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Increase of the resistance of human cervical carcinoma cells to cisplatin by inhibition of the MEK to ERK signaling pathway partly *via* enhancement of anticancer drug-induced NFκB activation☆

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Abstract

In this study, we showed that suppression of the MEK–ERK transduction pathway by a selective inhibitor, 2′-amino-3′-methoxyflavone (PD98059), increased drug resistance of SiHa cells to cisplatin, but not to another common anticancer drug, doxorubicin. The downstream mechanism of this discrepant cellular response was investigated. Both cisplatin and doxorubicin activated nuclear ERK2 and nuclear transcription factor κB (NFκB) of SiHa cells. However, suppression of the MEK–ERK2 pathway by PD98059 resulted in a further enhancement of cisplatin-induced NFκB activation, while no further regulation of NFκB was noted in doxorubicin-treated cells. The activation of NFκB by cisplatin or doxorubicin was not due to the degradation of cytoplasmic IκBα, as demonstrated by western blotting. Transfection of a dominant negative IκBα resulted in a markedly diminished PD98059-induced cisplatin resistance in SiHa cells. Our results suggest that the MEK–ERK signaling pathway plays a role in the chemosensitivity of SiHa cells, and suppression of this pathway increases cisplatin resistance partly *via* an increase of NFκB activation. The mechanism responsible for the discrepant effect of PD98059 on NFκB activation and hence the chemosensitivity of SiHa cells towards cisplatin and doxorubicin remains to be investigated.

Keywords: ERK; NFκB; PD98059; Drug resistance; Cervical cancer

1. Introduction

MAPKs are important signal transduction molecules involved in the cellular response to various stresses [1,2]. Three major MAPK families have been characterized, including ERKs, c-Jun N-terminal kinases or stress-activated protein kinases (JNKs/SAPKs), and p38 kinase [3,4]. ERKs are generally thought to transduce mitogenic signals and to be involved in cell growth and differentiation [1,2,5]. Upon exposure to stress, ERKs may play a role in protecting some types of cells [6]. The dynamic equilibrium between ERKs and other apoptosis-related signal transduction pathways may determine the ultimate fate of cells [7]. However, several recent reports have suggested that activation of ERKs induces cellular apoptosis and correlates with chemosensitivity of the tumors [8–14]. This discrepancy suggests that the relationship between the activity of ERKs and the cellular response to various stresses may be dependent upon individual cellular context and levels of stress. An illustrative example is that suppression of ERK2 sensitizes HeLa cells to oxidative stress but increases resistance of oligodendrocytes towards hydrogen peroxide [9,15].

The classical Ras/ERK pathway transduces signals from the membrane to the cell nucleus. However, since most anticancer drugs primarily cause DNA damage, it seems

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Abbreviations: MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; TBE, 89 mM boric acid, 2 mM EDTA, 89 mM Tris–Cl; TNF, tumor necrosis factor; NFκB, nuclear transcription factor κB; EMSA, electromobility shift assay.

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most likely that these signals initiate from inside the nucleus. Furthermore, the duration of ERK activation in the nucleus is an important determinant of its function [1]. Thus, specific characterization of the behavior of ERK in the nucleus may help clarify the role of ERK in response to anticancer drugs.

NFκB was first recognized as an anti-apoptotic molecule in studies where cells without NFκB activity exhibited greater sensitivity to TNFα and chemotherapeutic agents [16,17]. However, several reports have indicated recently that NFκB may promote cell death [14,18–20]. These conflicting studies suggest that the effects of most 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 the complicated interaction among these signal transduction pathways. The cross-talk between NFκB and other signal transducing molecules remains, for the most part, unknown.

Recently, Wang *et al.* [21] described that ERK2 is activated by cisplatin and that this activation is necessary for cisplatin-induced cell death. At the same time, Carter and Hunninghake [22] reported that an active MEK/ERK pathway may repress NFκB-driven gene expression in response to cytokine stimulation. Since ERK is activated by most anticancer drugs (for a recent review, see [23]), it is of interest to investigate whether ERK plays a role in the regulation of NFκB activation under the stress of DNA-damaging anticancer drugs.

In this study, we used two representative chemotherapeutic drugs, doxorubicin and cisplatin, which cause DNA damage by either inhibiting topoisomerase II or forming DNA adducts. We demonstrated that nuclear ERK2 was activated by both drugs and that suppression of the MEK to ERK2 pathway by an MEK1 inhibitor, PD98059, resulted in an increase of drug resistance towards cisplatin but not towards doxorubicin. We provided evidence that the cause of this drug resistance was partly through the enhancement of cisplatin-induced NFκB activity.

2. Materials and methods

2.1. Cell culture, treatment, and cell fractionation

Human cervical carcinoma SiHa and hepatoblastoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and incubated in a humidified incubator with 5% CO_2, at 37°.

For anticancer drug treatment, cells cultured overnight were pretreated with or without 10 μM 2′-amino-3′-methoxyflavone (PD98059) for 1 hr, and treated with 500 nM doxorubicin (Dox) or 20 μM cisplatin (CDDP) for 3 hr, and then fractionated.

For fractionation, the cells were swollen in an ice-cold hypotonic solution [1 mM KCl, 0.2 mM $MgCl_2$, 4 mM Tris buffer (pH 7.6), 0.2 mM phenylmethylsulfonyl fluoride] for 10 min, and then were lysed by adding an equal volume of the same buffer containing 1% Triton X-100 and vortexing vigorously for 20 s. The nuclei were pelleted by centrifugation in a KUBOTA 1700 microcentrifuge (13,000 rpm for 5 min at 4°). The purified nuclei were lysed with SDS–PAGE sample buffer, and the concentration of nuclear protein was determined using the Bio-Rad reagent. Whole cell lysate was used for some experiments as indicated in the figure legends. After various treatments, the cells were washed with ice-cold PBS, and were scraped in ice-cold PBS. The cells were pelleted by centrifugation in a bench-top centrifuge (1500 rpm for 5 min) and lysed with SDS–PAGE sample buffer. The lysates were sonicated to break bulk DNA. After boiling for 5 min, the protein concentration was determined.

2.2. Cytotoxicity assay

The cytotoxicity of doxorubicin and cisplatin was determined by a tetrazolium-based semiautomated colorimetric assay (MTT assay). Cells (4000/well) were seeded in a 96-well plate. After overnight culture, cells were treated with or without 10 μM PD98059 for 1 hr, and then with various concentrations of doxorubicin or cisplatin for 3 days as indicated in the figures. Cell numbers were evaluated by the MTT assay with an ELISA reader at OD_{540}.

2.3. Western blotting

Western blotting was performed as described previously [24]. All antibodies used in this study were purchased from Santa Cruz Biotechnology. The antibodies, as indicated in the figures, were used with a dilution factor of between 2000- and 4000-fold (0.5–1 μg/mL), and the final images were developed with a chemiluminescence reagent.

2.4. EMSA

The EMSA method was used to determine the DNA binding activity of NFκB. Nuclear extracts were prepared according to the method described by Andrews and Faller [25]. The oligo-deoxynucleotide probe, 5′-GGATTGGGACTTTCCCCTCC-3′, for NFκB binding was end-labeled with [^{32}P]. After incubation with 10 μg nuclear extract at room temperature for 30 min, the electromobility of the probe was analyzed on a 5% nondenaturing polyacrylamide gel containing 0.25 × TBE. NFκB activation induced by TNFα was run in parallel as a positive control, and the NFκB–DNA complex was confirmed by adding anti-NFκB (p65) antibody (supershift experiment).

2.5. Immunocomplex kinase assay

The immunocomplex kinase assay was performed as described previously [26]. Briefly, purified nuclei were lysed with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5%

deoxycholate, 0.1% SDS, 50 mM Tris buffer, pH 8.0) containing a mixture of protease inhibitors (Boehringer Mannheim Biochemicals). After a round of normal serum pre-clearing, the protein concentration was measured by a Bio-Rad protein concentration determination kit (based on the Bradford method). Nuclear proteins (150 μg) were subjected to the immunocomplex kinase assay. Anti-ERK2 or anti-p38 antibody (mouse monoclone; Santa Cruz) was added to the extract and incubated at 4° overnight. The immunocomplex was precipitated with protein G-agarose, and washed four times with RIPA buffer and once with sterilized ddH_2O. The complex was resuspended in kinase assay buffer [40 mM HEPES (pH 8.0), 2 mM dithiothreitol, 5 mM $MgCl_2$] containing 10 μCi [^{32}P]ATP and 1 μg PHAS-I, a specific substrate for ERK and p38. The mixture was incubated at 30° for 30 min. At the end of the incubation, the reaction was stopped by adding SDS–PAGE sample buffer. The immunoprecipitated complex was separated by 10% SDS–PAGE. The isotope-labeled substrate was visualized by exposure of the dried gel to X-ray film.

2.6. Plasmid construction and gene transfection

A dominant negative human IκBα mutant with amino acids 2–71 deleted was constructed under CMV promoter control (parental plasmid was pBK-CMV, Stratagene) as described previously [27]. The reporter plasmid (pRκB-Luc) was constructed by linking the luciferase gene to five NFκB binding sites and the TATA element (based on pRC plasmid, Clontech). The plasmids were transfected into SiHa cells with Lipofectant 2000 (Life Technologies, Inc.) according to the suggestions of the manufacturer. For dominant negative human IκBα transfection, the transfected cells were selected by exposure to 250 μg/mL of G418 for 20 days. The stable clones were pooled and used for chemosensitivity analysis. For reporter gene

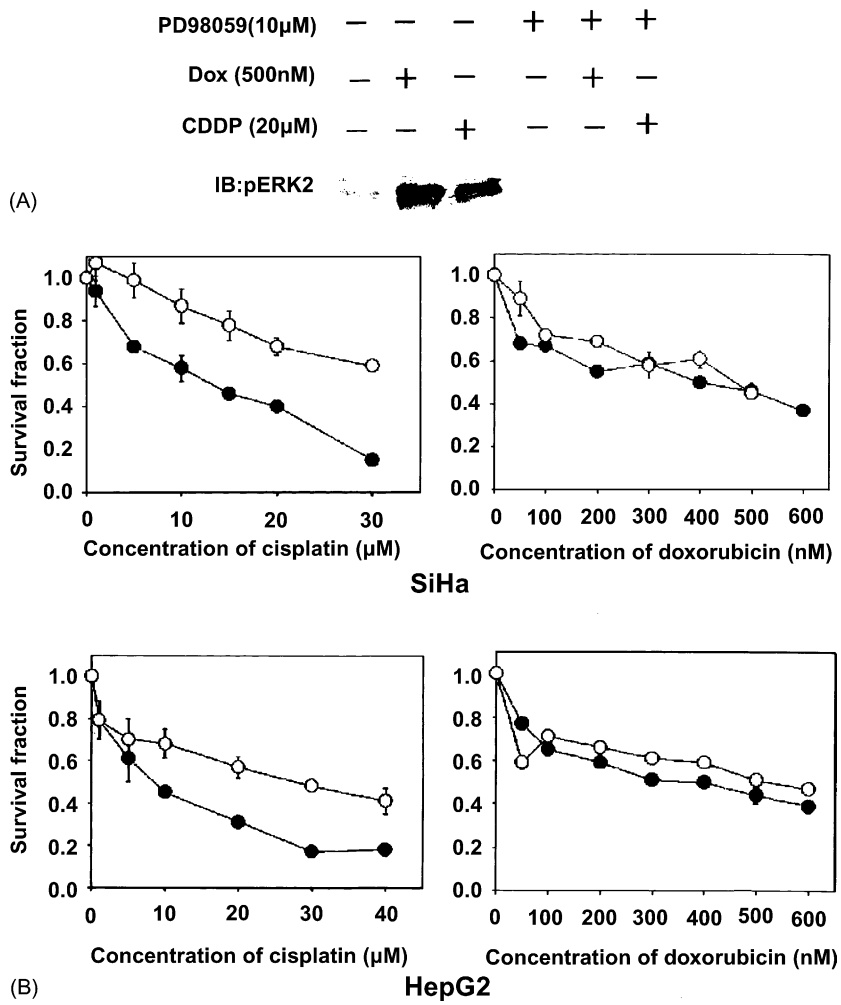

Fig. 1. (A) Nuclear ERK2 immunocomplex kinase assay. SiHa cells were pretreated for 1 hr with or without 10 μM PD98059 and then were treated with cisplatin (CDDP) or doxorubicin (Dox) for 3 hr, as indicated. Nuclei were isolated and subjected to immunoprecipitation with anti-ERK2 antibody. The immunocomplex kinase assay was performed using PHAS-I as the exogenous substrate. (B) Evaluation of the cytotoxicity of cisplatin and doxorubicin by the MTT assay. SiHa and HepG2 cells were pretreated with 10 μM PD98059 for 1 hr and then were co-treated for 72 hr with various concentrations of doxorubicin or cisplatin, as indicated. Treatment with 10 μM PD98059 alone caused a 20% reduction, and the data were normalized accordingly. Each point represents the average (±SD) of at least three independent experiments. Key: (●) cells treated with only cisplatin or doxorubicin; and (○) cells pretreated with PD98059.

transfection, the transfectants were selected by exposure to 400 μg/mL of hygromycin for 2 weeks. The single cell colony was cloned from a low cell-density culture.

2.7. Luciferase activity assay

For the luciferase assay, cells were seeded into a 24-well plate, and cultured overnight. After various treatments the cells were incubated for 6 hr. Cell lysates were prepared to determine the activity of luciferase according to the instructions given in a luciferase reporter gene assay kit (Packard).

3. Results

3.1. Activation of nuclear ERK2 activity by doxorubicin and cisplatin

To elucidate whether ERK2 was activated by anticancer drug challenge, nuclear ERK2 activation after drug treatment was determined by western blotting using a phospho-ERK2 specific antibody. The results showed that treatment for 3 hr with 500 nM doxorubicin or 20 μM cisplatin could activate nuclear ERK2, and that this activation could be blocked by 10 μM PD98059 (Fig. 1A).

3.2. Effect of ERK2 on the toxicity of cisplatin or doxorubicin in SiHa cells

To evaluate the biological significance of ERK2 on the cellular response to cisplatin and doxorubicin, the activity of ERK2 was suppressed by pretreating cells with 10 μM PD98059, and then the toxicity of cisplatin or doxorubicin in SiHa cells was determined by MTT assay. As shown in Fig. 1B, pretreatment with PD98059 increased the resistance of SiHa cells towards cisplatin but had little effect on the cytotoxicity of doxorubicin.

A similar toxic effect of PD98059 on hepatoblastoma HepG2 cells was observed, suggesting that it is not restricted to a single cell type.

3.3. Further enhancement of cisplatin- but not doxorubicin-induced NFκB activation by PD98059

Next, to explore whether NFκB is involved in PD98059-induced resistance towards cisplatin, we examined the effect of PD98059 on the activation of NFκB in cisplatin- or doxorubicin-treated cells. A 1-hr pretreatment with PD98059 enhanced the DNA binding activity of NFκB in cisplatin-treated cells (Fig. 2A). In contrast, PD98059 had only a slight effect on NFκB activation in doxorubicin-treated cells (Fig. 2B). The effect of PD98059 on NFκB activation was elucidated further by a NFκB-directed luciferase activity assay (Fig. 2C). Both cisplatin and doxorubicin activated NFκB to about 1.5- to 1.7-fold of the untreated control. Pretreatment with PD98059

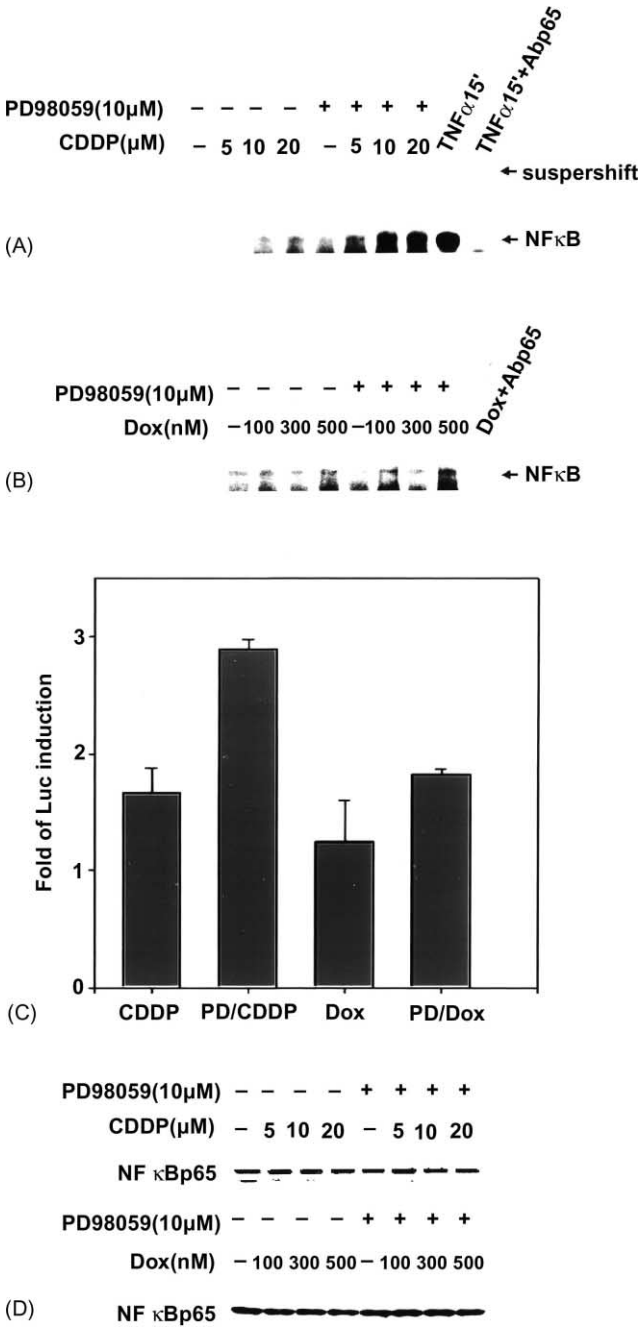

Fig. 2. (A, B) Determination of NFκB activity by EMSA. SiHa cells were pretreated with or without 10 μM PD98059 for 1 hr and then with various concentrations of cisplatin (CDDP; panel A) or doxorubicin (Dox; panel B) for 3 hr, as indicated. Nuclear extracts were prepared and used for the EMSA assay. Nuclear extracts from cells treated for 15 min with 10 ng/mL of TNFα, with or without anti-p65(RelA) antibody, or from cells treated for 3 hr with 500 nM doxorubicin, with anti-p65(RelA) antibody, were used to verify the NFκB–DNA complex (supershift). (C) NFκB directed luciferase activity assay. NFκB directed luciferase reporter plasmid (pRκB-Luc) transfected SiHa cells were treated with (a) 20 μM cisplatin (CDDP), (b) 10 μM PD98059 pretreatment and 20 μM cisplatin (PD/CDDP) for 1 hr, (c) 500 nM doxorubicin (Dox), or (d) 10 μM PD98059 pretreatment and 500 nM doxorubicin (PD/Dox) for 6 hr. Cells were then subjected to the luciferase activity assay. Fold of induction (means ± SD) was calculated from three independent experiments and expressed with respect to the untreated control. (D) Western blotting of NFκB. The nuclear fraction from SiHa cells receiving various treatments was subjected to western blotting to determine the amount of NFκB.

increased cisplatin-induced NFκB activation to around 2.9-fold, whereas no further increase of NFκB activation was observed in PD98059-pretreated, doxorubicin-treated cells.

The level of NFκB protein was determined by western blotting; the results showed no change of NFκB protein after the indicated treatment (Fig. 2D).

3.4. Anticancer drug-induced NFκB activation without marked IκBα degradation

A well-documented pathway for NFκB activation is through the degradation of cytoplasmic IκBα [28]. This pathway was examined in cells treated with genotoxic agents (Fig. 3). Treatment with TNFα showed a dramatic decrease of IκBα as a positive control. However, no obvious degradation of IκBα was observed in doxorubicin- or cisplatin-treated cells, regardless of whether or not they were pretreated with PD98059. The level of NFκB (p65) was also determined; no obvious change was observed.

3.5. Decrease of PD98059-induced cisplatin resistance in dominant negative IκBα transfected cells

To further explore the role of NFκB in PD98059-induced drug resistance, SiHa cells were transfected with a dominant negative IκBα mutant. As shown in Fig. 4A, no induction of NFκB activation was found with either 20 μM cisplatin or combined treatment with PD98059 and 20 μM cisplatin. The effect of PD98059 on the cytotoxicity of cisplatin also was examined. Pretreatment with PD98059 had much less effect on the resistance to cisplatin in dominant negative IκBα-transfected cells (Fig. 4B) than in parental cells (Fig. 1B).

3.6. Lack of effect of p38 on cisplatin-induced NFκB activation

Carter and Hunninghake [22] proposed that the negative regulation of ERK on NFκB activation may be produced, in part, *via* suppression of p38. Therefore, the activation of p38 was also examined in cisplatin-treated SiHa cells. As shown in Fig. 5, p38 was activated by cisplatin with a similar time course of ERK2 activation, peaked at 3 hr, and decreased thereafter.

To explore whether p38 plays a role on NFκB activation, SiHa cells were pretreated with 1 μM 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), a specific inhibitor of p38, and then were treated with various concentrations of cisplatin for 3 hr. NFκB activation was determined by EMSA. As shown in Fig. 6, blockade of p38 activity resulted in little change of cisplatin-induced NFκB activation.

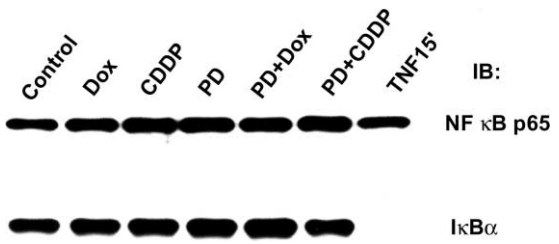

Fig. 3. Western blot analysis of IκBα degradation in light of anticancer drug-induced NFκB activation. SiHa cells were treated with 500 nM doxorubicin (Dox) or 20 μM cisplatin (CDDP) for 3 hr, or with 10 μM PD98059 (PD) for 4 hr, or a 1-hr pretreatment with 10 μM PD98059 followed by a 3-hr treatment with 500 nM doxorubicin (PD + Dox), or 20 μM cisplatin (PD/CDDP), or a 15-min treatment with 10 ng/mL of TNFα. The cell lysates were then prepared and subjected to western blotting with anti-NFκB (p65) or anti-IκBα antibody.

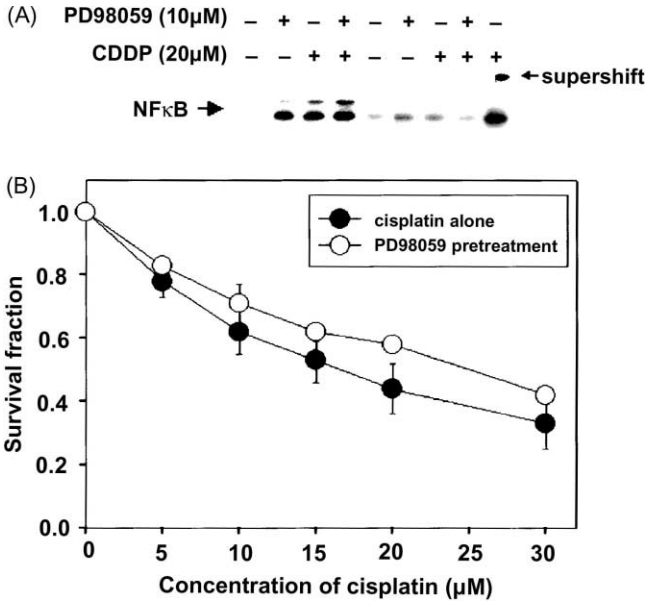

Fig. 4. Effect of PD98059 on the cytotoxicity of cisplatin (CDDP) on dominant negative IκBα-transfected SiHa cells. (A) Transfection of dominant negative IκBα. SiHa or dominant IκBα-transfected SiHa cells (SiHa/dnIκB) were pretreated with or without 10 μM PD98059 for 1 hr and then were co-treated with 20 μM cisplatin for another 3 hr. Nuclear extracts were prepared, and the NFκB activation was assayed by EMSA. The extract from 20 μM cisplatin-treated cells was incubated with anti-NFκB (p65) and run in the right lane as a positive control. (B) Effect of PD98059 on the cytotoxicity of cisplatin on SiHa/dnIκB cells. SiHa/dnIκB cells were pretreated with 10 μM PD98059 for 1 hr and then co-treated with various concentrations of cisplatin for 72 hr, as indicated. Survival of the cells was evaluated by the MTT assay. The results (means ± SD) were calculated from at least three independent experiments.

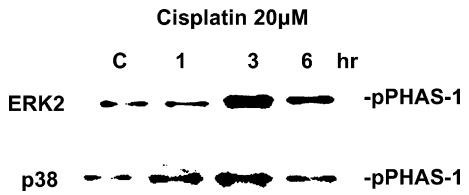

Fig. 5. Immunocomplex kinase assay of ERK2 and p38 following cisplatin treatment. SiHa cells were treated with 20 μM cisplatin for the indicated times. At each time point, nuclear proteins were extracted. The activation of ERK2 or p38 was determined by the immunocomplex kinase assay, using PHAS-I as the substrate.

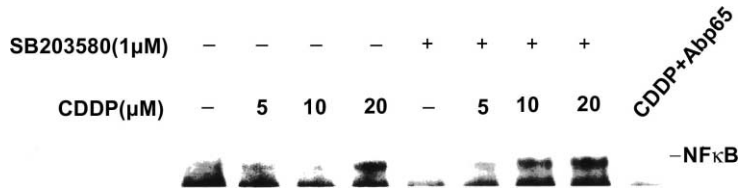

Fig. 6. Effect of p38 on cisplatin-induced NFκB activation, determined by EMSA. SiHa cells were pretreated with 1 μM SB203580 for 1 hr and then were co-treated with various concentrations of cisplatin as indicated for a further 3 hr. Nuclear proteins were extracted, and NFκB activation was determined by EMSA. Nuclear proteins from 20 μM cisplatin-treated cells were incubated with 1 μg anti-NFκB (p65) antibody as a control.

4. Discussion

In this report, we show that suppression of nuclear ERK2 activity increases the resistance of cervical carcinoma cells to cisplatin, but not to another common anticancer drug, doxorubicin. We also demonstrated that this cisplatin resistance may come from further enhancement of drug-induced NFκB activation by suppression of the MEK–ERK pathway. Pretreatment with PD98059 enhanced DNA binding activity as well as transcriptional activity of cisplatin-activated NFκB, as evidenced by EMSA and the NFκB-driven reporter gene assay. This is different from the report of Carter and Hunninghake [22]. They showed that activated ERK2 had no effect on the DNA-binding activity of NFκB but only inhibited the transactivation activity of NFκB. One possible explanation for this discrepancy is the different types of stimuli that had been applied. They used lipopolysaccharide, a cytoplasmic membrane-acting agent, to stimulate the cells, while cisplatin, an agent targeting DNA in the nucleus, was used in this study. Suppression of MEK/ERK may have a substantially different effect on NFκB, which is activated by different routes of the signal transduction pathways.

Although the mechanism is unclear, NFκB could indeed protect cells from some insults [16–20]. Our results suggested that the increase of drug resistance towards cisplatin by suppression of the MEK to ERK signaling pathway was mainly through further activation of NFκB, since the PD98059-induced drug resistance was reduced markedly in dominant negative IκBα-transfected cells. It may be argued that the dominant negative IκBα-transfected cells are less sensitive to cisplatin than the parental cells. However, a similar observation was reported recently, in which dominant negative IκBα-transfected cells showed no change of chemosensitivity to several different anticancer drugs [29]. It may be of interest to ask how much NFκB activation is necessary to protect cells from death. In our results, cisplatin increased NFκB activation by about 1.7-fold compared with the untreated control, and PD98059 pretreatment further increased the activation to 2.9-fold. A 1.7-fold increase of NFκB activity might not be enough to protect cells from the cisplatin insult; a similar effect was also observed in doxorubicin-treated cells. However, a 2.9-fold increase of NFκB activity reduced the cytotoxicity of cisplatin. The biological significance of this increase in NFκB activation is unclear at present. Persons *et al.* [30] showed that PD98059 suppresses cisplatin-induced ERK2 activation and results in decreased p53 protein stability. Our previous report also showed that nuclear ERK2 phosphorylates p53 and up-regulates the transcriptional activity of p53 [26]. Furthermore, a reciprocal inhibition between NFκB and p53 was reported by Webster and Perkins [31]. Thus, a possible explanation for the increase in cisplatin resistance of cells pretreated with PD98059 is that the further enhanced NFκB activation partly overcomes the apoptotic signal induced by the genotoxic agent.

The role of ERK2 in the cellular response to extracellular stress is controversial and may be cell context-dependent. For example, suppression of stress-induced ERK2 activation may increase drug resistance of cancer cells towards some anticancer drugs. Cui *et al.* [32] reported that suppression of ERK activity sensitizes ovarian carcinoma to cisplatin. Similarly, Persons *et al.* [33] and Hayakawa *et al.* [34] reported that PD98059 increases the toxicity of cisplatin in ovarian carcinoma cells. In contrast, the present study showed that PD98059 increases the drug resistance of SiHa cells towards cisplatin. A similar conclusion was achieved by Wang *et al.* [21] in HeLa cells. It is interesting to note that PD98059 sensitizes all tested ovarian cancer cells towards cisplatin while inducing drug resistance in all tested cervical carcinoma cells, suggesting a cell type- and tissue-dependent effect of ERK2. Little is known about the molecular basis of this differential effect of ERK2 in response to genotoxic stress. Our previous study [26] showed that several uncharacterized nuclear proteins were induced by doxorubicin and bound to ERK2 in SiHa cells. We hypothesized that these doxorubicin-induced and ERK2-bound nuclear proteins might play an important role in the guidance of the downstream effect of ERK2. Identification of these proteins and their expression in different cancer cells may help clarify the relationship between ERK2 activation and drug sensitivity in cancer cells.

The mechanism responsible for the discrepant cellular response to cisplatin and doxorubicin remains unclear. However, it might be partially related to the multiple-targeting effect of doxorubicin. Doxorubicin causes DNA strand breaks as well as oxidative stress in the cells [35]. The effect of suppression of the MEK–ERK pathway on these two different types of damage is uncharacterized in SiHa cells. However, in another cervical carcinoma cell

line, HeLa, suppression of ERK2 activity actually sensitizes cells to oxidative stress [15]. It is possible that ERK2 plays an opposite role in the cellular response to DNA damage and oxidative stress; thus, PD98059 appeared to have no effect on the sensitivity of cells to doxorubicin.

A large body of evidence shows that NFκB activation is regulated primarily by NFκB forming a complex with IκB in the cytoplasm. Following stimulation, IκBα is phosphorylated and subsequently subjected to ubiquitination and proteasomal degradation [36–39]. However, our results showed that, under doxorubicin and cisplatin treatment, significant degradation of IκBα and nuclear translocation of NFκB were not necessary for the activation of NFκB. This observation suggests that damaged DNA may activate NFκB *via* some uncharacterized pathway in the nucleus, although the downstream molecules remain largely unknown. A previous study made a similar observation that wortmannin blocks the activation of NFκB by DNA-PK, a nuclear enzyme, but not by TNFα, a membrane receptor-binding ligand [40].

In summary, our results suggest that ERK2 may play an important role in mediating the cellular response to cisplatin through the regulation of NFκB activation, although the mechanism by which suppression of the MEK–ERK signaling pathway further enhances cisplatin-induced NFκB activation is unclear. How the DNA damage signal is relayed to the MEK–ERK pathway and how the cross-talk is conducted between the MEK–ERK pathway and NFκB need to be studied further.

Acknowledgments

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