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ORIGINAL ARTICLE

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N-acetyl-*L*-cysteine enhances chemotherapeutic effect on prostate cancer cells

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Abstract Transcription factor nuclear factor κB (NF-κB) controls gene expression of a number of genes, including cytokines such as interleukin-6 (IL-6), granulocyte-macrophage (GM)-CSF, and interleukin-8 (IL-8). IL-6 is known to play important roles in the growth of prostate cancer cells, activation of androgen receptor, and prostate-specific protein expression. NF-κB is activated by extracellular signals such as proinflammatory cytokines, chemotherapeutic reagents, and radiation. Here we demonstrate that cisplatin (CDDP) and etoposide (VP-16) induce nuclear translocation of NF-κB in prostate cancer cell lines, followed by secretion of IL-6. We also demonstrated that the growth of hormone-independent prostate cancer cell lines can be inhibited by the anti-NF-κB reagent N-acetyl-*L*-cysteine (NAC). These observations indicate that NF-κB can be a target of new adjuvant therapy against hormone refractory prostate cancer.

Keywords Prostate cancer · Nuclear factor-κB · Interleukin-6

Introduction

Resistance of tumors to chemotherapies is a common clinical problem in human cancer, especially hormone-resistant prostate cancer. Resistance to chemotherapies may already exist before the initiation of therapy because of overexpression of the multidrug resistance gene product MDR1, which functions to export a variety of chemotherapies from tumor cells. Moreover, chemoresistance (acquired or inducible) may develop in response to chemotherapy by mostly unknown mechanisms.

Interleukin (IL)-6 plays a significant role in genitourinary carcinomas. It is reported that IL-6 promotes the growth of prostate carcinoma. Furthermore, it regulates prostate-specific antigen protein secretion in prostate carcinoma cells by inducing the androgen receptor. NF-κB is an inducible cellular transcription factor that regulates a wide variety of cellular genes, including IL-6 [1]. NF-κB consists of two major subunits, p50 and p65. In resting cells, NF-κB exists as an inactive form in the cytoplasm associated with the inhibitory molecule IκB [2, 3, 4, 5]. NF-κB can be activated by extracellular signals such as proinflammatory cytokines, TNF, and IL-1. Upon stimulation by these signals, IκB is separated from NF-κB, which then moves into the nucleus, where it activates target genes. It was recently reported that both the phosphorylation and redox pathways are involved in the activation of NF-κB [4, 6].

Chemotherapy and radiation are also known to activate NF-κB, and this response not only potently suppresses the apoptotic potential of these stimuli but induces the IL-6 secretion. In this study, we analyzed the effect of NF-κB inhibition using an anti-NF-κB reagent, N-acetyl-*L*-cysteine (NAC), on the viability of prostate cancer cells upon chemotherapy. The present findings establish that the inhibition of NF-κB activation by NAC substantially enhances the effects of chemotherapy by cisplatin (CDDP) or etoposide (VP-16). Thus, NF-κB activation serves as a principal mechanism for inducible resistance to chemotherapy, and inhibition of NF-κB could be a new adjuvant approach for treatment of intractable cancer.

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Materials and methods

Cells and culture conditions

Cultured human prostate cancer cell lines, PC-3 and DU145, were obtained from the American Type Culture Collection (ATCC, USA). The androgen-responsive prostate cancer cell line LNCaP was purchased from Dainihon Laboratory Products (Osaka, Japan). PC-3 and DU145 were maintained in Eagle's MEM supplemented with 10% fetal bovine serum. The LNCaP was maintained in RPMI1640 medium supplemented with 10% fetal bovine serum.

Immunostaining

Semiconfluent PC-3, DU145, or LNCaP cells on Lab-Tek tissue culture chamber slides (Nunc, Naperville, Ill., USA) were fixed with acetone for 10 min at –20°C and washed three times with PBS without dications (PBS[-]). They were subsequently incubated with primary antibodies, mAbs, against the p65 or p50 subunit of NF-κB for 1 h at 37°C. After washing three times with 0.05% TritonX-PBS(-), they were incubated with the secondary antibody for 20 min at 37°C. The slides were further washed with PBS(-) three times and then mounted with buffered glycerol for fluorescent microscopic examination. Primary and secondary antibodies were diluted 1:100 in PBS(-) containing 3% BSA. The p65 and p50 subunits were stained using the primary rabbit antibodies and rhodamin-conjugated guinea pig antirabbit IgG as the secondary antibody.

Immunoblotting

Semiconfluent prostate cancer cells were collected and lysed in modified RIPA buffer (20 mM Tris·HCl [pH 7.4], SDS 0.1%, Triton X 100 1%, sodium deoxycholate 1%). The protein concentration was determined by BCA assay. After separation by sodium dodecyl sulfate horizontal polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to Immobilon-P transfer membranes (Millipore, Bedford, Mass., USA). The membranes were blocked, probed with monoclonal antibody (1:1000) against thioredoxin, and developed using a Western blot kit (Kirkegaard and Perry, Gaithersburg, Md., USA). Antihuman thioredoxin antibody was obtained by A. Mitsui of the Ajinomoto Basic Research Institute.

IL-6 ELISA

The IL-6 concentration in the supernatant of cultured tumor cells was quantitated by enzyme-linked immunosorbent assay (ELISA). A total of 10^6 cells were incubated in medium for 12 h and pelleted, and the supernatants were collected for determination of IL-6 concentration. The IL-6 ELISA is based on a double-antibody sandwich technique and has been shown to be highly sensitive and specific. The mAb mouse anti-IL-6 (Genzyme, Cambridge, Mass., USA) was used as the first coating antibody. For the second antibody, the polyclonal rabbit anti-IL-6 antibody was used. The wells of 96-well ELISA plates (Coster, Cambridge, Mass., USA) were coated with 50 µl of mAb for at least 1 day and then stored for up to 4 weeks at 4°C. To set up the assay, antibody-coated plates were washed three times and blocked with PBS containing 1% bovine serum albumin for 1 h. The plates were washed twice, and 50 µl of the cell supernatant or the recombinant human IL-6 standard was added to the wells. After overnight incubation, the plates were washed three times, and 50 µl of polyclonal anti-IL-6 antibody was added to each well. After 1 h of incubation, alkaline phosphatase-conjugated goat antirabbit IgG (Caltag, South San Francisco, Calif., USA) was added to each well and incubated for an additional 1 h. Finally, the plates were washed and incubated with the substrate (Sigma 104). The plates were read after 2 h at 405 nm using a Titertek Multiscan MCC/240 ELISA reader.

Cytotoxicity assay

The antiproliferative effects of CDDP, VP-16, and NAC were assessed by mean transit time (MTT) assay. Briefly, 100 µl of target cell suspension (1×10^5 cells) were added to each well of a 96-well, flat-bottomed microtiter plate (Costar), and each plate was incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation, 100 µl of the first agent or complete medium for the control were added, and each plate was incubated for 6 h. After this period, the culture medium was removed, the cells were washed twice with PBS, and they were incubated at 37°C with 200 µl of the second agent or complete medium for additional 21 h. After incubation, 20 µl of MTT working solution (5 mg/ml, Sigma) were added to each culture well and the plate was incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was removed from the well and replaced with 100 µl isopropyl alcohol (Sigma) supplemented with 0.05 M HCl. The absorbance of each well was measured with a microculture plate reader at 540 nm. Percentage cytotoxicity was calculated as:

$$\%\ \text{of cytotoxicity} = 1 - \frac{(\text{Absorbance of experimental wells})}{(\text{Absorbance of control wells})} \times 100$$

Statistical analysis

Statistical significance of the antiproliferative effect of the anticancer drugs with or without NAC was determined using Student's t-test. $P < 0.05$ was considered significant. Calculations of synergistic cytotoxicity were determined as described by Berenbaum [7].

Results

Induction of nuclear translocation of NF-κB in prostate cancer cells by chemotherapy

Immunostaining was performed to detect NF-κB in prostate cancer cells using the monoclonal antibodies against NF-κB subunits. In the nonstimulated three prostate cancer cell lines, NF-κB was found localized in the cytoplasm as stained by the anti-p65 antibody. However, when PC-3 and DU145 were treated with CDDP or VP-16 (1 µg/ml), NF-κB was found to be translocated to the nucleus (Fig. 1 A). In LNCaP, the translocation to the nucleus of NF-κB was not remarkable (Fig. 1B). Similarly, nuclear translocation of p50 following CDDP or VP-16 was also demonstrated with anti-p50 antibody (data not shown). When these cells were pretreated with NAC before the treatment with CDDP or VP-16, the nuclear translocation of NF-κB was inhibited in a dose-dependent manner for NAC (Fig. 1A).

Induction of IL-6 secretion after NF-κB activation

We then examined whether PC-3, DU145, and LNCaP cell lines secrete IL-6 upon the NF-κB activation induced by CDDP or VP-16. The cell culture supernatants were determined for the IL-6 concentration. As shown Fig. 2, PC-3 and DU145 secreted IL-6 constitutively; however, the level of IL-6 secretion from LNCaP was lower than for PC-3 and DU145.

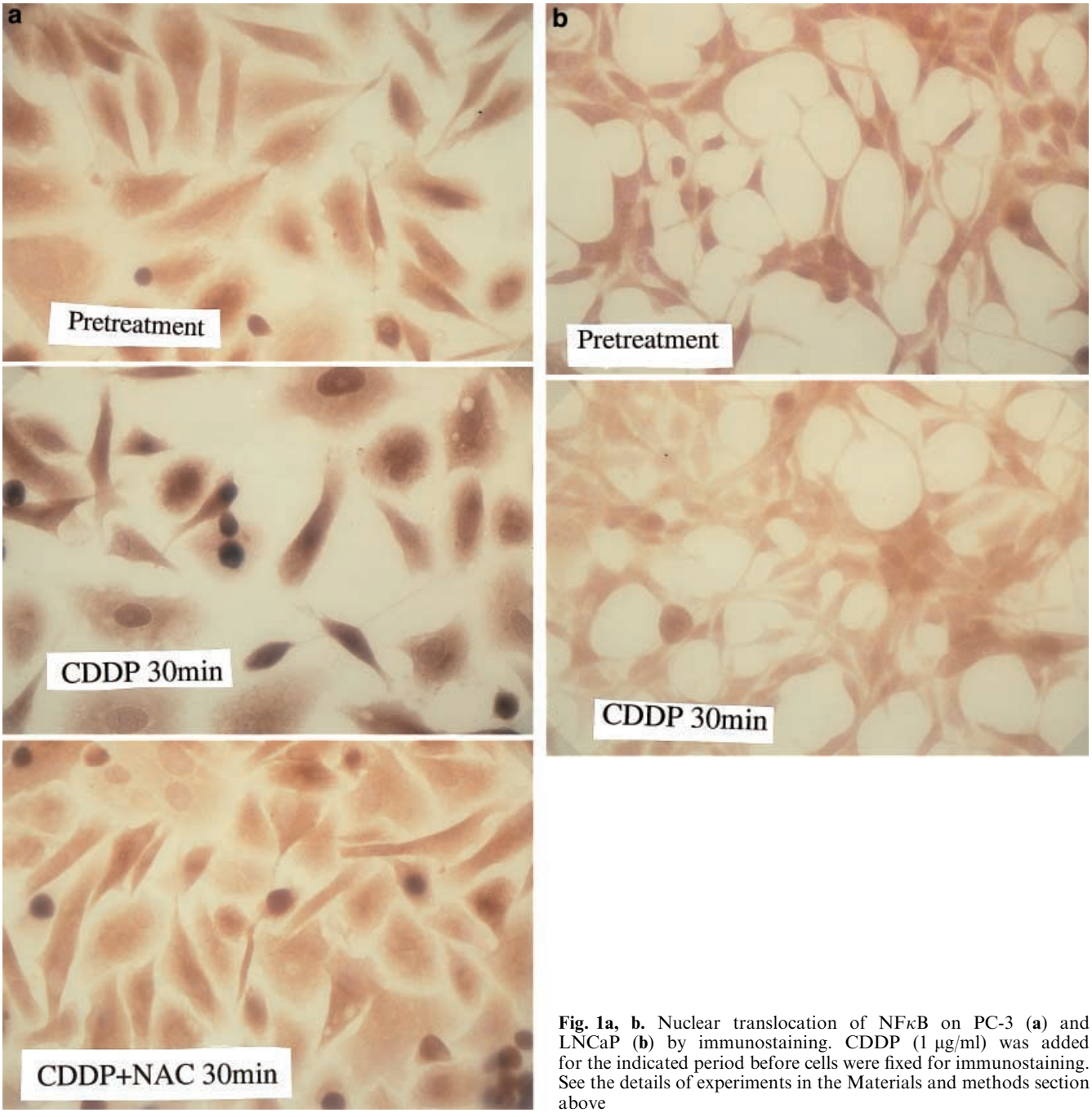

Fig. 1a, b. Nuclear translocation of NFκB on PC-3 (**a**) and LNCaP (**b**) by immunostaining. CDDP (1 μg/ml) was added for the indicated period before cells were fixed for immunostaining. See the details of experiments in the Materials and methods section above

The level of IL-6 secretion increased markedly for CDDP or VP-16 in PC-3 and DU145 cells. No IL-6 induction was observed in LNCaP cells.

Inhibition of IL-6 secretion by NAC

We then investigated the effect of NAC on the IL-6 level in the cell culture supernatant in the presence of CDDP or VP-16. Various concentrations of NAC were added to the prostate cancer cells, and after 24 h the cell culture supernatant was determined for IL-6 concentration by ELISA. As shown in Fig. 2, secretion of IL-6 was greatly inhibited by NAC in PC-3 and DU145 cells. NAC inhibited the IL-6 secretion at a concentration of less than 1 mM.

Sensitization of PC-3 and DU145 cell lines to CDDP and VP-16 after treatment with NAC

It was reported that both PC-3 and DU145 were found to be relatively resistant to chemotherapeutic drugs when compared with other carcinoma cell lines [8].

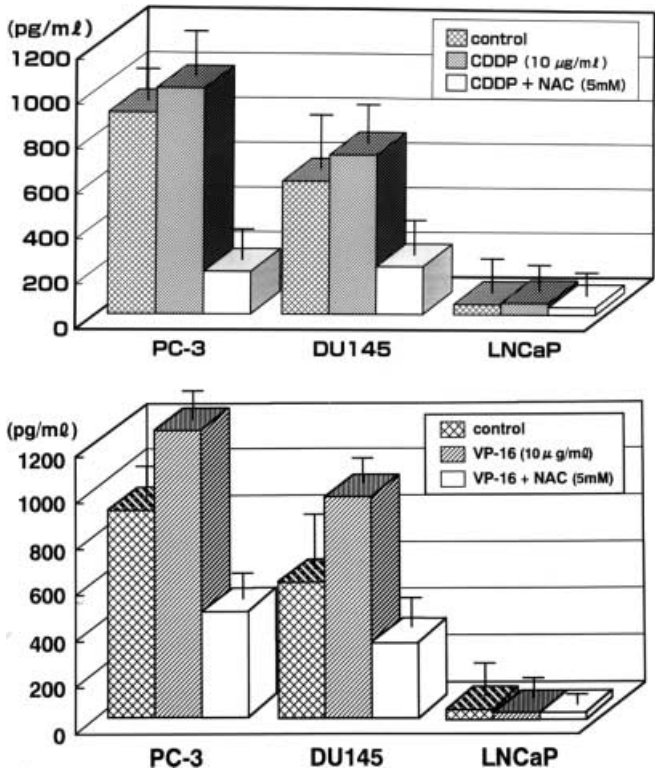

Fig. 2. Induction of IL-6 secretion after CDDP and VP-16 treatment and inhibition of IL-6 secretion by NAC. The cells were incubated for 4 h with CDDP (10 μg/ml) and VP-16 (10 μg/ml), washed, counted, and then incubated at 1×10^5 cells/ml for an additional 24 h with or without NAC (5 mM). The presence of IL-6 protein in the supernatant was quantitated by ELISA

Other studies have suggested that IL-6 can act as a resistance-promoting factor [9, 10]. Furthermore, in these two cell lines, IL-6 secretion is induced by NF-κB activation but not in LNCaP. Thus, we examined the effect of NAC on the cell viability of PC-3, DU145, and LNCaP cells upon treatment with CDDP or VP-16. Various concentrations (0–5 mM) of NAC were used in combination with anti-cancer drugs (10 μg/ml) and the cytotoxicity was measured after 48 h of culture. Clearly, there was a significant augmentation of cytotoxicity by CDDP or VP-16 in the presence of NAC (1–5 mM) on PC-3 and DU145 (Fig. 3). However, no detectable effect of NAC was observed in LNCaP. The 50% cytotoxicity concentration as determined by MTT assay for NAC was 10.1 mM. Though NAC suppresses the expression of IL-6, it is not directly cytotoxic to prostate cancer cells.

Inhibition of thioredoxin expression by NAC

Western blot analysis was carried out with the protein extracts obtained from PC-3 cells. NAC (0.2–5 mM) inhibited thioredoxin expression induced by VP-16 (1 μg/ml) in a dose-dependent manner in PC-3 (Fig. 4).

Discussion

Several cytokines, cell adhesion molecules, growth factors, and their receptors have been characterized in prostate-derived cell lines and human cancer specimens [11, 12]. These included transforming growth factor β, epidermal growth factor, and insulin-like growth factor.

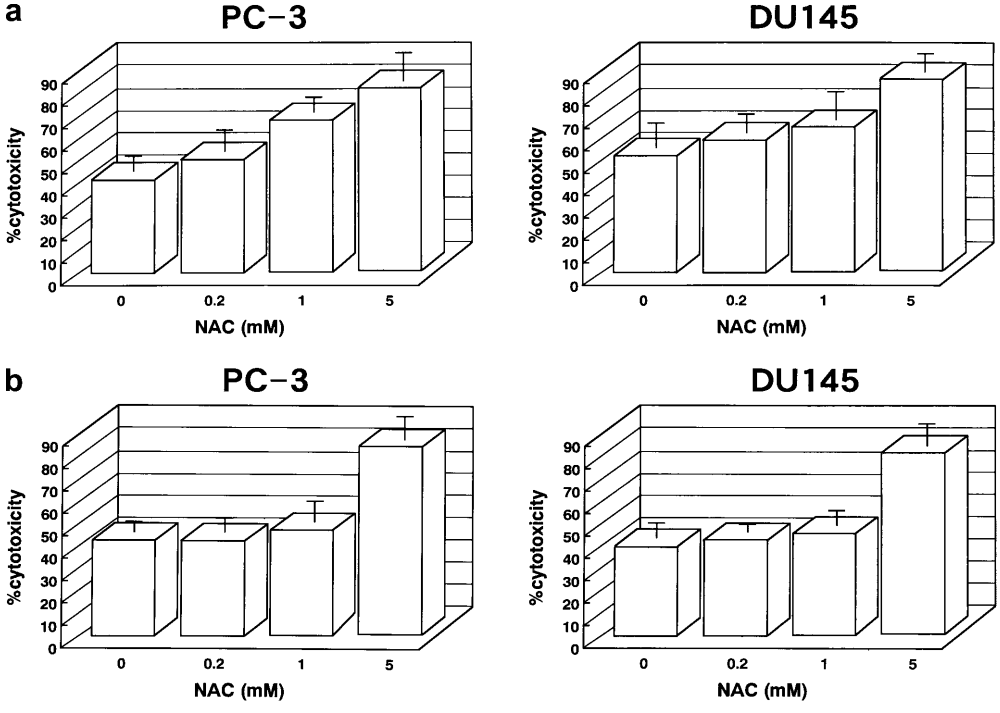

Fig. 3a, b. NAC enhances chemotherapeutic percentual cytotoxicity to both the PC-3 and DU145 cell lines as measured by MTT assay. **a** NAC augmented the cytotoxicity of CDDP at 1 and 5 mM after CDDP treatment, ($P<0.05$). **b** NAC augmented the cytotoxicity of VP-16 at 5 mM after VP-16 treatment ($P<0.05$). *Bars* ±SD

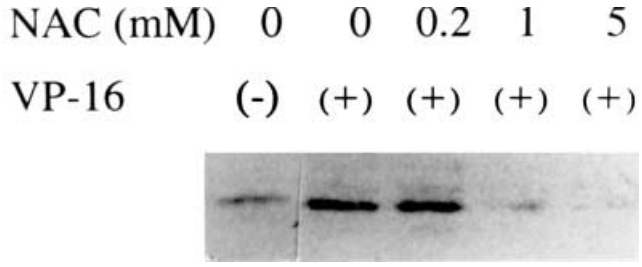

Fig. 4. NAC (0.2–5 mM) inhibited thioredoxin expression induced by VP-16 (1 μg/ml) in a dose-dependent manner in PC-3. Western blot analysis was carried out with the protein extracts obtained from PC-3 cells at 30 min after addition of VP-16

These growth factors function via an autocrine mechanism in prostate cancer, and their secretion in normal and neoplastic hormone-responsive tumors is regulated by androgens [13, 14]. Borsallino et al. reported that IL-6 secretion by DU145 and PC-3 hormone-resistant prostate cancer cell lines can contribute to the autonomous growth of these cell lines [15]. They suggested that IL-6 synthesized by prostate carcinoma cell lines might be responsible for the protection of tumor cells from the cytotoxicity of chemotherapeutic drugs. In fact, in patients with metastatic prostatic carcinoma, the level of circulating IL-6 was elevated and correlated well with the tumor burden [16]. Moreover, it was reported that IL-6 regulated the expression of prostate-specific protein in prostate carcinoma cells through activation of the androgen receptor [17]. Mori et al. [18] demonstrated that dexamethasone, one of the NF-κB inhibitors, upregulated the expression of membrane and soluble IL-6 receptors in prostate cancer cells. These findings suggest that IL-6 plays important roles in the progression of prostate cancer.

We previously reported that the adhesion of tumor cells to IL-1β-treated human umbilical vein endothelial cells can be inhibited by anti-NF-κB reagents such as NAC, aspirin, or pentoxifylline [19]. In the present study, we used NAC as an anti-NF-κB reagent because the cytotoxicity of NAC was lowest in these three reagents. The action of NAC is considered to be due to its antioxidative properties. NAC has been shown to scavenge reactive oxygen intermediate (ROI) and to increase intracellular glutathione levels.

Interestingly, thioredoxin, a cellular thiol along with glutathione and metallothionein, has been implicated in the regulation of some redox-responsive transcription factors such as NF-κB, AP-1, and glucocorticoid receptor [20, 21, 22]. It is indicated not only that thioredoxin relates to the intracellular activation of NF-κB but also that it works from an extracellular level. Though it is known that thioredoxin is also discharged extracellularly and strengthens the action of IL-1 and TNF, the mechanism is not yet clear. Yokomizo et al. [23] demonstrated that cellular levels of thioredoxin are associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. In the present study, we demonstrated that NAC inhibited thioredoxin expression induced by chemotherapeutic drugs in a dose-dependent manner in prostate cancer cell lines (Fig. 4). Therefore, it is possible that the effects of NAC on the induction of IL-6 were mediated by downregulation of thioredoxin and inhibition of the NF-κB activation. Many points remain unknown concerning the mechanism of the expression of IL-6. NF-IL6 and NF-κB are thought to be important transcription factors. Different cell stimuli have been reported to activate these transcription factors. Therefore, it is impossible that IL-6 is expressed by activation of NF-IL6, even in the presence of NF-κB in the cytoplasm.

Recently, many investigators reported that NF-κB can be a target of prostate cancer therapy. Muenchen et al. [24] suggested that inhibition of NF-κB by IκB, super-repressor caused TNF-a-induced apoptosis in prostate cancer cells. Wang et al. reported that antitumor therapy was enhanced through increased apoptosis by inhibition of NF-κB using adenovirus vector expressing IκBα [25].

In conclusion, chemotherapeutic drugs induced NF-κB activation in prostate cancer cell lines and IL-6 secretion, and this induction of IL-6 by NF-κB activation was inhibited by NAC. Furthermore, the effect of chemotherapeutic drugs was markedly enhanced by NAC. It is necessary that the effect of other anti-NF-κB reagents such as aspirin, pentoxifylline, glucocorticoid, and oleandrin be investigated. Thus, NAC can be a new adjuvant therapy against prostate cancer.

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