicillin, 50 µg/mL streptomycin, 50 µmol/L 5-fluoro-2'-deoxyuridine (Invitrogen), 150 µmol/L uridine, and 30 ng/mL of nerve growth factor (Harlan Bioproducts for Science, Inc.) in 3% CO₂ at 37°C.

Cell viability assays. To assay cell viability, trypan blue exclusion analysis was performed as previously described (17). Cells were resuspended using a 0.05% trypsin-EDTA solution. Equal volumes of the cell suspension and 0.4% (w/v) trypan blue in PBS were mixed, and the cells were scored under phase contrast microscope.

ROS measurement. Sensory neuronal cell cultures were treated with cisplatin for 24 h. After washing with PBS, the cells were incubated with 10 µmol/L carboxy-H₂DCFDA (Invitrogen) in fresh PBS for 60 min (22). Excessive probe was washed off with PBS, and cells were incubated in PBS for another 60 min at 37°C. The cells were harvested with trypsin, and fluorescence of the labeled cells was measured by using a Coulter EPICS XL flow cytometer (Coulter). An average of 10,000 cells from each sample was counted.

Ape1siRNA transfection. To decrease Ape1 in sensory neuronal cell cultures, Ape1siRNA and SCsiApe1 (scrambled control) were used as described previously (17, 23). Briefly, on the fourth day in culture, the growth media was replaced with 0.5 mL of Optimem I medium containing 10 µL of the transfecting reagent Neuroporter in the absence or presence of the 21-mer oligonucleotide double-stranded small interfering RNA (siRNA) to Ape1 (Ape1siRNA) or scrambled Ape1siRNA (SCsiRNA). Fresh medium (0.5 mL) without antibiotics was added after 24 h of incubation, and after an additional 24 h, the medium was replaced with normal medium containing antibiotics and cell growth maintained.

Adenoviral infection. Adult sensory neuronal cell cultures were grown for 8 d and then infected with either the WT Ad5 HA-Ape1 (WT-Ape1), the redox deficient/DNA repair competent Ad5 HA-C65A (C65-Ape1), DNA repair deficient/redox competent Ad5 HA-N226A+R177A (N226+R177-Ape1), or vector control Ad5 IRES2EGFP (Vector) adenovirus at 150 plaque-forming unit per cell for 24 h by adding the virus directly to the growth medium, as previously described (17). The N226A + R177A Ape1 double mutant has been shown to decrease the ability of this mutant Ape1 to bind to AP sites in DNA (24). The constructs used contained the human Ape1 sequence so that the rat Ape1siRNA oligonucleotides could not bind to it because of sequence differences between human and rat. However, the codons are highly conserved, as well as their functions. The hemagglutinin epitope (HA) tag was added to the amino terminus of Ape to distinguish exogenous transgene overexpression from endogenous Ape1 gene levels and has been routinely used by us (17). After 24 h, the medium containing adenovirus was aspirated and replaced with culture medium. Infection efficiencies of transduced cells were determined 24 h after infection by fluorescence microscopy.

Cisplatin treatment. Adult sensory neuronal cultures were grown in culture for 11 d, with or without siRNA transfection and with or without adenovirus infection (vector, WT-Ape1, 226 + 177-Ape1, C65-Ape1), the cells were washed, treated with cisplatin at various concentrations, and analyzed as described in Results. Cisplatin (Sigma-Aldrich) was prepared fresh for each experiment by dissolving in *N,N*-dimethylformamide (Sigma-Aldrich) with the final *N,N*-dimethylformamide concentration of <0.005%.

Western blot analysis. Cells in culture were harvested and lysed in an ice-cold radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Inc.). Protein concentration was quantified using the detergent-compatible (25), Lowry-based protein assay (Bio-Rad Laboratories). Protein (20–40 µg) was electrophoresed in SDS gel loading buffer on a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was transferred to a polyvinylidene

difluoride (PVDF) membrane. The membrane was incubated with a primary antibody against Ape1 (17, 26), phosphorylated histone H2AX (Ser¹³⁹; Upstate Cell Signaling Solutions), phosphorylated p53 (Ser¹⁵; Cell Signaling Technology), p53, Gadd45a (C-4; Santa Cruz Biotechnology, Inc.). Generally, antibodies were used at dilutions of 1:1,000 or 1:1,500. After incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.), antibody binding was detected using chemiluminescence (Roche Diagnostics Corp.), and equal loading was confirmed by probing with β-actin monoclonal antibody (NeoMarkers, Inc.).

Cell apoptosis analysis. Apoptosis was quantitated using flow cytometry after cell treatment using Annexin V-APC and 7-amino-actinomycin D (7-ADD) and the Annexin V Apoptosis Detection kit (BD Biosciences) according to the manufacturer's instructions. Apoptotic cells were defined as those positive for Annexin V with or without 7-ADD staining.

CGRP. Release studies were performed on the cells as previously described (17). Briefly, the neuronal cultures were washed once with HEPES buffer consisting of (in mmol/L) 25 HEPES, 135 NaCl, 3.5 KCl, 2.5 CaCl₂, 1 MgCl₂, 3.3 D-glucose, and 0.1% bovine serum albumin (pH 7.4), maintained at 37°C, and then incubated for successive 10-min intervals with 0.4 mL of the same buffer in the absence or presence of drugs. Basal or resting release was determined by exposing the cells to HEPES buffer alone, whereas stimulated release was determined by exposing the cultures to 30 nmol/L capsaicin. Cells then were reexposed to HEPES buffer without drugs for one or two 10-min incubations to reestablish resting release. During incubations, the cells were maintained in at 37°C. After each incubation, the buffer was removed to measure the amount of CGRP using RIAs, as previously described (17). At the end of each release experiment, the cells are scraped and sonicated in 0.4 mol/L HCl and an aliquot was taken to measure total CGRP content in the cultures using RIA. Total content is determined by adding the total amount released in all incubations to the amount remaining in the cells. The release data is calculated as a percentage of the total peptide content in the cells. Because CGRP was measured by RIA, results were expressed as CGRP-like immunoreactivity (iCGRP).

Statistical analysis. Data are expressed as the mean ± SE for at least three independent experiments from separate harvests. Statistical analysis was performed using ANOVA (**P* < 0.05 versus control group).

Results

Effects of Cisplatin on Sensory Neuronal Cultures

Cisplatin-induced cytotoxicity. In the first series of experiments, we determined the cytotoxic effect of cisplatin on sensory neuronal cultures by exposing cultures to various concentrations of cisplatin for 24 hours and then measuring cell viability using the trypan blue exclusion assay. As shown in Fig. 1A, exposing sensory neurons to cisplatin causes a concentration-dependent decrease in cell survival. Cisplatin (25 µmol/L) for 24 hours had no significant effect on cell viability, whereas with 400 µmol/L cisplatin, only 4 ± 0.01% of the cells survived. The calculated LD₅₀ for cisplatin in these experiments was 80 µmol/L.

Cisplatin-induced ROS generation. Although it is clear that cisplatin results in DNA adducts in sensory neurons (6, 7), recent evidence suggests that the drug also can produce ROS in nonneuronal cells (27). To determine if cisplatin could produce ROS in sensory neurons, cultures were exposed to increasing concentrations of cisplatin for 24 hours and ROS was measured using carboxy-H₂DCFDA and fluorescence-activated cell sorting (FACS) analysis as described in Materials and Methods. In a manner analogous to cell viability studies, 25 µmol/L cisplatin was not effective, i.e., it did not increase ROS, whereas with concentrations of 50, 100, and 200 µmol/L cisplatin, ROS was significantly higher than untreated controls. As a positive control in these experiments, cultures were exposed to 100 µmol/L tert-butyl

hydroperoxide (TBHP) for 1 hour (28), but the increase in ROS was not as high as that observed with higher concentrations of cisplatin. These data support our contention that ROS and oxidative DNA damage are important components of the deleterious effects of cisplatin on neuronal cells.

Cisplatin-induced increase in Ape1 expression. Several studies have shown that ROS enhanced Ape1 expression in gastric epithelial, ovary, macrophages, and liver cells (29, 30). The question of whether chemotherapeutic agents, especially those typically classified as DNA cross-linking agents alter Ape1 expression has not been addressed. Therefore, we examined whether Ape1 expression in sensory neuronal cultures would be altered by exposing cells to cisplatin. For these experiments, cultures were exposed to various concentrations of cisplatin for 24 hours and Ape1 protein expression was analyzed using immunoblotting. As shown in Fig. 1C, Ape1 protein levels were not significantly increased when cells were exposed to 6.25 or 12.5 $\mu\text{mol/L}$ cisplatin, but were significantly higher than controls at 25 $\mu\text{mol/L}$ and higher. Increased Ape1 expression levels were also observed in cells 48 hours after treatment with cisplatin (data not shown).

Reduced Expression of Ape1 Enhances Cisplatin-Induced Cytotoxicity and ROS Production in Sensory Neuronal Cultures

Because cisplatin increased ROS and Ape1 expression in sensory neurons, we examined whether reducing Ape1 expression in sensory neuronal cultures would augment cisplatin-induced cell death. Exposing neuronal cultures to Ape1siRNA significantly reduced the expression of Ape1 compared with levels in cultures exposed to SCsiRNA (see Fig. 2A for a representative Western blot). This reduction of Ape1 resulted in a statistically significant increase in cisplatin-induced cytotoxicity compared with the SCsiRNA controls (Fig. 2B). The increase in cell killing was $\sim 20\%$ greater when Ape1 levels were decreased compared with cells treated with SCsiRNA. This result shows that reduction of Ape1 expression in sensory neuronal cells significantly increases cisplatin-induced cytotoxicity.

We also examined ROS production in cultures exposed to SCsiRNA or Ape1siRNA. In these experiments, Ape1siRNA treatment reduced Ape1 expression by 70% compared with cultures exposed to SCsiRNA (data not shown). This decrease in Ape1

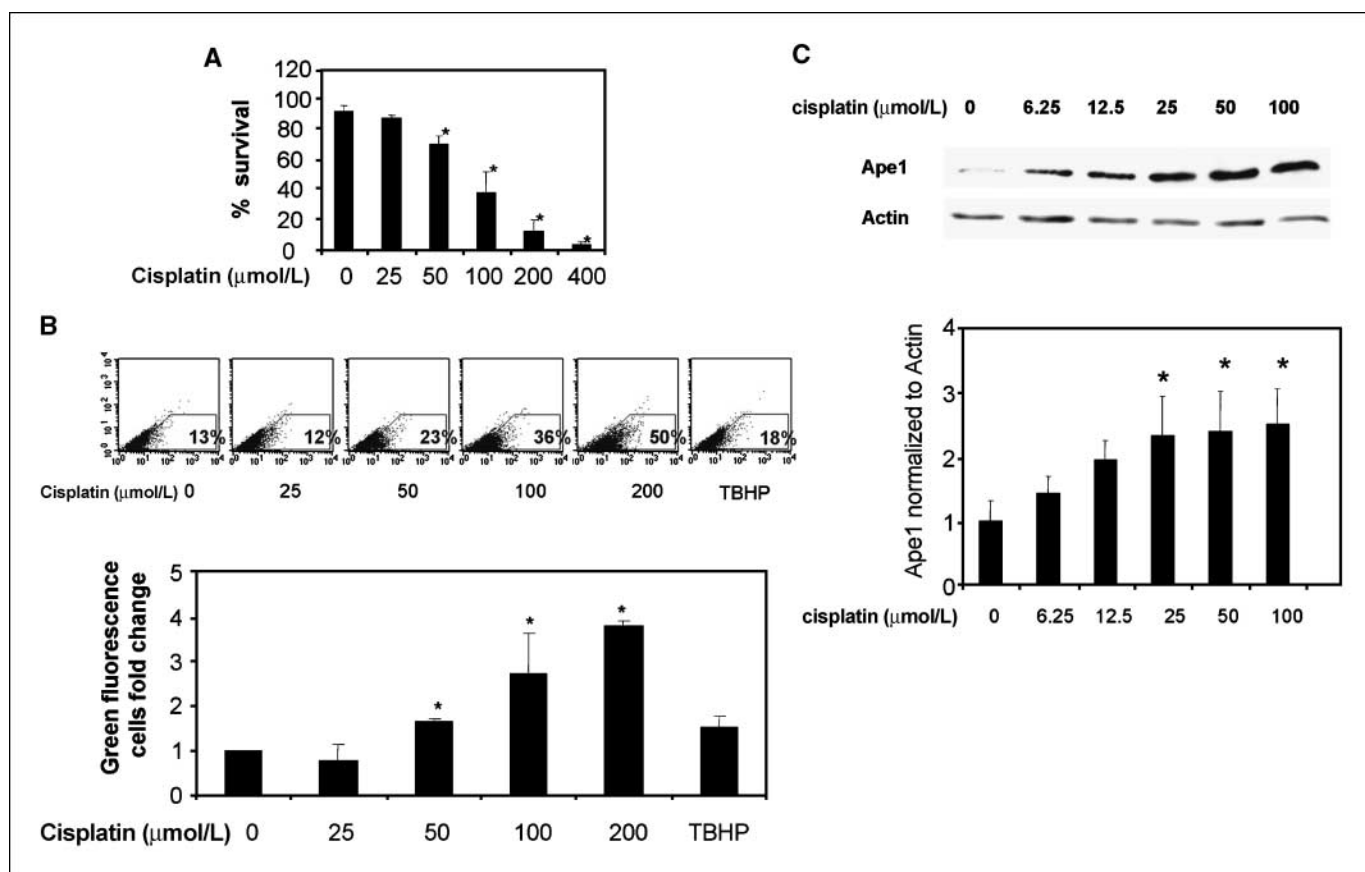


Figure 1. Effect of cisplatin treatments on sensory neuronal cells. *A*, sensory neuronal cell cultures were incubated with different concentrations of cisplatin for 24 h. Cytotoxicity was determined using the trypan blue exclusion assay, as described. *Columns*, mean for three independent harvests of cells; *bars*, SE. Asterisk, statistically significant (* , $P < 0.05$) difference from control. *B*, ROS generation in sensory neuronal cell cultures after cisplatin treatment. After incubation with cisplatin for 24 h, sensory neuronal cell cultures were loaded with 10 $\mu\text{mol/L}$ carboxy-H₂DCFDA and incubated for 1 h. Carboxy-H₂DCFDA is an indicator for ROS and only fluoresces when hydrolyzed by esterases and oxidation occurs within the reaction system. *Top*, fluorescence of the oxidized form of carboxy-H₂DCFDA as analyzed in a representative flow cytometry experiment. As a positive control for oxidative stress, cells were treated for 1 h with 100 $\mu\text{mol/L}$ TBHP. *Bottom*, summary of three experiments expressed as the amount of change in fluorescent cells after cisplatin treatment. *Columns*, mean for three independent harvests of cells; *bars*, SE. Statistically different points from controls are indicated with an asterisk (* , $P < 0.05$). *C*, Ape1 protein expression levels after cisplatin treatment. *Top*, representative Western blot of the expression of Ape1 after sensory neuronal cell cultures were incubated with different cisplatin concentrations for 24 h; *bottom*, summary of Ape1 expression after cisplatin treatment normalized to the amount of actin detected by densitometry. *Columns*, mean for three independent harvests of cells; *bars*, SE. Statistically different points from controls are indicated with an asterisk (* , $P < 0.05$).

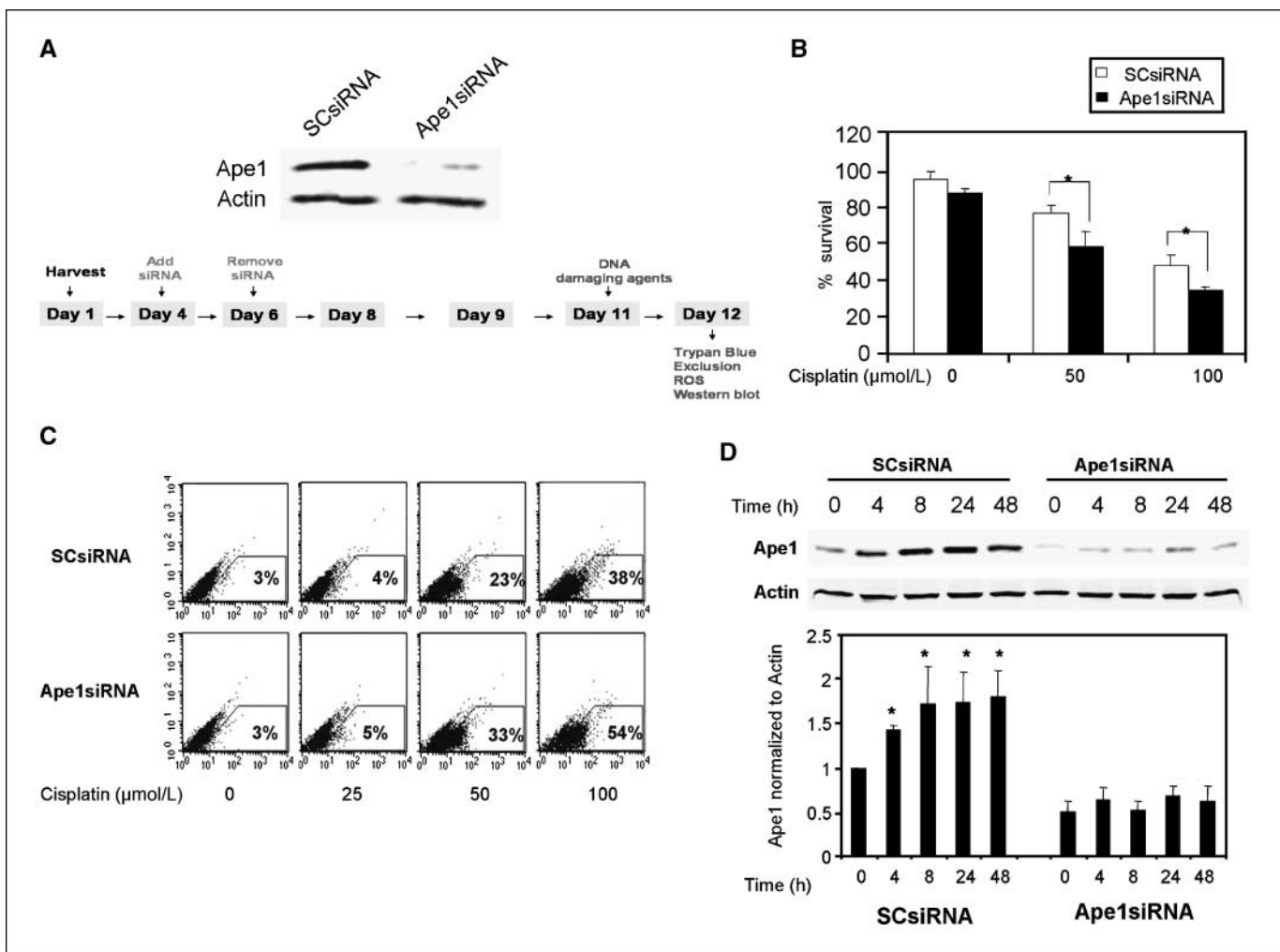


Figure 2. Effect of Ape1 siRNA knockdown on sensory neuronal cells after cisplatin treatment. *A*, sensory neuronal cells were incubated with 100 nmol/L Ape1siRNA on day 4 in culture, and the Ape1siRNAs were removed on day 6. Ape1 expression was analyzed on day 11 by Western blot. SCsiRNA (100 nmol/L) was used as negative control. *B*, Ape1siRNA knockdown and SCsiRNA transfected sensory neuronal cells were incubated with cisplatin (0, 50, and 100 μmol/L) for 24 h. The ordinate represents the percentage of cell survival measured by trypan blue exclusion. Columns, mean for three independent harvests of cells; bars, SE. Statistically different points from controls are indicated with an asterisk (*, $P < 0.05$). *C*, ROS generation. The fluorescence of the oxidized form of carboxy-H2DCFDA was analyzed by flow cytometry in SCsiRNA and Ape1siRNA knockdown cells. These data are representative of four individual experiments. *D*, Ape1 protein expression levels in SCsiRNA and Ape1siRNA knockdown DRG cells after cisplatin (50 μmol/L) treatment at indicated time points (*left*) as analyzed by Western blot. The summary of Ape1 expression was normalized to the amount of actin detected by densitometry (*right*). Columns, mean for three independent harvests of cells; bars, SE. Statistically different points from controls are indicated with an asterisk (*, $P < 0.05$).

expression resulted in a significant increase in the ROS generated by a 24-hour exposure to 50 or 100 μmol/L cisplatin to 10% and 16% (Fig. 2C), respectively, compared with SCsiRNA-treated cultures.

Cisplatin treatment (50 μmol/L) enhanced the level of Ape1 expression in cultures from 4 to 48 hours after a 24-hour exposure in cultures treated with SCsiRNA (Fig. 2D) in a manner comparable with that observed in previous experiments (Fig. 1A), but this did not occur in cultures exposed to Ape1siRNA.

Ape1 Overexpression in Sensory Neuronal Cells in Cultures Protects against Cisplatin-Induced Cytotoxicity

Because reducing Ape1 expression in sensory neuronal cultures augments cisplatin-induced toxicity, we determined whether overexpressing Ape1 could be neuroprotective. WT-Ape1, C65-Ape1, or vector control adenoviral constructs containing an IRES site and

an enhanced green fluorescent protein construct were used to infect sensory neuronal cell cultures pretreated with either SCsiRNA or Ape1siRNA. The cells were transfected with Ape1siRNA on days 4 to 6 in culture, infected with WT-Ape1 or the C65-Ape1 adenovirus on day 8, and treated with cisplatin (50 μmol/L or 100 μmol/L) on day 11, and cell viability was assessed using trypan blue after 24 hours of exposure to cisplatin. In all experiments, we confirmed expression of the transgene proteins by performing Western blots on cultures at the end of each experiment and using HA antibody. As shown in representative Western blot in Fig. 3A, after transfecting with Ape1siRNA, the expression of Ape1 was lower than Ape1 levels in cultures treated with SCsiRNA. After infection with viral vectors, however, the Ape1 expression was higher (Fig. 3A). When cells treated with SCsiRNA and viral vector control were exposed to cisplatin, there was a significant reduction in cell survival (Fig. 3B, *left*). Cell viability was reduced from 95% in cells not exposed to cisplatin to $65 \pm 5\%$ and $51 \pm 3\%$ after 50 and

100 $\mu\text{mol/L}$ cisplatin, respectively. With a reduction in Ape1 expression, vector-treated cells showed an augmentation of cisplatin-induced neurotoxicity (Fig. 3B, right) and these results are analogous to the finding presented above. Overexpressing either WT-Ape1 or C65-Ape1 significantly rescued cells from killing by cisplatin. This occurred independent of whether the cells had reduced Ape1 levels secondary to siRNA treatment. However, the protective ability of C65-Ape1 was not equivalent to that of the WT-Ape1. For example, in cells treated with Ape1siRNA, overexpressing WT-Ape1 increased cells viability after 100 $\mu\text{mol/L}$ cisplatin to $50 \pm 6\%$, whereas the C65-Ape only increased viability to $32 \pm 3\%$.

Overexpression of Ape1 Reduces Cisplatin-Induced Apoptosis in Sensory Neuronal Cultures

Apoptosis has been observed in sensory neurons after cisplatin treatment *in vitro* and *in vivo* (31, 32). To ascertain whether Ape1 affects cisplatin-induced apoptosis in sensory neuronal cultures, we performed apoptosis assays using Annexin V/propidium iodide flow cytometry analysis. The time line for these experiments is

analogous to the previous studies, but after 24 hours of exposure to 100 $\mu\text{mol/L}$ cisplatin GFP-positive cells were gated and cells undergoing apoptosis were detected by Annexin and 7-ADD staining. As shown in the representative experiment in Fig. 4A and the summary data from six experiments in Fig. 4B, $22 \pm 4\%$ of cells from cultures exposed to SCsiRNA, viral vector, and cisplatin were apoptotic. In cells treated with Ape1siRNA, vector, and cisplatin, $46 \pm 6\%$ were apoptotic. Overexpressing C65-Ape1 did not significantly rescue the cells from cisplatin-induced apoptosis, whereas overexpressing WT-Ape1 significantly decreased the number of apoptotic cells independent of whether the cells were exposed to SCsiRNA or Ape1siRNA (Fig. 4). Although we do not see a significant difference between the overexpression of C65-Ape1 compared with the vector control, there seem to be a trend in the direction of the C65-Ape1 providing protection.

Effect of Ape1 on Cisplatin-Altered Evoked Release of iCGRP from Sensory Neurons

We examined the effects of cisplatin in the absence or presence of Ape1 manipulations on the release of iCGRP from sensory

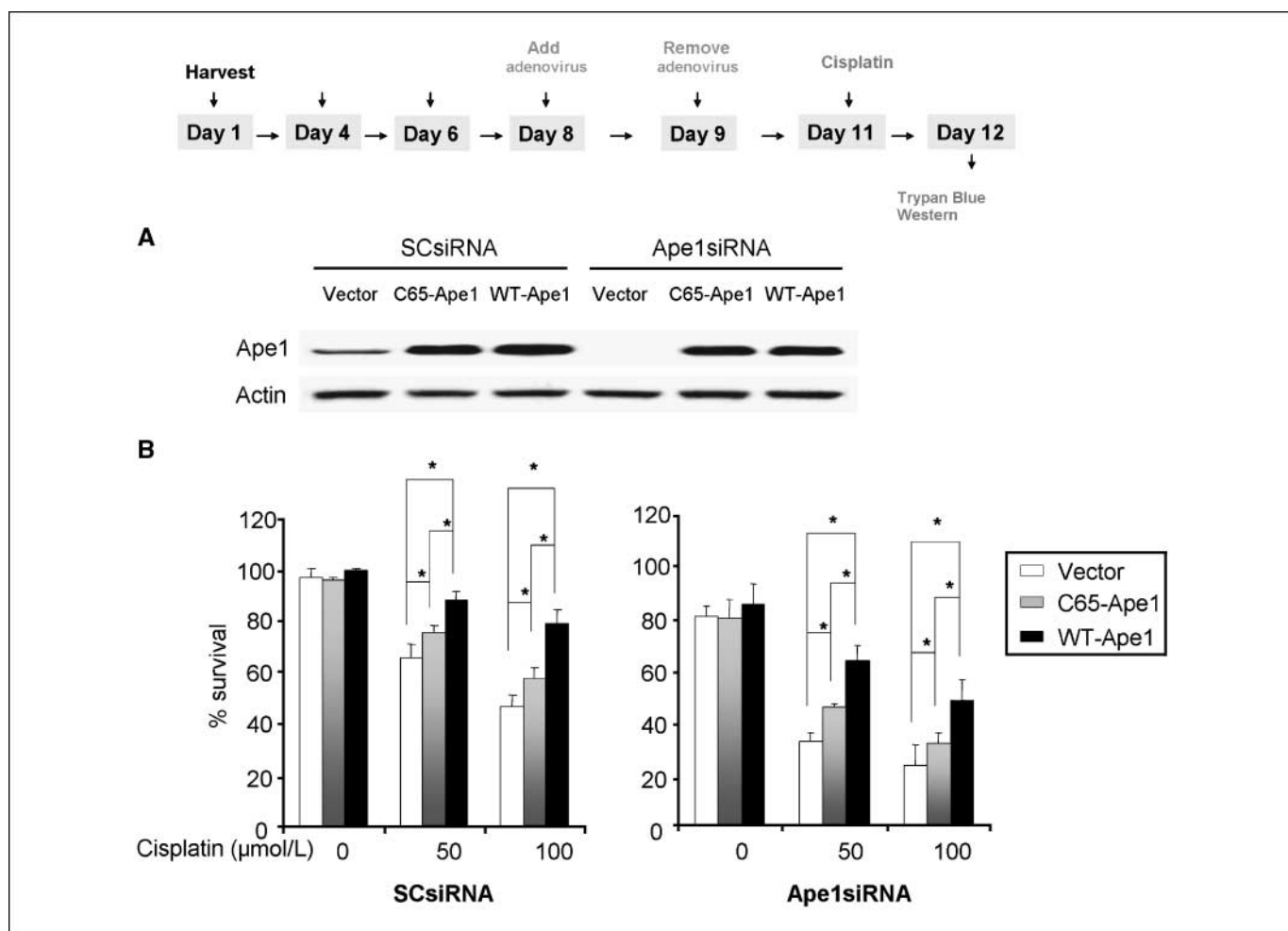


Figure 3. Effect of Ape1 overexpression on sensory neuronal cell viability after cisplatin treatment. *A*, sensory neuronal cells were incubated with 100 nmol/L Ape1siRNA on day 4 in culture, and the Ape1siRNAs were removed on day 6. The cells were then infected with one of three adenoviral constructs; vector control, WT-Ape1, or C65-Ape1 on day 8 for 24 h. The level of adenovirus infection was measured on day 11 by fluorescence microscope. Ape1 expression was analyzed on day 11 by Western blot. *B*, survival of sensory neuronal cells after cisplatin treatment with or without Ape1 knockdown and the addition of transgene Ape1 constructs. The ordinate represents percentage of cells surviving at 24 h after various doses of cisplatin treatment as measured by trypan blue exclusion in DRG cells, without (left) or with (right) Ape1 knockdown. Columns, mean for three independent harvests of cells; bars, SE. Statistically different points from controls are indicated with an asterisk (*, $P < 0.05$).

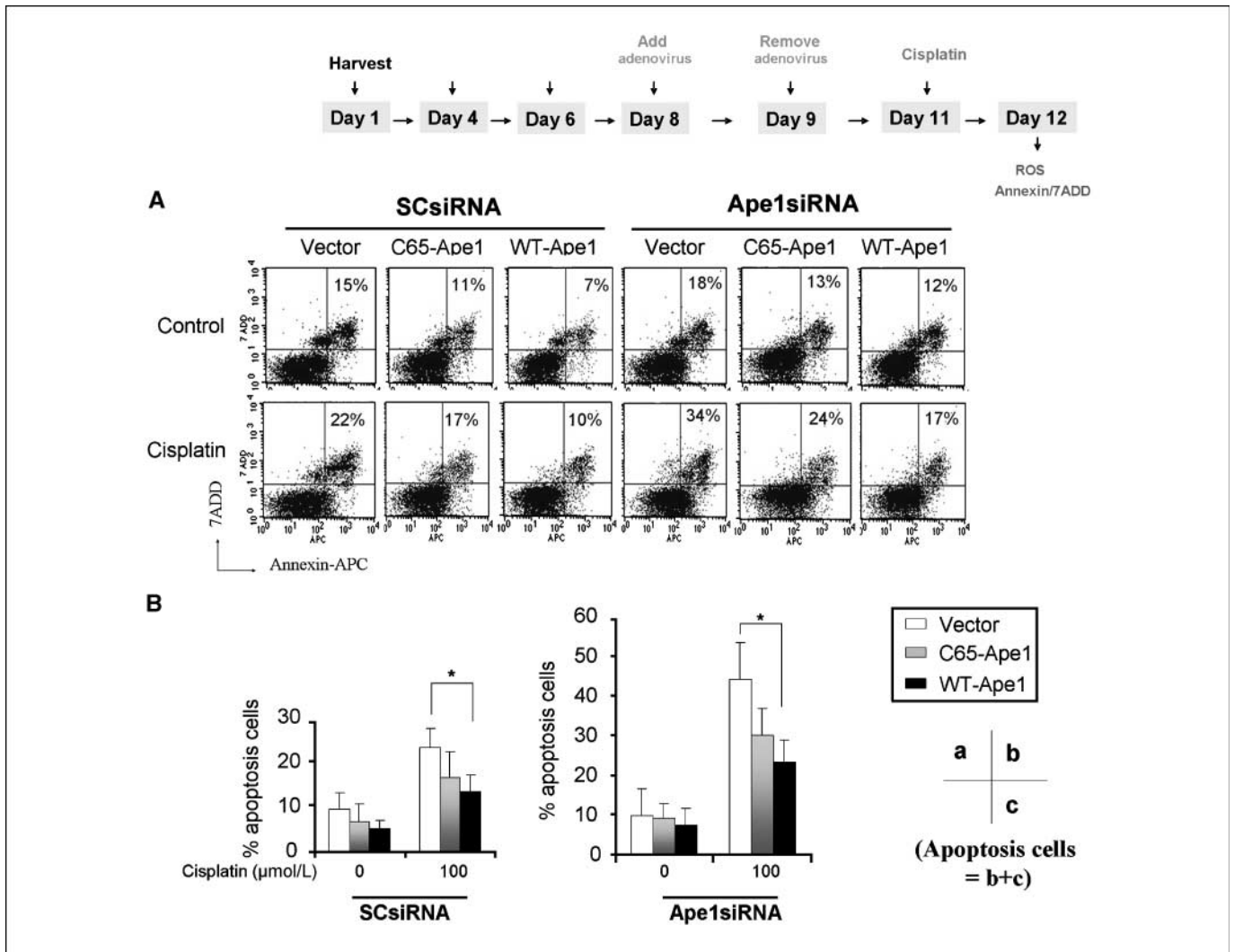


Figure 4. Effect of Ape1 levels on the apoptosis levels of sensory neuronal cells after cisplatin treatment. *A*, sensory neuronal cell cultures were incubated with Ape1siRNA or SCsiRNA control oligos and were then infected with either WT-Ape1 or C65-Ape1. The cells were then treated with cisplatin (100 μmol/L) for 24 h. The level of cells undergoing apoptosis was detected by Annexin V and 7-ADD staining and FACS analysis as discussed in Materials and Methods. *B*, quantification of the data presented in *A*. The percentage of cells undergoing apoptosis (*c*, bottom right) plus late apoptosis (*b*, top right) is shown. Statistically different points from controls are indicated with an asterisk (*, $P < 0.05$).

neurons in culture as an integrated functional output from these cells. For these studies, sensory neuronal cultures in the absence or presence of Ape1 manipulations were exposed to various concentrations of cisplatin for 24 hours, then release studies were performed as outlined in Materials and Methods. For internal consistency, the release studies were always performed on cells grown in culture for 12 days. Exposing sensory neurons in culture to various concentrations of cisplatin results in a concentration-dependent decrease in the capsaicin-evoked release of iCGRP (Fig. 5A). Cisplatin (3 μmol/L) did not alter release compared with untreated cells ($11.3 \pm 0.3\%$ of total iCGRP content for controls versus $12.9 \pm 0.9\%$ for treated cells). In contrast, both 10 and 30 μmol/L cisplatin reduced the capsaicin-evoked release to $6.3 \pm 0.2\%$ and $3.5 \pm 0.1\%$ of total content, respectively. Cisplatin treatment had no significant effect on basal release of iCGRP from sensory neurons. Basal release in untreated cells was 7.7 ± 2.0 fmol/well/10 min and was 7.0 ± 0.1 , 7.4 ± 2.0 , and 7.1 ± 0.2 fmol/well/10 min after 3, 10, or 30 μmol/L cisplatin, respectively (data not shown). For the remaining studies, we used a concentration

of 10 μmol/L cisplatin because it produces a significant decrease in iCGRP release but does not cause cell death in the cultures (see Fig. 1).

When neuronal cultures were exposed to SCsiRNA or Ape1siRNA in the absence of cisplatin treatment, there was no significant effect on capsaicin-evoked release compared with untreated controls (Fig. 5B). When cells were exposed to SCsiRNA, which did not reduce levels of Ape1, 10 μmol/L cisplatin for 24 hours significantly reduced cisplatin-evoked release to $7.0 \pm 0.2\%$ of total iCGRP content, which is to the values observed in control cells treated with 10 μmol/L cisplatin (Fig. 5A). Reducing Ape1 expression using siRNA, however, significantly augmented the ability of cisplatin to reduce peptide release to $4.8 \pm 0.3\%$ of total content (Fig. 5B).

As in previous studies, we next determined if overexpression of Ape1 or Ape1 mutants could reverse the effects of cisplatin on iCGRP release. As in previous experiments, cultures were first treated with siRNAs, infected with viral vectors, exposed to 10 μmol/L cisplatin for 24 hours, then basal and capsaicin-evoked

iCGRP release measures. When cells treated with SCsiRNA or Ape1siRNA were infected with the vector control, cisplatin treatment resulted in a significant decrease in capsaicin-evoked release of iCGRP in a manner analogous to untreated cells (Fig. 5C). In contrast, overexpression of WT-Ape1 or C65-Ape1 significantly attenuated the effects of cisplatin (Fig. 5C). The addition of siRNA and viral vectors in cells not exposed to cisplatin did not alter basal or capsaicin evoked release of iCGRP (data not shown). In addition, no treatment regimen significantly affected basal release.

These data suggest that the repair component of Ape1 is involved in reversing the effects of cisplatin on peptide release from sensory neurons. To further substantiate this notion, experiments were performed using overexpression of another mutant Ape1, N226A + R177A-Ape1 (24). This mutant has the redox component of WT-Ape1, but no repair function (24). When this mutant was overexpressed in sensory neuronal cultures, it had no effect on the ability of cisplatin to reduce capsaicin-evoked release of iCGRP (Fig. 5C).

Effect of Ape1 on DNA Damage and Expression of Transcription Factors in Sensory Neuronal Cultures

Although the data above indicate that Ape1 is neuroprotective against cisplatin-induced neurotoxicity, the question, what is the

mechanism of this protection, remains, thus to determine if exposing sensory neurons to cisplatin would alter the phosphorylation of histone H2A.X as an indicator of double-strand breaks (26) and transcription factors that are downstream of Ape1 that are under redox control by Ape1, such as the transcription factor p53 (33). Sensory neuronal cultures were treated with either SCsiRNA or Ape1siRNA and followed by 50 $\mu\text{mol/L}$ cisplatin, and the cells were harvested after 8, 24, and 48 hours exposure. When cells treated with SCsiRNA were exposed to cisplatin, there was a small increase in H2AX phosphorylation and phosphorylation of p53 at the Ser¹⁵ position over time (Fig. 6A and B). Figure 6A shows a representative Western blot, whereas Fig. 6B shows summary results from three experiments. These increases were not significantly different from cultures not exposed to cisplatin (time zero). There also was no significant change in Gadd45a expression as measured by Western blotting. In contrast, in cells treated with Ape1siRNA, phosphorylation of H2AX was significantly higher after 48 hours of cisplatin exposure (Fig. 6A and B). Phosphorylation of p53 was also significantly higher in cultures with reduced Ape1 expression after 24 and 48 hours of cisplatin treatment. Gadd45a protein was also greatly augmented at 24 hours in the Ape1siRNA knockdown sensory neuronal cells compared with SCsiRNA controls. This time course indicates an increase in the p53

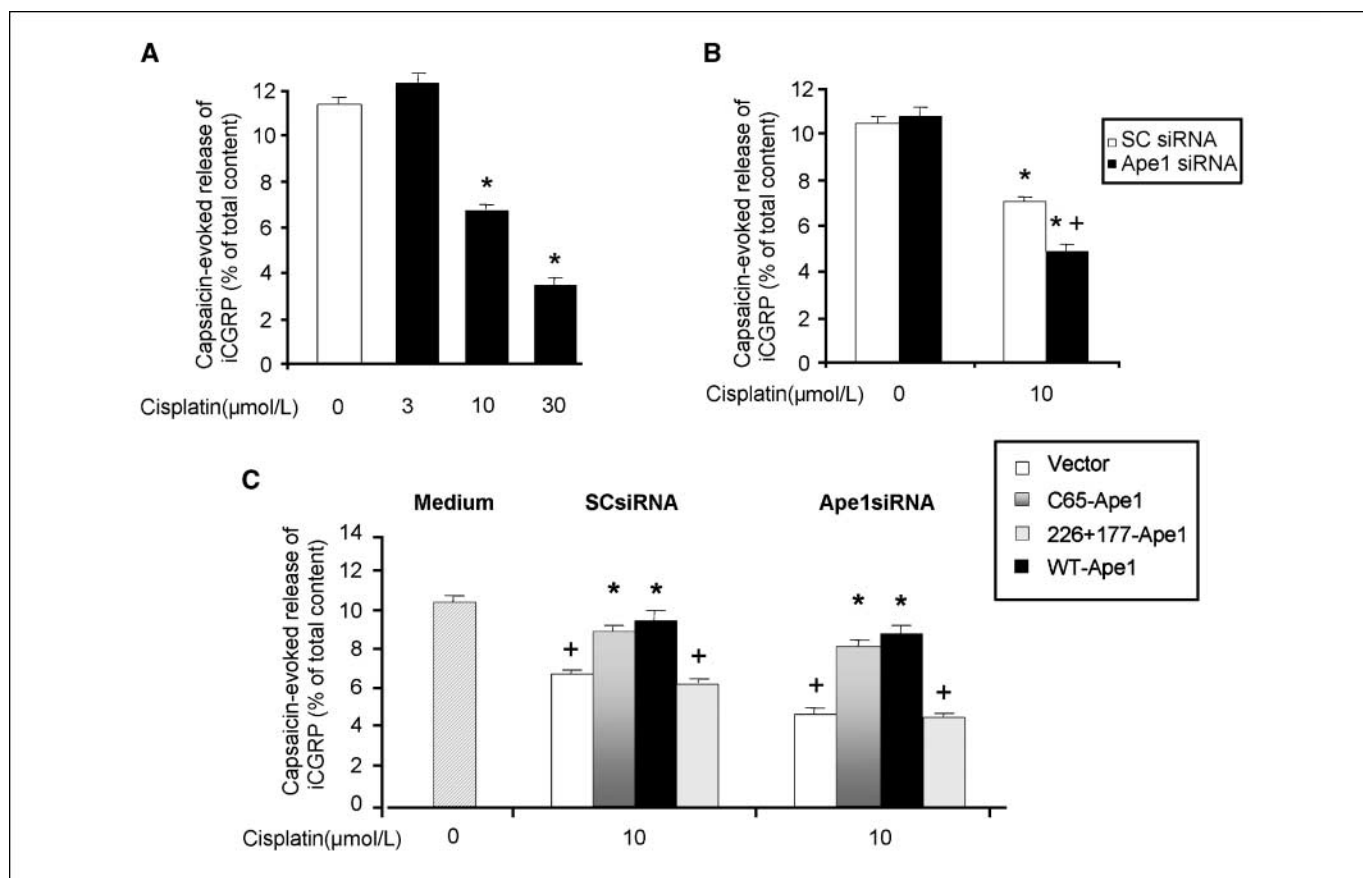


Figure 5. Effect of altered Ape1 levels on cisplatin-induced iCGRP release from sensory neuronal cells. *A*, sensory neuronal cells were treated for 24 h with increasing amounts of cisplatin. There was a decrease in the amount of iCGRP released in the capsaicin-evoked release of iCGRP. *Columns*, mean of the percentage of total content of iCGRP after exposure to 30 nmol/L capsaicin for nine wells of neurons; *bars*, SE. *B*, sensory neuronal cells were treated with cisplatin, Ape1-siRNA or SC-siRNA and/or virus as indicated. Compared with untreated controls, SCsiRNA-treated and Ape1siRNA-treated cells had a statistically significant (*, $P < 0.05$) decrease in release after exposure to cisplatin. *C*, sensory neuronal cells were treated with cisplatin at 10 $\mu\text{mol/L}$ and either SCsiRNA or Ape1-siRNA and then either adenoviral vector control or adenovirus with either WT-Ape1, C65-Ape1, or N226A + R177A Ape1. There was a significantly significant increase in the release in the C65A-Ape1 and WT-Ape1 transgenes compared with the vector or N226A + R177A-Ape1 (*, $P < 0.05$).

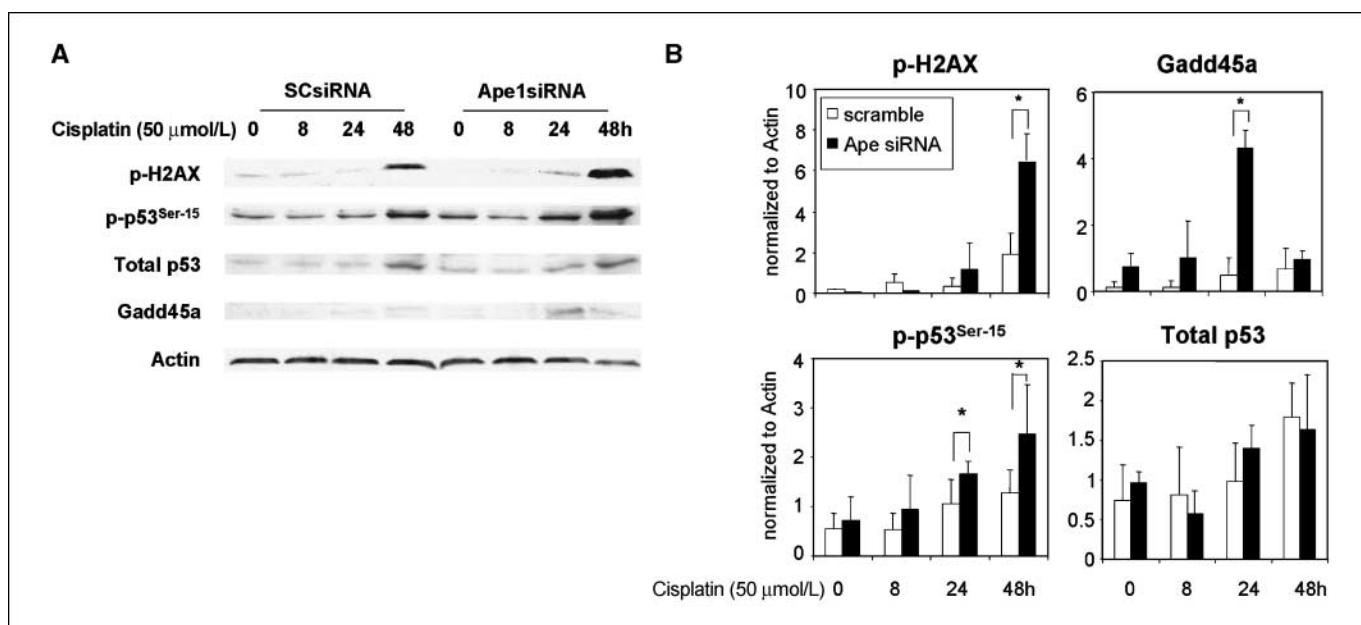


Figure 6. Effect of altering Ape1 levels on the p53 signaling and dsb pathway after cisplatin treatment of sensory neuronal cells. *A*, a representative Western blot analysis of the protein levels of p53 and Gadd45a and the phosphorylation of p53^{Ser15} and phosphorylation levels of H2AX. Sensory neuronal cell cultures were incubated with Ape1siRNA or SCsiRNA control oligos and were then treated with 50 μ mol/L cisplatin for 48 h. *B*, the protein levels of three independent experiments after normalization to actin. *Columns*, mean for at least three independent harvests of cells; *bars*, SE. Differences were considered significant at * $P < 0.05$.

response pathway leading to an increase in Gadd45a after p53 activation 24 to 48 hours after cisplatin treatment and with the reduction of Ape1 in cells.

Discussion

This report is the first data demonstrating a role of a BER protein, Ape1, in sensory neuronal survival and function after cisplatin treatment. Furthermore, we show that it is largely Ape1 repair function that is involved in the response of sensory neuronal cells to cisplatin stress and that cisplatin causes an alteration of the p53 signaling pathway. We chose to examine the effects of cisplatin on sensory neurons because the neuropathy caused by this anticancer drug is largely sensory and often involves paresthesia and/or overt pain (34). Furthermore, platinum binding to DNA in sensory neurons is high (3, 6) and exposing isolated sensory neurons to cisplatin causes apoptosis (32). In this report, we observed a concentration-dependent increase in sensory neuronal cell killing and apoptosis after cisplatin, confirming the previous studies. Of interest, however, is the fact that a 10 μ mol/L over 24 hours significantly reduced the release of iCGRP from sensory neurons. We did not observe any significant change in the content of iCGRP in the cultures after exposure to cisplatin (data not shown); thus, the cisplatin-induced alterations in release were not secondary to cell death or loss of transcription. Our observation of a decrease in evoked release of iCGRP after cisplatin exposure, are similar to *in situ* studies demonstrating that treating rats with 1.5 mg/kg cisplatin once per day for 5 days results in a decrease in stimulated-release of the neuropeptides substance P, somatostatin, and CGRP from the lower trachea and main bronchi (35). The cisplatin-induced decrease in CGRP release from sensory neurons suggests a loss of function of these neurons that could have important consequences. The release of CGRP from peripheral terminals of small diameter sensory neurons is associated with

local vasodilation that contributes to neurogenic inflammation (36). Indeed, CGRP is a potent vasodilator and a decrease in its release could negatively affect local blood flow and contribute to symptoms of peripheral neuropathy. At central terminals of sensory neurons, CGRP may contribute to thermal sensation as well as nociception. Thus, a decrease in its release in response to stimuli could result in reduced sensory input. The potential effect of a decrease in CGRP release on nociception still needs to be determined. Cisplatin has been shown to induce ROS generation in a selected number of cell systems (10, 37) and the increase in ROS could damage lipids, proteins, and nuclear and mitochondrial DNA (mtDNA), leading to cell death (38). Oxidative damage has been suggested as the main cause of cisplatin-induced renal cell death and several antioxidants and radical scavengers alleviate cisplatin-induced nephrotoxicity *in vitro* and *in vivo* (39, 40). Recently, it was shown that inhibiting mitochondrial respiration results in an enhancement of cisplatin toxicity to leukemic cells (10) and an imbalancing of the BER pathway in mitochondria using the OGG1 protein leads to enhanced cisplatin toxicity (10). In line with these studies, our results show a significant increase in intracellular ROS concentration in sensory neuronal cells follow cisplatin exposure (Figs. 1 and 2). The basal increase in ROS production in sensory neuronal cells parallels a significant increase in Ape1 expression; the first time an induction of Ape1 has been seen after cisplatin treatment. Furthermore, reducing Ape1 expression augments cisplatin-induced ROS production. This correlates with previous studies in tumor cell lines that showed cisplatin apoptosis is mediated by ROS production and Ape1 overexpression can suppress this ROS production (18, 41, 42).

The question remains as to the mechanisms by which cisplatin induced toxicity in sensory neurons. Previous work shows a correlation between the platinum binding to DNA and the toxicity in sensory neurons (3, 6). This would imply that the nucleotide excision repair pathway is important in regulating cisplatin-induced

toxicity and in mice deficient in NER function; there is an increase in accumulation of unrepaired cross-links and in neurotoxicity (7). Our current findings, however, show that Ape1 plays a role in reducing cisplatin-induced neurotoxicity. We show that reducing Ape1 by siRNA increased cisplatin-induced ROS production (Fig. 2) and also increased cell killing and apoptosis (Figs. 3 and 4) and decreased iCGRP release (Fig. 5). Moreover, overexpression of Ape1 in DRG cells or siRNA knockdown DRG cells resulted in increased protection from cisplatin. Our current results further suggest that the repair component of Ape1 plays a major role in its ability to protect against cisplatin-induced toxicity. In most studies, both WT-Ape1 and the C65-Ape1 repair competent/redox-deficient protein protected the cells, although overexpression of the C65 mutant was effective at an intermediate level. In contrast, the N226A + R177A-Ape1, which has the redox component of WT-Ape1, but no repair function (24), was not effective in blocking the ability of cisplatin to decrease iCGRP release.

To begin to determine the mechanistic and signaling pathways that may be involved in the response of DRG cells to cisplatin-induced stress and the role of Ape1 in this process, we studied the role of the p53 stress response pathway. We chose this pathway to begin our analyses given the previously shown interactions between Ape1 and p53 in tumor and normal, dividing cells (33, 43) and because the p53 tumor suppressor protein plays a major role in cellular response to DNA damage and other genomic aberrations, particularly in mitotically growing cells. p53 is a nuclear phosphoprotein and phosphorylation of Ser¹⁵, a key phosphorylation target during the p53 activation process, has been shown as being critical for p53-dependent transactivation (44). DNA damage induces phosphorylation of p53 at Ser¹⁵ and leads to reduced interaction between p53 and its negative regulator, oncoprotein MDM2 (45, 46). To begin to examine this pathway, we performed studies using Western blot analysis and found that altering Ape1 levels leads to alterations in the amount of phosphorylated p53 and an increase in Gadd45a protein levels (Fig. 6). Our results indicate an induction of p53 phosphorylation at Ser¹⁵ after the alteration of Ape1 levels and cisplatin treatment, which is the first time a relationship between Ape1 protein levels and p53 phosphorylation has been observed. This change in p53 phosphorylation was correlated with an induction of Gadd45a and implicates this pathway as the primary signaling pathway involving Ape1, cisplatin, and ROS in DRG cells. Gadd45a, which is involved in the DNA repair,

maintenance of genomic stability, cell cycle control, and apoptosis, and its induction is primarily p53 dependent (47). The p53-GADD45a pathway is also intimately linked to the repair of DNA damage related to cross-linking agents or those that are normally repaired by the NER pathway (33, 48). Therefore, our findings reported here are consistent with alterations in Ape1 levels and functions impinging on DRG survival and function by altering both BER and NER pathways.

While we present a number of novel findings in this report (induction of Ape1 by cisplatin, knocking down Ape1 levels leading to increased ROS in postmitotic cells, role of Ape1 redox and repair functions in cisplatin survival, and function of sensory neurons and the perturbation of the p53-Gadd45a signaling pathway by Ape1), we have not completely delineated all the mechanisms that may be acting with Ape1 perturbation in the DRG neuron. One of the areas that will require additional study involves the role of the mitochondria in these cells and in the DRG response to chemotherapeutic agents. For example, in a recent report, apoptosis induced by cisplatin was shown to require the production of ROS, but was independent of damage to nuclear DNA (49). Additionally, mtDNA has been shown to be naturally more sensitive to oxidative damage than nuclear DNA (50). Therefore, studying the effects of cisplatin on the mitochondrial genome and role of Ape1 in the repair of mtDNA damage after cisplatin treatment warrants further investigation. It may turn out that the main response of DRG neurons to cisplatin treatment is mediated through mitochondrial ROS production, such that altering Ape1 leads to a suboptimal response of the mitochondria and a chain of events resulting in DRG dysfunction or death.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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