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Involvement of nuclear transcription factor-κB in low-dose doxorubicin-induced drug resistance of cervical carcinoma cells☆

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Abstract

Administration of suboptimal doses of anticancer drugs not only fails to control tumor but often results in increased drug resistance of tumor cells. However, little is known about the effects of transient exposure to a minimally cytotoxic dose of chemotherapy on the development of drug resistance of the tumors. Previous studies have shown that upregulation of drug-exporter proteins (ATP-binding-cassette proteins) may be one of the key mechanisms involved in inducible drug resistance. In this study, we demonstrated that upregulation of NF-κB is another possible mechanism. SiHa cells were exposed to low-dose doxorubicin (100 nM; IC_{30}) for 3 hr, and then were continuously cultured in drug-free culture media (designated as SiHa/DR cells). SiHa/DR cells at up to 9 passages showed increased resistance to doxorubicin and cross-resistance to cisplatin. Results of quantitative real-time PCR and flow cytometry assay indicated that the increased drug-resistance in SiHa/DR cells was not due to upregulation of drug-exporter proteins or to the decrease of intracellular concentration of anticancer drugs. Both the basal and drug-induced NF-κB activity were shown to be increased in SiHa/DR cells by EMSA and NF-κB-driven luciferase reporter gene assay. Suppression of NF-κB activation by transfection of a dominant negative IκBα prevented the development of drug resistance, indicating that the upregulated NF-κB activity was positively correlated with the low-dose doxorubicin-induced drug resistance. These results suggest that even a transient exposure to a small dose of anticancer drugs may induce drug resistance in some cancer cells via upregulation of NF-κB activity.

Keywords: Doxorubicin; Cisplatin; Drug-resistance; NF-κB; Cervical carcinoma; Low-dose anticancer drugs

1. Introduction

Avoiding the use of an insufficient dose of anticancer drugs is a basic tenet of medical oncology. It is generally agreed that use of a suboptimal dose of anticancer drug would not only fail to provide tumor control, but often result in the development of increased non-specific drug resistance of tumor cells. Although previous reports have suggested that upregulation of the multidrug exporters, the ABC family proteins, may be responsible for some of the inducible drug resistance [1,2], the mechanisms underlying the cross resistance of tumors to other ABC-unrelated anticancer drugs remain largely unknown [3]. Recently, compelling evidence suggests that anticancer drugs activate many signal transduction pathways, some of which may be linked to the development of drug resistance of tumor cells (for a review, see [4]).

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Abbreviations: NF-κB, nuclear transcription factor-κB; ABC family, ATP-binding-cassette; EMSA, electromobility shift assay.

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NF-κB is widely involved in various cellular responses to extracellular stimuli. Recent study has shown that activation of NF-κB is tightly associated with cell transformation and tumorigenesis [5–7]. Overexpression of NF-κB or mutation of its natural inhibitor, IκBα, has been found in several tumors [8–10]. Furthermore, NF-κB activation is closely related to drug-resistance of tumor cells, partly due to its associated increase of the apoptosis threshold [11–13]. Suppression of NF-κB activation may inhibit tumor growth and increase the efficacy of chemotherapy [14–18]. However, whether NF-κB is involved in the development of drug resistance induced by the use of low-dose anticancer drugs has not yet been investigated.

We previously reported that NF-κB is activated by a wide spectrum of anticancer drugs [19]. In addition, we found that NF-κB may regulate the chemosensitivity of cervical carcinoma, SiHa cells [20]. In this study, we addressed the question of whether NF-κB activation is involved in low-dose anticancer drug-induced drug-resistance. Our results indicate that NF-κB activation is the major mechanism responsible for low-dose doxorubicin-induced drug-resistance, and suggest that suppression of NF-κB might serve as a new strategy to improve the efficacy of cancer chemotherapy.

2. Materials and methods

2.1. Cell culture

Human cervical carcinoma SiHa cells were cultured in DMEM supplemented with 10% fetal calf serum, and incubated in a humidified incubator with 5% CO_2 at 37°. SiHa cells transfected with dominant negative IκBα (SiHa/dnIκB) or NF-κB-driven luciferase reporter gene (SiHa/κB-Luc) were established and maintained as previously described [20].

2.2. Cytotoxicity assay

The cytotoxicity of anticancer drugs was determined by a tetrazolium-based semiautomated colorimetric assay (MTT assay) as previously described [20]. Briefly, cells were seeded in a 96-well plate with 4000 cells per well. After overnight culture, cells were treated with various doses of anticancer drugs and cultured for 3 days. The cell numbers were evaluated with an ELISA reader at OD_{540}.

2.3. Quantitative real-time PCR (qr-PCR)

The expression of ABC family proteins was quantitatively determined using a TaqMan quantitative real-time PCR assay (Perkin-Elmer Applied Biosystems), according to the manufacturer's instructions and a previous report [21]. The oligonucleotide primers used for MDR-1 amplification were 5′-GCCTGGCAGCTGGAAGACAAATACACAAAATT (forward primer) and 5′-CAGCAGCAGCTGACAGTCCAAGAACAGGACT (reverse primer), with the dual-labeled fluorescent probe 5′-(FAM) CCCGACTTACAGATGATGTCTCCA (TRAMA). The primers for MRP were 5′-TCTACCTCCTGTGGCTGAATCTG (forward) and 5′-CCGATTGTCTTTGCTCTTCATG (reverse), with the dual-labeled fluorescent probe 5′-(FAM) TGGTCCTCATGGTGCCCGTCAAT (TRAMA). The primers for BCRP were 5′-TTTCCAAGCGTTCATTCAAAAA (forward) and 5′-TACGACTGTGACAATGATCTGAGCTA (reverse), with the dual-labeled fluorescent probe 5′-(FAM) TTGCTGGGTAATCCCCAGGCCTCT (TRAMA). The housekeeping gene, GAPDH, was used as an internal control. The primers and dual-labeled fluorescent probes were purchased from Perkin-Elmer Applied Biosystems.

2.4. Drug retention analysis by flow cytometry

The retention of doxorubicin in SiHa cells was analyzed by flow cytometry. Cells were treated with 1 μM doxorubicin for 3 hr, and then washed twice with cold PBS. The cells were trypsinized, neutralized by complete medium and resuspended with PBS. The relative intracellular concentration of doxorubicin was determined by Becton Dickinson FACScan.

2.5. Western blotting

The method of Western blotting was described previously [22]. The cytosolic or nuclear lysates were prepared according to previously reported methods [19,20]. All antibodies used in this study were purchased from Santa Cruz Biotechnology. The antibodies were used with a dilution factor between 2000 and 4000 (0.5–1 μg/mL). The final images were developed with a chemiluminescence reagent.

2.6. Electromobility shift assay (EMSA)

The EMSA method was used to determine the DNA binding activity of NF-κB. Nuclear extracts were prepared according to previously described method [20]. The oligodeoxynucleotide probe, 5′-GGATTGGGACTTTCCCCTCC, for NF-κB binding was end-labeled with ^{32}P. Following incubation with 10 μg nuclear extract at room temperature for 30 min, the electromobility of the probe was analyzed in 5% native polyacrylamide gel. The NF-κB–DNA complex was identified by adding anti-NF-κB p65 antibody (supershift experiment).

2.7. Luciferase activity assay

For luciferase assay, the 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 luciferase activity according to the instructions of a Luciferase reporter gene assay kit (Packard).

3. Results

3.1. Durable multidrug-resistance induced by low-dose doxorubicin

To characterize the response of tumor cells to low-dose anticancer drugs, a model of transient drug exposure was developed in SiHa cervical carcinoma cells. SiHa cells were treated with 100 nM doxorubicin (IC_{30}) for 3 hr, and then continuously cultured in drug-free complete media. The cells that had been transiently exposed to doxorubicin were designated as SiHa/DR. SiHa/DR cells had growth characteristics that were similar to untreated SiHa cells (data not shown). The chemosensitivity of these cells was determined at passage 3, 6 and 9 (SiHa/DR p3, p6 and p9). The results showed that a single low-dose, short-term doxorubicin treatment increased drug-resistance to doxorubicin. SiHa/DR cells were also shown to increase resistance to another DNA damage agent, cisplatin, although with different structure and mechanism. The induced drug-resistance persisted at least up to 9 passages, but gradually decreased in intensity along with the increased passage numbers (Fig. 1).

3.2. Low-dose doxorubicin-induced drug-resistance without increase of the expression of ABC family genes or decrease of drug retention

To determine whether ABC family was involved in the low-dose doxorubicin-induced multidrug resistance, MDR, MRP, and BCRP expression was detected by qr-PCR. As shown in Fig. 2A, the expression of these genes was not increased in SiHa/DR cells. The involvement of ABC proteins was further excluded by the findings of drug-retention analysis. SiHa and SiHa/DR cells were treated with 1 μM doxorubicin for 3 hr, and the relative concentration of intracellular doxorubicin was determined by flow cytometry. There was no difference in the intracellular concentration of doxorubicin between SiHa and SiHa/DR cells (Fig. 2B), suggesting that the ABC drug-exporters were not responsible for the drug-resistance of SiHa/DR cells.

3.3. Irrelevance of Bcl-2, MLH1, and topoisomerase II to low-dose doxorubicin-induced drug resistance

In addition to the ABC family, several representative drug resistance-related proteins, such as Bcl-2, MLH1, and topoisomerase II [23–25], were screened by Western blot analysis for their involvement in this low-dose doxorubicin-induced drug resistance of SiHa/DR cells. As shown in Fig. 3, the amount of these proteins was similar between SiHa and SiHa/DR cells, suggesting that they played little role in low-dose doxorubicin-induced drug resistance.

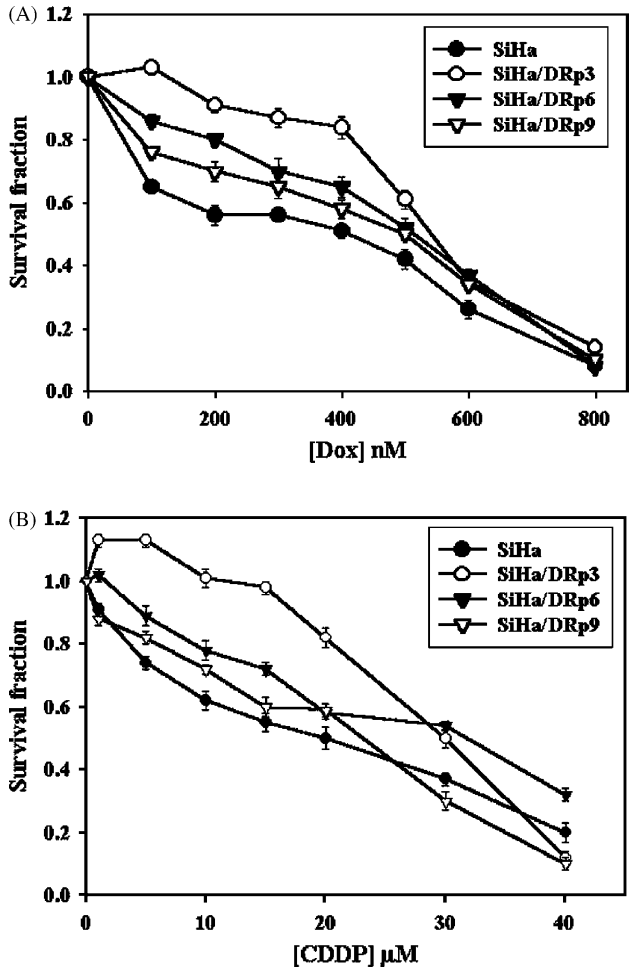

Fig. 1. Cytotoxicity of doxorubicin or cisplatin on SiHa and SiHa/DR cells. SiHa and SiHa/DR cells (at passages 3, 6, and 9) were treated with various concentrations of doxorubicin or cisplatin for 72 hr. The number of surviving cells was evaluated by MTT assay. Each point represents the average of at least four independent experiments. Standard deviation bars are indicated.

3.4. Increase of basal and anticancer drug-induced NF-κB activation in SiHa/DR cells

To investigate whether NF-κB activation plays a role in the drug-resistance of SiHa/DR cells, the basal level of NF-κB activity was first examined by EMSA. As shown in Fig. 4A, the basal level of NF-κB activity in early passage of SiHa/DR cells was markedly increased, and this NF-κB activity gradually decreased to near the control level with increased numbers of passage.

To directly assay the transcriptional activity of NF-κB induced by low-dose doxorubicin, SiHa cells were transfected with an NF-κB-driven luciferase reporter gene and the activity of NF-κB was determined by luciferase activity assay. The reporter gene-transfected SiHa cells (SiHa/κB-Luc) were exposed to low-dose (100 nM) doxorubicin for 3 hr and then cultured in drug-free medium. These cells were designated as SiHa/κB-Luc/DR cells. SiHa/κB-Luc and different passages of SiHa/κB-Luc/DR cells were treated with various concentrations of doxorubicin for

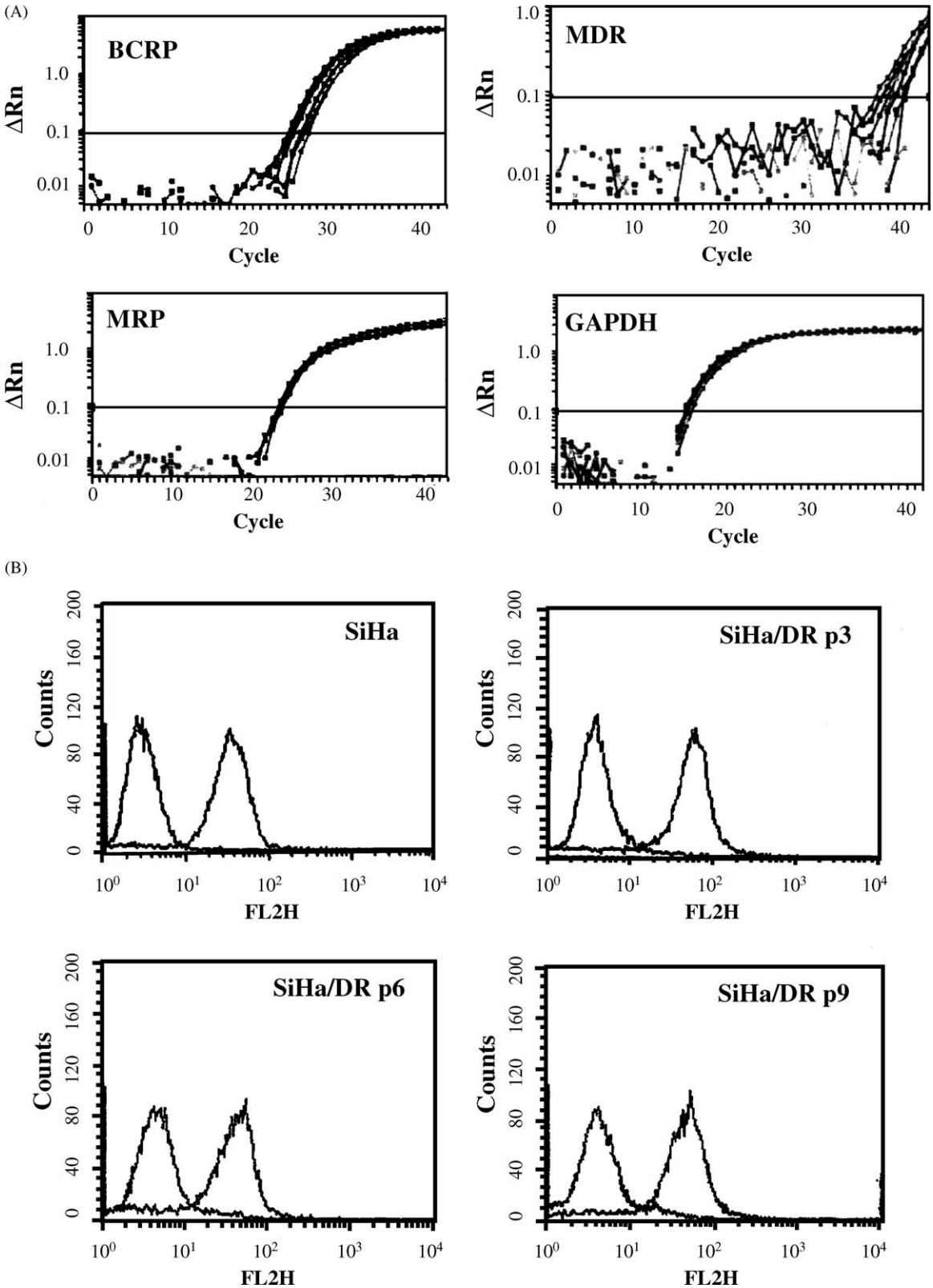

Fig. 2. (A) Quantitative real-time PCR assay of ABC family genes. The expression of ABC family genes, including MDR-1, MRP, and BCRP, in SiHa and SiHa/DR cells (at passages 3, 6, and 9) was analyzed by quantitative real-time PCR. The expression of GAPDH run in parallel was used as an internal control. No specific label is given for each curve, since they merged together. (B) Drug-retention test of SiHa and SiHa/DR cells. SiHa and SiHa/DR cells (at passages 3, 6, and 9) were treated with 1 μM doxorubicin for 3 hr, and the relative intracellular concentration of doxorubicin was analyzed by FASC. Left curve: untreated control; right curve: doxorubicin treated.

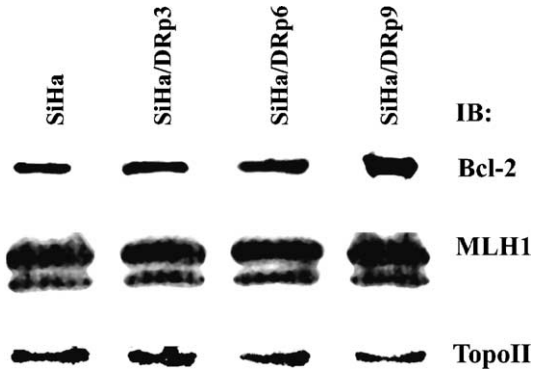

Fig. 3. Western blotting of Bcl-2, MLH1, and topoisomerase II in SiHa and SiHa/DR cells. Whole cell lysates were prepared from SiHa and SiHa/DR cells. Aliquots of protein (30 μg) were subjected to Western blotting with anti-Bcl2, anti-MLH1 or anti-topoisomerase II antibody.

6 hr, and then the activity of luciferase was evaluated. As shown in Fig. 4B, the basal level of NF-κB activity in early passage of SiHa/κB-Luc/DR cells was markedly increased but gradually decreased to near the control level with increased numbers of passage, consistent with the results of EMSA (comparing D0 bar of each passage). The inducibility of NF-κB activation by doxorubicin only increased slightly in SiHa/κB-Luc/DR cells.

The effect of cisplatin on NF-κB activation in SiHa and SiHa/DR cells was also examined by reporter gene assay. In contrast to doxorubicin, the cisplatin-inducible NF-κB activity in SiHa/DR cells was much higher than in control SiHa cells (Fig. 4C). NF-κB activation was highest in SiHa/DR p3 cells, and then decreased with the later passages.

3.5. Doxorubicin or cisplatin activated NF-κB without marked IκBα degradation and NF-κB nuclear translocation

A well-characterized pathway of NF-κB activation is through degradation of its cytoplasmic inhibitor, IκBα [26,27]. To determine whether low-dose doxorubicin upregulates NF-κB activity by facilitating IκBα degradation, the protein levels of cytosolic IκBα and nuclear NF-κB were determined by Western blotting. As shown in Figs. 5 and 6, neither doxorubicin- nor cisplatin-induced marked IκBα degradation or accumulation of nuclear NF-κB.

3.6. Doxorubicin-induced drug resistance prevented by dominant negative IκBα

The involvement of NF-κB in drug-induced resistance was further characterized by transfection of a dominant negative IκBα into SiHa cells (SiHa/dnIκB). As shown in Fig. 7A and B, expression of dominant negative IκBα reduced the protein level of nuclear NF-κB and blocked the cisplatin-induced NF-κB activation, although the expression of the transfected dominant negative IκBα

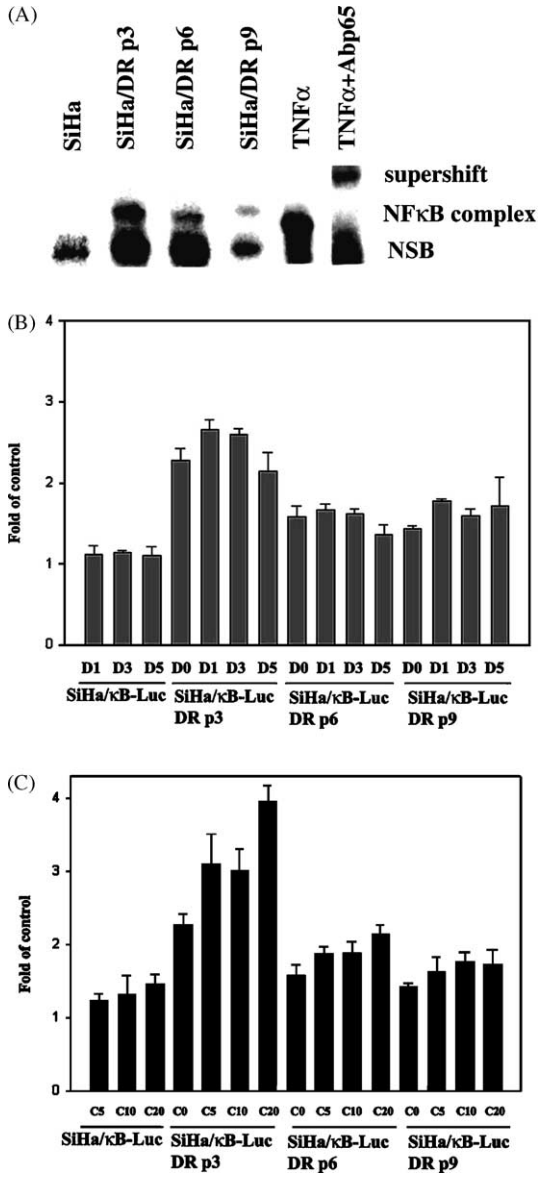

Fig. 4. (A) Increase of the basal level of NF-κB activity by low dose doxorubicin. Nuclear extracts were prepared from SiHa or SiHa/DR cells at different passages and used for EMSA assay. Nuclear extracts prepared from SiHa cells treated with 10 ng/mL TNFα for 15 min and incubated with or without 1 μg anti-p65 antibody were used as a positive control. NSB: nonspecific binding. (B) Determination of doxorubicin-induced NF-κB activation in SiHa and SiHa/DR cells by NF-κB directed luciferase activity assay. SiHa and SiHa/κB-Luc/DR cells at different passages were treated with various concentrations of doxorubicin (D1: 100 nM; D3: 300 nM; D5: 500 nM) for 6 hr and then subjected to luciferase activity assay. (C) Determination of cisplatin-induced NF-κB activation in SiHa and SiHa/DR cells by NF-κB directed luciferase activity assay. SiHa and SiHa/κB-Luc/DR cells at different passages were treated with various concentrations of cisplatin (C5: 5 μM; C10: 10 μM; D20: 20 μM) for 6 hr and then subjected to luciferase activity assay. The relative activity was normalized to the protein concentration. Folds of induction were calculated from three independent experiments and are expressed with respect to untreated control. The standard deviation bars are indicated.

was relatively low compared with the endogenous IκBα. SiHa/dnIκB-DR cells were established by transient exposure of SiHa/dnIκB cells to low-dose doxorubicin as with SiHa/DR cells. The cytotoxicity of doxorubicin or cisplatin

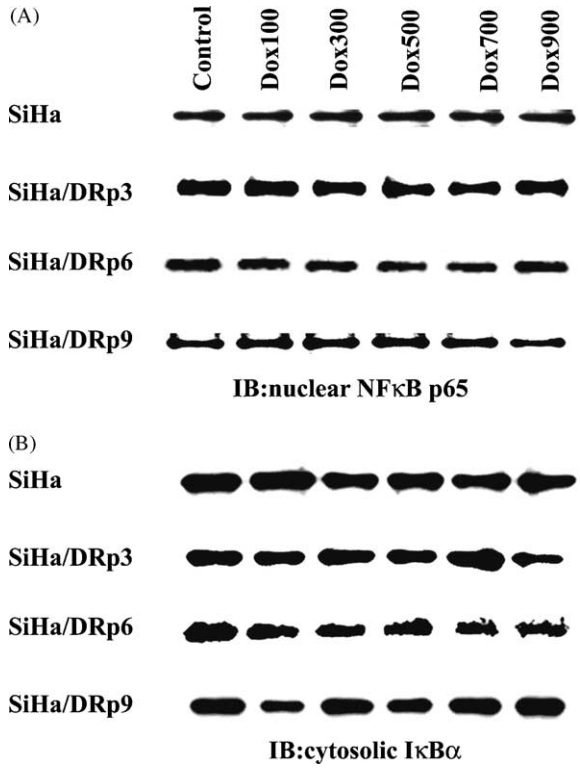

Fig. 5. Western blotting of nuclear NF-κB p65 and cytosolic IκBα in doxorubicin-treated SiHa or SiHa/DR cells. SiHa and SiHa/DR cells were treated with various concentrations of doxorubicin (Dox, 100–900 nM) for 3 hr. The nuclear and cytosolic lysates were prepared and subjected to Western blotting with (A) anti-p65 or (B) anti-IκBα antibody.

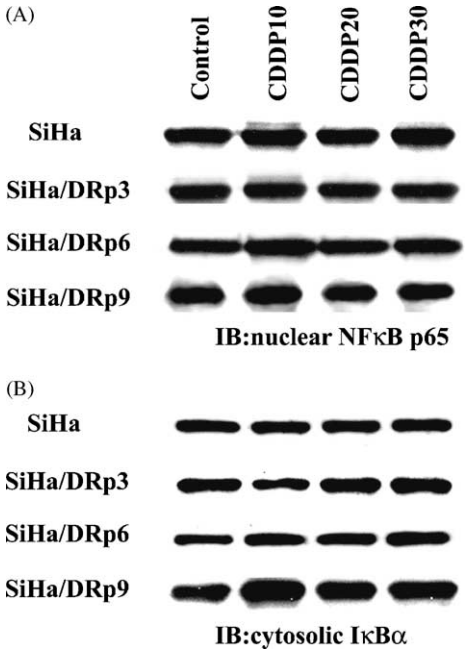

Fig. 6. Western blotting of nuclear NF-κB p65 and cytosolic IκBα in cisplatin-treated SiHa or SiHa/DR cells. SiHa and SiHa/DR cells were treated with various concentrations of cisplatin (CDDP, 10–30 μM) for 3 hr. The nuclear and cytosolic lysates were prepared and subjected to Western blotting with (A) anti-p65 or (B) anti-IκBα antibody.

was assayed at the third passage of SiHa/dnIκB-DR cells. As shown in Fig. 7C, drug resistance to doxorubicin or cisplatin was not induced in SiHa/dnIκB cells.

4. Discussion

The development of drug-resistance in tumor cells is the major problem in chemotherapy. In most experimental systems, drug-resistant tumor cells are established by continuous exposure to escalating doses of anticancer drugs. In these cells, a major mechanism involved in the development of drug-resistance is the expression of ABC family proteins [28]. However, tumor cells selected by a specific anticancer drug usually develop multi-drug resistance to ABC-unrelated anticancer drugs. The occurrence of drug-resistant tumor cells cannot be explained simply by the expression of ABC family proteins. Continuous drug exposure may induce a response similar to a summation of repeated short-term exposure, and the expression of ABC family proteins may not be the initial step in the development of drug-resistance by cancer cells. This study provides evidence that a low-dose, short-term exposure to doxorubicin may induce a durable, and to some degree, a reversible drug-resistance. Further, since high-dose anticancer drugs kill most of the cells, the remaining viable cells represent a small population of cells with possible intrinsic drug-resistance. A genuine comparison of possible mechanism of high-dose- or low-dose-induced drug-resistance is therefore difficult. In this study, we did not find changes of the ABC family proteins and several representative drug resistance-related proteins, including BCl-2, MLH1, and topoisomerase II. Specifically, we demonstrated that a dynamic change of NF-κB activity was in parallel with the transient drug-resistance induced by low-dose doxorubicin. We speculate that alteration in the expression of some target proteins are one of the major mechanisms that result in high-dose anticancer drugs-induced drug resistance. In contrast, alteration of the activity but not the expression of target proteins may play an important role in low-dose anticancer drugs-induced drug resistance. However, the mechanism of NF-κB activation by low-dose doxorubicin and the possible different mechanism of drug resistance induced by low- or high-dose chemotherapy remain to be elucidated. Also, whether other anticancer drugs, at the low-dose ranges, may have similar effect on the induction of drug-resistance via upregulation of NF-κB needs further characterization.

Although NF-κB acts as either an anti-apoptotic or a pro-apoptotic molecule, depending upon cell type and the nature of stress [29–31], the level of NF-κB activity is associated with the drug-resistance in most tumor cells, and inhibition of NF-κB may sensitize tumor cells to chemotherapy [5,16,32,33]. In the present study, a dynamic change of basal and inducible NF-κB activity was tightly associated with the chemoresistance of tumor cells. Interestingly, the increased basal or inducible NF-κB activity played a different role in the development of drug resistance

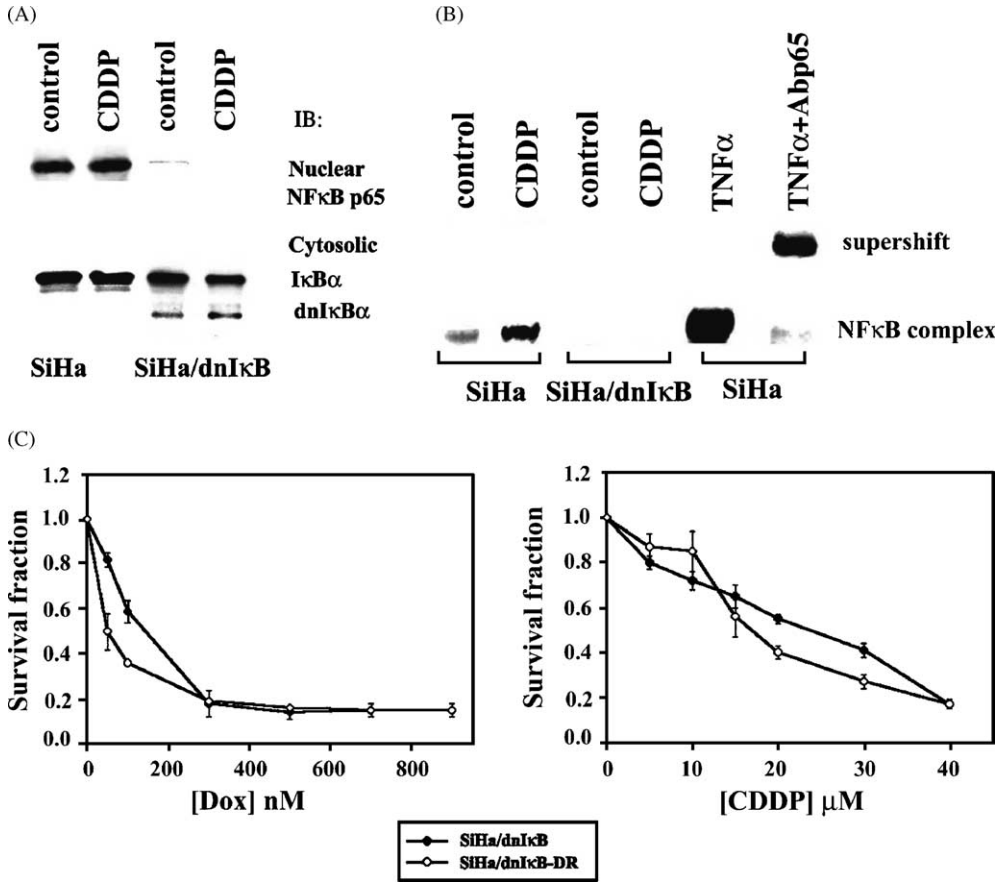

Fig. 7. (A) Western blotting of nuclear NF-κB p65 and cytosolic IκBα in SiHa and dominant negative IκBα transfected cells. SiHa and dominant negative IκBα transfected SiHa cells (SiHa/dnIκB) were treated with 20 μM cisplatin (CDDP) for 3 hr. The nuclear and cytosolic lysates were prepared and subjected to Western blotting with anti-p65 or anti-IκBα antibody. (B) Inhibition of cisplatin-induced NF-κB activation in SiHa/dnIκB cells. SiHa and SiHa/dnIκB cells were treated with 20 μM cisplatin for 3 hr. Nuclear extracts were prepared and used for EMSA assay. Nuclear extracts prepared from SiHa cells treated with 10 ng/mL TNFα for 3 hr and incubated with or without 1 μg anti-p65 antibody were used as a positive control. (C) Cytotoxicity of doxorubicin or cisplatin on SiHa/dnIκB and SiHa/dnIκB-DR cells. The cytotoxicity of doxorubicin or cisplatin on SiHa/dnIκB and SiHa/dnIκB-DR (at passage 3) cells was assayed by MTT. Each point represents the average of at least four independent experiments. The standard deviation bars are indicated.

to doxorubicin or cisplatin. Doxorubicin did not induce an obvious NF-κB activation in SiHa or SiHa/DR cells (Fig. 4), suggesting that drug-induced NF-κB activation is not a major factor responsible for the development of resistance to doxorubicin. Instead, these results suggest that the basal level of NF-κB activity is important for the development of increased resistance, as inhibition of NF-κB by a dominant negative IκBα sensitized SiHa cells to doxorubicin (the IC_{50} for doxorubicin shifted from 500 nM of parental SiHa to 150 nM of SiHa/dnIκB, Figs. 1 and 7C). A similar observation was reported recently by Arlt *et al.*, who concluded that basal but not inducible NF-κB activity is the major factor responsible for the resistance to VP16 and doxorubicin in pancreatic carcinoma cells [33]. In contrast, as shown by the comparison of the survival curves of SiHa and SiHa/dnIκB (Figs. 1 and 7C), suppression of basal NF-κB activity did not increase the chemosensitivity to cisplatin. Instead, the higher inducibility of NF-κB activity in early passage SiHa/DR cells was the major mechanism responsible for their resistance to cisplatin (Fig. 4C), since suppression of NF-κB activation by dominant negative IκBα simultaneously diminished the low-dose doxorubicin-induced resistance to cisplatin. Although the molecular mechanism responsible for this differential effect of NF-κB on drug resistance is unclear, it might be related to the activation of different signal transduction pathways by anticancer drugs with different targets.

Our data suggest that marked degradation of IκBα and subsequent nuclear translocation of NF-κB are not necessary for anticancer drug-induced NF-κB activation. The conventional IKK/IκBα/NF-κB activation pathway appeared to be intact in SiHa cells, since TNFα effectively induced IκBα degradation and NF-κB nuclear translocation (data not shown). Recently, several pieces of evidence suggest that other mechanisms besides IκBα trapping also modulate NF-κB activity. For example, the phosphorylation of subunit p65 is required for the transcriptional activity of NF-κB [34–36]. On the contrary, protein phosphatase X has also been reported to directly interact with p65 subunit and activate NF-κB [37]. How the signal generated by anticancer drug is relayed to induce NF-κB activation remains to be further elucidated.

In this study, expression of dominant negative IκBα diminished cisplatin-induced NF-κB activation. We hypothesized that cisplatin may directly activate pre-existing nuclear NF-κB. This hypothesis is supported by results obtained after transfection of dominant negative IκBα into SiHa cells. The expression of dominant negative IκBα reduced the steady-state protein level of nuclear NF-κB, and thereby inhibited cisplatin-induced NF-κB activation (Fig. 7A). Nevertheless, we did not observe an increase of the amount of nuclear NF-κB in SiHa/DR cells, suggesting that there are uncharacterized mechanisms in the nucleus which further modulate NF-κB activation. Several nuclear proteins have been found to modulate NF-κB activity. For example, protein phosphatase X [37], DNA-PK [38] and ATM [39], have been reported to increase NF-κB activity, and ERK2 was reported to negatively regulate NF-κB activity [20,40]. Whether transient treatment with low-dose doxorubicin also changes some of these proteins remains to be determined. In addition, whether the protein level of nuclear NF-κB may indicate the response of tumor cells to anticancer drugs is interesting but needs further characterization.

Although doxorubicin does not effectively activate NF-κB, the basal level of NF-κB activity was increased by a 3 hr, low-dose doxorubicin treatment (Fig. 4A and B). The underlying mechanism responsible for this discrepancy is unclear, however, one possible explanation is that, after removing doxorubicin, NF-κB is activated during cell ongoing DNA repair. This speculation is indirectly supported by a recent study, which showed that NF-κB is correlated with Ku autoantigen, a DNA double-strand break binding protein and an important DNA double-strand break repair protein [32].

In conclusion, this study demonstrated that a low-dose, transient doxorubicin treatment increased basal and drug-inducible NF-κB activity, in conjunction with an increase of drug-resistance of tumor cells. Our data suggest that this increased basal and drug-inducible NF-κB activity acts as the major mechanism for the development of this drug-resistance.

Acknowledgments

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