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NF-κB activation is required for cisplatin-induced apoptosis in head and neck squamous carcinoma cells

Seong Bum Kim[a,1], Jong Soo Kim[a,1], Ji Hye Lee[a], Won Joon Yoon[a], Dong Seuk Lee[b], Myoung Seok Ko[a], Byung Suk Kwon[a], Dae Hwa Choi[c], Hong Rae Cho[c], Byung Ju Lee[a], Dae Kyun Chung[b], Hyeon Woo Lee[d], Jeong Woo Park[a,*]

[a] *Department of Biological Sciences, University of Ulsan, Nam-Gu, Ulsan 680-749, Republic of Korea*
[b] *School of Biotechnology and Institute of Life Science and Resources, and RNA Inc., Kyung Hee University, Yongin-gun, Kyungki-do, Republic of Korea*
[c] *Department of Surgery, Ulsan University Hospital, College of Medicine, University of Ulsan, Ulsan 682-060, Republic of Korea*
[d] *Department of Pharmacology, School of Dentistry, Kyung Hee University, Republic of Korea*

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Abstract **This study demonstrates a requirement for NF-κB activation in *cis*-diamminedichloroplatinum (cisplatin)-induced apoptosis in human head and neck squamous cell carcinoma (HNSCC) cell lines. This conclusion was supported by the following observations: cisplatin induced IκBα degradation and NF-κB-dependent transcriptional activation prior to cell death; pyrrolidine dithiocarbamate (PDTC), a chemical inhibitor of NF-κB activation, prevented apoptosis; lactacystin, an inhibitor of IκBα degradation, also prevented apoptosis; and finally, the expression of a super-repressor mutant IκBα blocked apoptosis. The expression of tumor necrosis factor α (TNFα) was promoted by cisplatin treatment and was suppressed by PDTC treatment. In addition, a neutralizing antibody against TNFα protected cells from cisplatin-induced apoptosis. These findings suggest that NF-κB activation is required for cisplatin-induced apoptosis and TNFα may play an important role in NF-κB-mediated apoptosis in cisplatin-treated HNSCC cell lines.**

Keywords: Human head and neck squamous cell carcinoma; Cisplatin; Apoptosis; NF-κB; TNFα

1. Introduction

cis-Diamminedichloroplatinum (cisplatin) is a DNA-damaging agent which has been used therapeutically in head and neck cancer, both alone and in combination with other chemotherapeutic agents or radiation therapy [1,2]. Apoptosis induced by cisplatin is generally considered to be the result of its ability to damage DNA [3], but the mechanisms by which the DNA damage triggers apoptosis remain unclear. There is evidence that cisplatin induces the activation of NF-κB and that this NF-κB activity results in an increase in cisplatin resistance [4]. Consistent with this, many studies have supported the general view that NF-κB activation delivers a survival signal that protects cells from apoptosis [5,6] by promoting the expression of survival factors, such as certain members of the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, and XIAP) [7] and the Bcl-2 homologues Bfl-1/A1 [8] and Bcl-xL [9].

In contrast, there are also many reports about a pro-apoptotic role for NF-κB. First, many potent NF-κB stimulators such as TNF [10], ceramide [11], H_2O_2, and serum deprivation [12,13] ultimately induce apoptosis. Second, both apoptosis and the activation of NF-κB are suppressed by the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) [14]. Third, the NF-κB-induced death receptor 4 (DR4), DR5 [15], TNF-related apoptosis-inducing ligand (TRAIL) [16,17], Fas [18], and Fas ligand (FasL) [19] all may promote cell death [19,20]. Fourth, the upstream promoter regions of several 'death genes' contain potential NF-κB binding motifs [13]. These accumulated experimental data strongly suggest that exogenous signals such as toxins or reactive oxygen species activate NF-κB. This activation results in the transcription of genes that later execute the apoptosis program [12,21,22].

These conflicting studies of the role of NF-κB in apoptosis suggest that the effects of many signaling molecules are dependent upon cell content and/or stress. Genotoxic drugs affect many signal transduction pathways, and the final outcome, cell survival or death, may be determined by complicated interactions between these signal transduction pathways.

This study examined the role of NF-κB activation in regulating cisplatin-induced apoptosis in human head and neck squamous cell carcinoma (HNSCC) cell lines, and the results from this study indicate that NF-κB activation is required for cisplatin-induced apoptosis. In addition, experiments described here demonstrate that TNFα, induced by NF-κB activity in the cisplatin-treated HNSCC cell lines, may play important roles in NF-κB-mediated apoptosis.

2. Materials and methods

2.1. Cell culture

The human HNSCC cell lines HN3 and HN4 were derived from patients with HNSCC [23] and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco-BRL), 100 U of penicillin, and 100 μg of streptomycin/ml. Cells

*Corresponding author. Fax: +82 52 259 1694.
E-mail address: jwpark@uou.ulsan.ac.kr (J.W. Park).

[1] These authors contributed equally to this work.

Abbreviations: Cisplatin, *cis*-diamminedichloroplatinum; PDTC, pyrrolidine dithiocarbamate; HNSCC, head and neck squamous cell carcinoma; TUNEL, TdT-mediated dUTP nick end labeling

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were cultured at 37 °C in a humidified chamber containing 5% CO_2. For the induction of apoptosis, cells were plated in 60-mm dishes 1 day prior to cisplatin treatment.

2.2. TUNEL staining

TdT-mediated dUTP nick end labeling (TUNEL) staining was conducted using an in situ cell death detection kit, TMR Red, according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals). Briefly, cells were plated in 60-mm dishes at 2×10^5 cells/ml DMEM. On the following day, the cells were treated with cisplatin, harvested, and fixed with 2% paraformaldehyde solution and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing twice with PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM $Na_2HPO_4 \cdot 7H_2O$; and 1.4 mM KH_2PO_4; pH 7.2), cells were incubated in a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and tetramethyl-rhodamine-dUTP. Cells were analyzed for fluorescence intensity using a FACS flow cytometer (Becton–Dickinson Inc.) and FluoView™ 500 confocal microscope (Olympus).

The effect of the caspase-8 inhibitor, z-IETD-fmk (Calbiochem, USA), on cisplatin-induced apoptosis was determined by adding 10 or 30 μM z-IETD-fmk to cells 1 h prior to treatment with 40 μM cisplatin.

2.3. Annexin V staining

Annexin V staining was conducted using an Annexin-V-FLUOS staining kit, according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals). Briefly, cells were washed twice with PBS and resuspended in a binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; and 2.5 mM $CaCl_2$) containing Annexin V. Cells were analyzed for fluorescence intensity using a FACS flow cytometer (Becton–Dickinson).

2.4. Caspase-8 activity assay

Caspase-8 activity was assayed using a Caspase-8 Colorimetric Assay Kit (BD ApoAlert™ Caspase Colorimetric Assay Kit), according to the protocol supplied by the manufacturer (BD Biosciences). Briefly, cells (2×10^6) were lysed in 50 μl lysis buffer for 10 min on ice. After centrifugation in a microcentrifuge at maximum speed for 10 min at 4 °C, supernatants were incubated with 5 μl of 4 mM caspase-8 substrate, IETD-pNa, for 1 h at 37 °C. Caspase-8 activity was determined by reading absorbance at 405 nm taken against a blank containing buffer and peptide alone.

2.5. Propidium iodide staining

Cells were plated in 60-mm dishes at 2×10^5 cells/ml DMEM. On the following day, the cells were treated with cisplatin for 6 h, fixed with 2% paraformaldehyde solution, and incubated with propidium iodide buffer (0.1% sodium citrate, 0.1% Triton X-100, and 0.1 mg/ml propidium iodide). Subsequently, nuclei of the cells were observed with a FluoView™ 500 confocal microscope (Olymphus).

2.6. Immunoblot analysis

Cells were washed twice with cold PBS, and 30–50 μg of protein was resolved by SDS–PAGE, transferred onto Hybond-P membranes (Amersham Biosciences Inc.), and probed with appropriate dilutions of the following primary antibodies: anti-IκBα (C-21) (Santa Cruz Biotechnology Inc.) and anti-caspase 8 (Ab3) (Oncogene™ Research Product). Immunoreactivity was detected using the ECL detection system (Amersham Biosciences). The films were exposed at multiple time points to ensure that the images were not saturated.

2.7. Exposure to TNFα or an anti-TNFα antibody

Cells were incubated in 10 or 50 ng/ml of TNFα (BD Biosciences Pharmingen) for 24 h at 37 °C or in 10, 50 or 100 μg/ml of anti-TNFα antibody (Was) (BD Biosciences Pharmingen).

2.8. Exposure to NF-κB inhibitor or an inhibitor of IκB degradation

NF-κB activation was inhibited by incubating cells in 120 μM PDTC (Sigma) or 10 μM lactacystin (Calbiochem, USA).

2.9. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared as described [24]. Double-stranded oligonucleotides containing a consensus binding site for c-Rel (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Santa Cruz Biotechnology) were 5′-end-labeled using polynucleotide kinase and [γ-^{32}P]dATP. Nuclear extracts (2.5–5 μg) were incubated with 1 μl of labeled oligonucleotide (20000 cpm) in 20 μl of incubation buffer (10 mM Tris–HCl, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 2% glycerol, and 1–2 μg of poly(dI–dC)) for 20 min at 25 °C. The specificity of NF-κB DNA-binding activity was confirmed by competition with mutant oligonucleotide. DNA–protein complexes were resolved by electrophoresis in 5% non-denaturing polyacrylamide gels and analyzed by autoradiography. For supershift experiments, nuclear extracts were incubated with 2 μg of anti-NF-κB p50 (E-10) (Santa Cruz Biotechnology) for 1 h on ice before the labeled probe was added.

2.10. Transfection

HN4 cells expressing the IκBα mutant αSR were established by transfection of 10 μg of pRcCMVIκBSR (Invitrogen) using Lipofectamine (Gibco-BRL), followed by selection with G418 (100 μg of G418/ml; Gibco-BRL). A control cell line, HN4/pRcCMV, was generated by transfection with pRcCMV vector.

2.11. Real-time PCR

Five micrograms of DNase I-treated total RNA was reverse transcribed using random priming and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye (Molecular Probes, Eugene, OR) on a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research Inc.) according to the manufacturer's instructions. Specificities of each primer pair were confirmed by melting curve analysis and agarose-gel electrophoresis. Primers were designed using the Primer3 software: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. PCR primer pairs were as follows: Fas: AGGACATGGCTTAGAAGTGG, CACCCCAAGTTAGATCTGGA; FasL: GGATGTTTCAGCTCTTCCAC, CATAGGTGTCTTCCCATTCC; TNFR1: GCTTCAGAAAACCACCTCAG, CACACGGTGTTCTGTTTCTC; TNFα: CTGTAGCCCATGTTGTAGCA, AGGTTGACCTTGGTCTGGTA; DR4: TGGAGTGACATCGAGTGTGT, GTTGTGAGCATTGTCCTCAG; DR5: AGCACTCACTGGAATGACCT, CACTCCAGGGTGTACAATCA; TRAIL: GCTGATCGTGATCTTCACAG, GGTTTCCTCAGAGGTTCTCA; TNFR-associated factor 2 (TRAF2): CTACAAGATGTGTCTGCGTAT, CTCTGAAAAGAGGATGAAGTC; Fas-associated death domain protein (FADD): CTCTCTGAGACTGCTAAGTAGG, AATAGGATTACCTTAGGAATAAGA.

3. Results

3.1. Cisplatin treatment induces apoptosis in HN4 cells

Cisplatin treatment results in apoptosis in many different cell types. To examine the ability of cisplatin to induce apoptosis in HN4 cells, cultures were treated with varying amounts of cisplatin for 12 h, after which they were stained with TUNEL and examined by FACS analysis. Cisplatin caused apoptosis of HN4 cells in a dose-dependent manner, with a concentration of 40 μM cisplatin resulting in death of greater than 62% of the cell population by 12 h of treatment (Fig. 1A). The kinetics of cisplatin-induced apoptosis was examined using a 40 μM dose. Cisplatin-induced apoptosis was detectable after 5 h of treatment of cisplatin at this dose and increased dramatically thereafter (Fig. 1B).

3.2. Cisplatin induces IκBα degradation and NF-κB activation

To determine whether or not cisplatin treatment enhances NF-κB activity, first the IκBα levels after treatment of HN4 cells with 40 μM cisplatin were determined. IκBα levels are of importance in the regulation of NF-κB activity under most conditions [25]. There was no significant change in the IκBα level in untreated HN4 cells over time. However, a marked degradation of IκBα was observed at 4 h after cisplatin treatment,

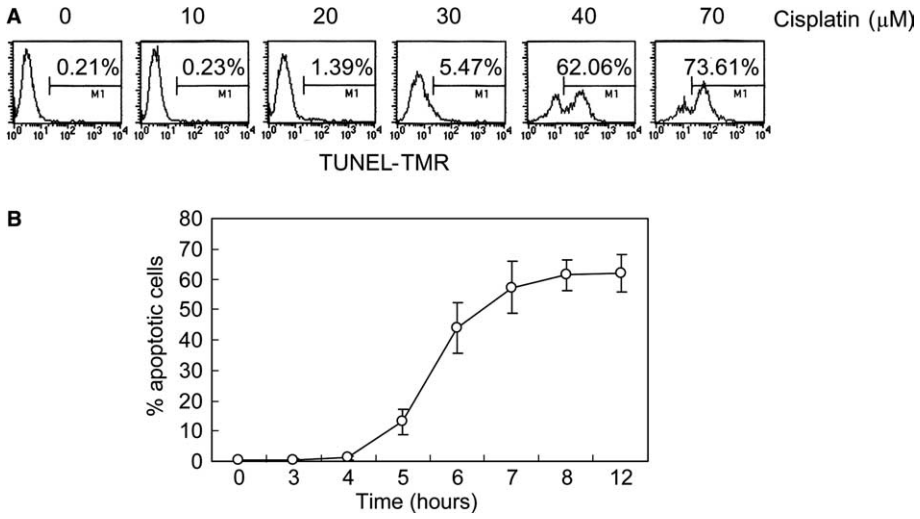

Fig. 1. Cisplatin treatment induces apoptosis of HN4 cells in a dose- and time-dependent manner. HN4 cells were treated either with different doses of cisplatin for 12 h (A) or 40 μM cisplatin for the indicated times (B). Cells were then fixed with 2% paraformaldehyde, stained with TUNEL, and analyzed by FACS. Cells analyzed under marker M1 are apoptotic (TUNEL positive). Data represent means ± S.D. of three independent experiments.

just before cisplatin-induced apoptosis began to be detected (Fig. 2A, left panel). After the initial degradation, the level of IκBα gradually recovered with time, which was consistently observed in three separate experiments.

An EMSA was performed with a specific NF-κB binding site probe using a nuclear extract collected 4 h after cisplatin treatment. Cisplatin-induced activation of NF-κB was demonstrated by the increased levels of DNA–protein complexes in cisplatin-treated HN4 cells compared to untreated HN4 cells (Fig. 2B). The DNA–protein complexes were dramatically reduced by treatment with PDTC or preincubation of the reaction mixture with an NF-κB p50-specific antibody (Fig. 2C), which suggests that the protein in the DNA–protein complex is NF-κB. Taken together, these results indicate that cisplatin treatment increases the NF-κB activity in HN4 cells.

3.3. Cisplatin-induced apoptosis is blocked by PDTC, an NF-κB inhibitor

To determine the role of NF-κB in the cisplatin-induced apoptosis in HN4 cells, NF-κB activation was blocked by pretreating cells with the NF-κB inhibitor PDTC for 30 min prior to the addition of 40 μM cisplatin. PDTC treatment

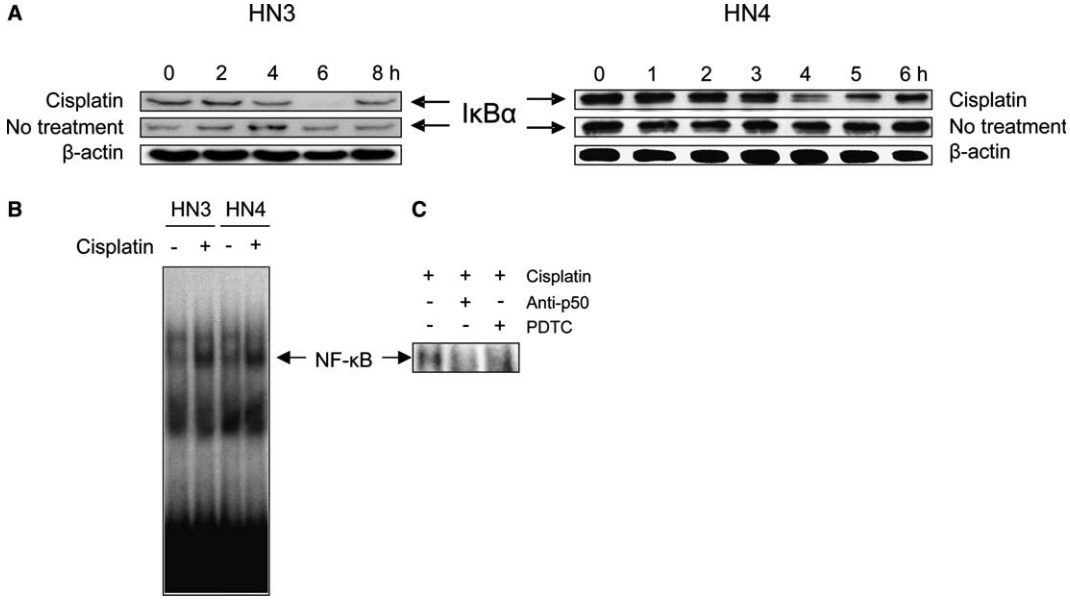

Fig. 2. Cisplatin treatment causes an increase in the DNA binding of NF-κB. (A) Cisplatin treatment increases IκBα degradation. HN3 and HN4 cells were treated with control medium (no treatment) or 40 μM cisplatin. Cells were collected at the indicated time after cisplatin treatment and total cell lysates (30 μg) were resolved by SDS–PAGE and immunoblotted to detect IκBα. (B) Nuclear extracts were collected from HN3 and HN4 cells 6 and 4 h after cisplatin treatment, respectively, and were incubated with an NF-κB DNA probe. DNA–protein complexes were significantly increased in cisplatin-treated HN3 and HN4 cells 6 and 4 h after cisplatin treatment, respectively. (C) The DNA–protein complexes were reduced by PDTC treatment or preincubation of the reaction mixture with an antibody to NF-κB p50.

suppressed the NF-κB binding activity induced by cisplatin, which confirmed the inhibitory effect of the PDTC on NF-κB activity (Fig. 2C). Next, the effect of PDTC on the cisplatin-induced apoptosis in HN4 cells was examined. Cells treated with cisplatin alone displayed typical features of apoptosis including condensation of nuclei (Fig. 3A). However, pretreatment of cells with PDTC markedly suppressed the morphologic changes induced by cisplatin (Fig. 3A, top). To confirm the suppressive effect of PDTC on cisplatin-induced apoptosis in HN4 cells, cells treated with cisplatin in the presence or absence of PDTC were stained by TUNEL, and apoptotic cells were observed with confocal microscopy. PDTC reduced the cisplatin-induced apoptosis of HN4 cells from 60% to 4% (Fig. 3A, bottom and Fig. 3B). These results suggest the possibility that, instead of a role in the protection of cells against cisplatin-induced apoptosis, NF-κB activity, upregulated by cisplatin treatment, may mediate cisplatin-induced apoptosis.

3.4. Cisplatin-induced apoptosis is blocked by proteasome and IκB inhibitors

Activation of NF-κB requires the phosphorylation and ubiquitin-mediated degradation of IκBα by the IKK complex. If the activation of NF-κB is essential for cisplatin-induced apoptosis, the treatment of lactacystin, a specific proteasome inhibitor, may protect the HN4 cells from the cisplatin-induced apoptosis by preventing the degradation of IκBα. The addition of 10 μM lactacystin prevented cisplatin-induced IκBα degradation in HN4 cells (Fig. 4A) and protected HN4 cells from cisplatin-induced apoptosis (Fig. 4B). These results support the possibility that NF-κB activation mediates the cisplatin-induced apoptosis.

3.5. Expression of dominant negative mutant IκBαSR blocks cisplatin-induced apoptosis

Although the above results demonstrated that inhibition of NF-κB activity was associated with blocking of cisplatin-induced apoptosis, they did not address whether NF-κB activity was directly involved in cisplatin-induced apoptosis in HN4 cells. Therefore, further experiments using a specific inhibitor of NF-κB activity were performed. These experiments made use of a mutant form of IκBα to block cisplatin-induced apoptosis in HN4 cells. To analyze the role of NF-κB activation by cisplatin treatment, a stable transfectant derivative of HN4 was established. The HN4/IκBαSR cell line stably expresses a non-degradable phosphorylation site mutant of IκBα (Iκϖ BαSR) [26]. As a negative control, an HN4/pRcCMV cell line, stably transfected with empty vector, was also created. We verified the activity of the transfected protein by showing that cisplatin-induced IκBα degradation was impaired in HN4/IκBαSR cells (Fig. 5A). Induction of apoptosis in HN4/pRcCMV and HN4/IκBαSR cells treated with cisplatin was analyzed by TUNEL staining. HN4/pRcCMV cells were very sensitive to cisplatin-induced apoptosis (Fig. 5B), with more than 60% of cells showing apoptosis when treated with 40 μM cisplatin. In IκBαSR-expressing cells, however, there was a pronounced decrease in the sensitivity to cisplatin, with only 5% of cells showing apoptosis (Fig. 5B). These results indicate that NF-κB activation is important in cisplatin-induced apoptosis.

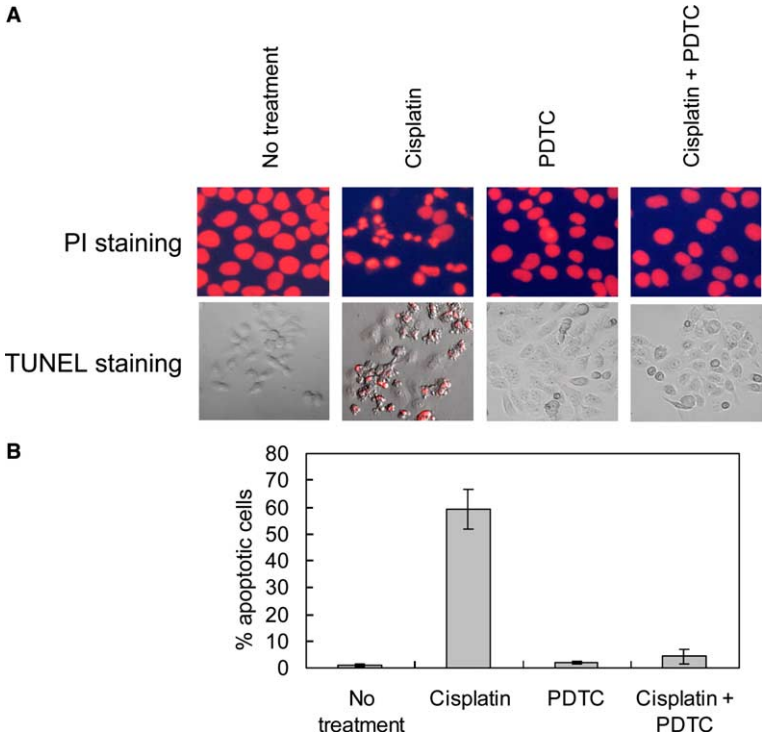

Fig. 3. PDTC inhibits cisplatin-induced apoptosis. HN4 cells were treated with control medium (no treatment), 40 μM cisplatin, 120 μM PDTC, or 40 μM cisplatin plus 120 μM PDTC for 6 h. Cells were stained with PI and the nuclei were examined using confocal microscopy. The nuclei of apoptotic cells are fragmented and condensed (A, top). Cells were fixed with 2% paraformaldehyde, stained with TUNEL, and were observed with confocal microscopy (A, bottom). (B) HN4 cells were collected 6 h after cisplatin treatment, fixed with 2% paraformaldehyde, stained with TUNEL, and were analyzed by FACS. The results are presented as the means ± S.D. from three separate experiments.

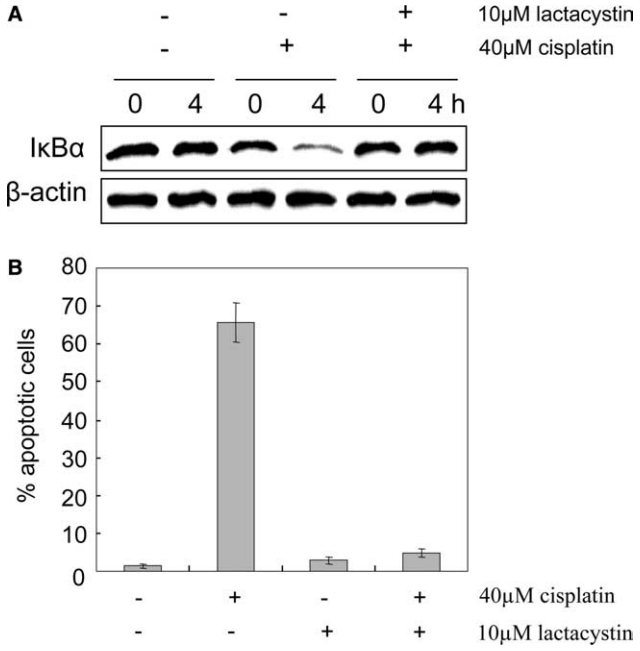

Fig. 4. Lactacystin inhibits cisplatin-induced apoptosis. (A) Immunoblot analysis of IκBα degradation. HN4 cells were treated with 40 μM cisplatin or 40 μM cisplatin plus 10 μM lactacystin. Cells were harvested after cisplatin treatment for 4 h and analyzed for the expression of IκBα by immunoblotting. (B) HN4 cells were treated with control medium (no treatment), 40 μM cisplatin, 10 μM lactacystin, or 40 μM cisplatin plus 10 μM lactacystin. Cells were then fixed with 2% paraformaldehyde, stained with TUNEL, and analyzed by FACS. The results are presented as the means ± S.D. from three separate experiments.

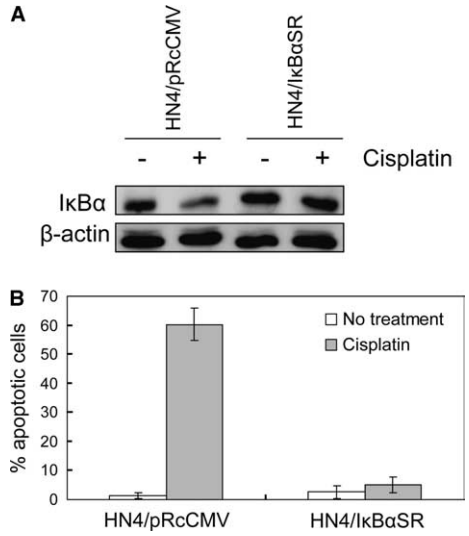

Fig. 5. IκBαSR inhibits cisplatin-induced apoptosis. (A) Immunoblot analysis of IκBα degradation. The stable transfectant cell lines HN4, HN4/IκBαSR, and HN4/pCMV were harvested after cisplatin treatment for 4 h and analyzed for the expression of IκBα by immunoblotting. (B) IκBα expression in HN4/IκBαSR inhibited cisplatin-induced apoptosis. Cells were treated with control medium (no treatment) or 40 μM cisplatin. Cells were then fixed with 2% paraformaldehyde, stained with TUNEL, and analyzed by FACS. The results are presented as the means ± S.D. from three separate experiments.

3.6. NF-κB activation is also required for cisplatin-induced apoptosis in another cell line

Previous work has shown that both the HN3 and HN4 cell lines are cisplatin sensitive [27]. To determine whether or not NF-κB activation is also required for the cisplatin-induced apoptosis in HN3 cells, we, first, determined the change in the IκBα level in cisplatin-treated HN3 cells over time. In three separate experiments, a significant decrease in the IκBα level was observed at 6 h after cisplatin treatment and, after then, the IκBα level gradually recovered at 8 h after cisplatin treatment (Fig. 2A, right panel). The decrease in the IκBα level at 6 h after cisplatin treatment coincided with the increase in the NF-κB activity determined by an EMSA using a nuclear extract collected 6 h after cisplatin treatment in cisplatin-treated HN3 cells (Fig. 2C). Next, we treated HN3 cells with cisplatin in the presence or absence of PDTC and lactacystin. Apoptotic cells were stained by TUNEL and annexin V. Similar to the HN4 cells, the addition of 10 μM lactacystin or 120 μM PDTC protected HN3 cells from cisplatin-induced apoptosis (Fig. 6). These results suggest the possibility that NF-κB activation mediates the cisplatin-induced apoptosis in HN3 as well as in HN4 cells.

3.7. Caspase-8 activity is required for cisplatin-induced apoptosis

A reduction in the levels of caspase-8, a biochemical feature of apoptosis, was detected within 6 h of cisplatin exposure by Western blot analysis (Fig. 7A). To confirm the ability of cisplatin to activate caspase-8, caspase-8 activity was examined in cell lysates from HN4 cells using in an in vitro chromogenic assay with IETD-pNa as a substrate. Cisplatin treatment induced a 3.1-fold increase in caspase-8 activity in HN4 cells (Fig. 7B). In addition, treatment with a caspase-8 inhibitor, z-IETD-fmk, suppressed the caspase-8 activity (Fig. 7B) and cisplatin-induced apoptosis (Fig. 7C) in HN4 cells. These results suggest that caspase-8 activity is required for the cisplatin-induced apoptosis of HN4 cells.

3.8. TNFα is responsible for cisplatin-induced apoptosis

Caspase-8 is normally activated by the engagement of death receptors which belong to the TNF receptor gene superfamily [28]. Thus, it is possible that the NF-κB activity upregulated in HN4 cells by cisplatin treatment may mediate apoptosis by inducing TNF and the TNF receptor (TNFR) superfamily. To determine whether members of this superfamily are induced by cisplatin and are responsible for the cisplatin-induced apoptosis in HN4 cells, the effect of cisplatin treatment on their expression levels was investigated. While cisplatin treatment did not result in any significant changes in the expression of several genes, including *CD95*, *DR4*, *DR5*, *TNFR1*, *FasL*, and *TRAIL*, the expression of *TNFα* was increased by 2.7-fold by cisplatin treatment, and its expression was suppressed by PDTC treatment (Table 1). These results strongly suggest that TNFα is induced by NF-κB activity and may play a critical role in cisplatin-induced apoptosis in HN4 cells.

To further examine the role of TNFα, levels of TNFα in the supernatant of cisplatin-treated HN4 cells were assayed, but no TNFα was detected (data not shown). It is possible that levels of TNFα which are too low to be detectable are still enough to induce the apoptosis. Thus, a neutralizing antibody against TNFα was added to attempt to protect the cells. The TNFα neutralizing antibody treatment resulted in a dose-dependent reduction of the cisplatin-induced apoptosis of HN4 cells

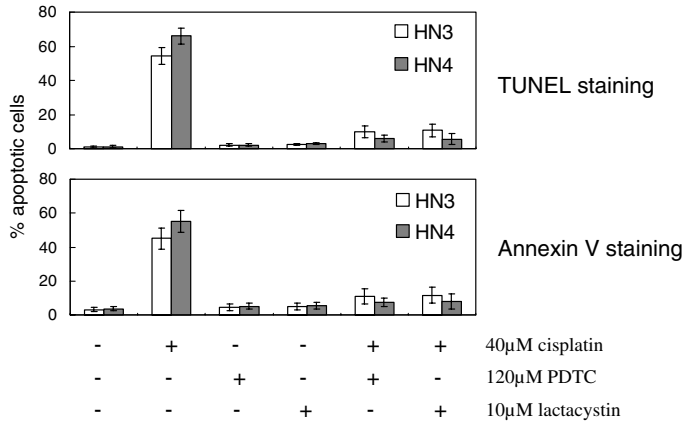

Fig. 6. PDTC and lactacystin inhibit cisplatin-induced apoptosis both in HN3 and HN4 cells. HN3 and HN4 cells were treated with control medium (no treatment), 40 μM cisplatin, 120 μM PDTC, 10 μM lactacystin, 40 μM cisplatin plus 120 μM PDTC, or 40 μM cisplatin plus 10 μM lactacystin for 6 h. Cells were then stained with TUNEL or annexin V, and were analyzed by FACS. The results are presented as the means ± S.D. from three separate experiments.

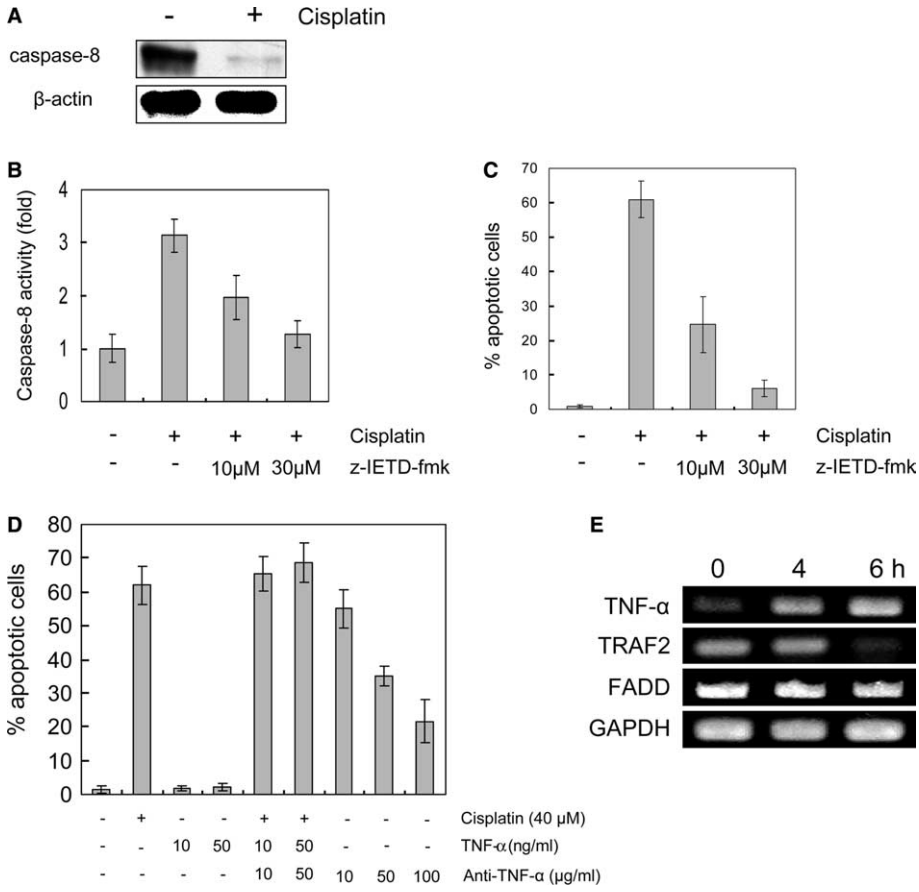

Fig. 7. TNFα is responsible for cisplatin-induced apoptosis. (A) Cleavage of caspase-8 in HN4 cells treated with 40 μM cisplatin. Cells were harvested at 6 h and cleavage was assessed by Western blot analysis using an anti-caspase-8 monoclonal antibody. (B) Caspase-8 activity was measured in cells lysates at 6 h after cisplatin treatment. The caspase-8 activity of untreated HN4 cells was defined as one. (C) Effect of the caspase-8 inhibitor z-IETD-fmk on cisplatin-induced apoptosis in HN4 cells. HN4 cells were treated with 40 μM cisplatin in the absence or presence of z-IETD-fmk (10 and 30 μM). Cisplatin-induced apoptosis was determined by TUNEL staining at 6 h after cisplatin treatment. Data represent means ± S.D. of three independent experiments. (D) An anti-TNFα antibody protected HN4 cells from cisplatin-induced apoptosis. HN4 cells were treated with the indicated concentration of cisplatin or anti-TNFα for 6 h or TNFα for 24 h. Cells were then collected, stained with TUNEL, and were analyzed by flow cytometry. The results are presented as the means ± S.D. from three separate experiments. (E) Semiquantitative RT-PCR analysis. Total RNA samples were isolated from HN4 cells after cisplatin treatment for 4 and 6 h. Cisplatin treatment suppressed the expression of TRAF2, as indicated by RT-PCR analysis.

Table 1
Real-time PCR analysis of selected death receptor genes and their ligands in the absence or presence of PDTC

Gene	Fold change			
	Cisplatin		Cisplatin + PDTC	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
CD95 (Fas)	0.86	0.92	0.38	0.25
TRAIL-R1 (DR4)	1.04	0.98	0.89	0.93
TRAIL-R2 (DR5)	0.65	0.72	0.67	0.59
TNFR1	0.38	0.51	0.41	0.49
CD95L (FasL)	1.02	1.05	0.43	0.52
TRAIL	0.29	0.24	0.11	0.14
TNFα	2.27	3.05	1.16	0.95

The fold-change values for real-time PCR were calculated by the following equation: fold-change value = $2^{-(Ct(CDDP\text{- or CDDP + PDTC-treated HN4}) - Ct(untreated\ HN4))}$.

(Fig. 7D), and addition of 100 μg/ml anti-TNFα monoclonal antibody reduced the cisplatin-induced apoptosis to 21% (Fig. 7D). However, even though treatment with TNFα in combination with cisplatin caused a slight increase in the cisplatin-induced apoptosis, TNFα treatment alone did not induce apoptosis in HN4 cells (Fig. 7D), which suggests that TNFα is not enough to induce apoptosis in HN4 cells.

Binding of TNFα to its receptors can signal both cell survival and death by recruiting the intracellular proteins TRAF2 and FADD, respectively [29–31]. Thus, we determined whether or not cisplatin treatment induced any changes in the expression levels of TRAF2 and FADD. While there was no significant change in the expression of FADD, the expression of TRAF2 was significantly reduced by cisplatin treatment in HN4 cells (Fig. 7E). This change TRAF2 expression may be required for TNFα to induce cell death in HN4 cells. Taken together, the results described above suggest that the cisplatin-induced apoptosis may be mediated by NF-κB-induced TNFα.

4. Discussion

The purpose of this study was to investigate the role of NF-κB activity in the cisplatin-induced apoptosis of HNSCC cell lines. In this report, we showed that the cisplatin treatment induced IκBα degradation and NF-κB activation, prior to cell death. In addition, the small-molecule NF-κB inhibitor PDTC suppressed cisplatin-induced apoptosis, and a specific proteasome inhibitor, lactacystin, prevented cell death. Finally, inhibition of NF-κB activity by expression of mutant form of IκBα significantly reduced the cisplatin-induced apoptosis. Thus, cisplatin-induced NF-κB activation is required for the cisplatin-induced apoptosis in HNSCC cells.

This finding was unexpected because many studies have supported the general view that NF-κB activation delivers a survival signal that protects cell from apoptosis [5,6]. Consistent with a prosurvival function for NF-κB, NF-κB activation induced by cisplatin was found to protect cells from cisplatin-induced apoptosis [4]. In contrast, other recent studies have found evidence that NF-κB is a mediator of cell death [10–15]. NF-κB has been reported to mediate cell death by inducing the expression of the TNFR superfamily, including members such as CD95 (Fas) [18,32], TRAIL-R1 (DR4) [15], and TRAIL-R2 (DR5) [15,33], or their respective cognate 'death ligands', such as TRAIL [16,33,34]. The death signal from the surface death receptors recruits caspase 8, facilitating the connection from death receptor to effector caspases [28,35].

How NF-κB acts to kill HNSCC cells after cisplatin treatment is unclear. As described above, it is possible that NF-κB activates TNFR superfamily-mediated apoptosis. In HN4 cells, NF-κB, activated by cisplatin treatment, resulted in an increase in the expression of TNFα, which has been reported to be induced by NF-κB activity [36,37]. Binding of TNFα to its receptors initiates a signaling pathway by recruiting intracellular proteins which couple the receptors to intracellular signaling pathways. It is well known that the receptor-interacting protein (RIP), TRAF2, and FADD are important effector molecules of TNFR1 signaling [29–31]. RIP and TRAF2 mainly act as cell survival factors to protect against TNF-induced apoptosis via NF-κB activation [29,38,39]. On the other hand, FADD interacts with and activates caspase 8 to initiate the apoptotic cell death pathway [28,35]. In cisplatin-treated HN4 cells, our results showed that TNFα seemed to signal cell death. This was supported by the degradation of pro-caspase-8 seen with cisplatin treatment, the suppression of cisplatin-induced apoptosis by a caspase-8 inhibitor, and suppression of cisplatin-induced apoptosis by the neutralization of TNFα. We did not investigate in detail how TNFα contributes to cell death in cisplatin-treated HN4 cells. However, we observed a significant decrease in the expression of TRAF2, which has been reported to be a key molecule for survival signaling from TNFα [29,38,39]. Thus, it is possible that reduced expression levels of TRAF2 resulting from cisplatin treatment can lead to the apoptotic cell death of HN4 cells in response to TNFα.

TRAF2 has been reported to be an NF-κB target gene [40]. In our study, while the expression level of TNFα, another NF-κB target gene, was increased by cisplatin treatment, TRAF2 expression was decreased. It is possible that κB sites in TNFα and TRAF2 genes may exhibit different preferences for particular subunits comprising the NF-κB dimer, and cisplatin treatment may regulate the expression of these NF-κB target genes by activation of particular subunits of the NF-κB dimer. It will be important to determine which subunits of NF-κB are activated by cisplatin treatment and whether the activation of these subunits is necessary for the expression of TNFα and apoptosis in cisplatin-treated in HN4 cells.

Our study links cisplatin-induced apoptosis and the activation of NF-κB in HNSCC cells. Based upon our results, it is possible that NF-κB, activated by cisplatin, induces the expression of TNFα, which may be involved in the cisplatin-induced apoptosis. A better understanding of which subunits of NF-κB are activated by cisplatin treatment and analysis of the target genes of the particular subunits of NF-κB will enable us to define the mechanism underlying the cisplatin-induced apoptosis of HNSCC cells.

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