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N-Acetylcysteine Modifies *cis*-Dichlorodiammineplatinum-induced Effects in Bladder Cancer Cells

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We previously demonstrated a role of reactive oxygen species (ROS) in cytotoxicity induced by *cis*-dichlorodiammineplatinum (CDDP) in combination with glutathione (GSH) depletors in bladder cancer cells. However, the relationship between CDDP and ROS is still unclear, although many mechanisms of drug resistance have been well characterized. The present study was undertaken to investigate the effects of N-acetylcysteine (NAC), a GSH precursor, on the CDDP-induced effects in bladder cancer cells (KU1). The cytotoxic effects of CDDP were significantly blunted by NAC (1 m*M*) in KU1 cells. The IC_{50} of CDDP only (10.2±1.2 μM) is significantly lower than that of CDDP with NAC (IC_{50}: 20.3±1.6 μM) in KU1 cells. NAC also significantly increased the intracellular concentration of GSH in KU1 cells (37.2±1.6 nmol/10^6 cells), compared to controls (15.9±7.6 nmol/10^6 cells). While CDDP produced a significant increase in ROS as measured in terms of dichlorofluorescein (DCF) production in KU1 cells in a time-dependent manner, pretreatment with NAC significantly reduced CDDP-induced intracellular DCF in KU1 cells. Moreover, TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay showed that CDDP-induced apoptosis (31.1±3.8%) was significantly inhibited by pretreatment with NAC in KU1 cells (11.2±2.6%). These results demonstrated that NAC scavenges CDDP-induced ROS and inhibits CDDP-induced cytotoxicity, suggesting that ROS mediate the CDDP-induced cytotoxicity in bladder cancer cells.

Key words: CDDP — ROS — NAC

N-Acetylcysteine (NAC) was first introduced as a mucolytic agent in the 1960s[1] and has since become widely accepted as the antidote of choice for the prevention of hepatotoxicity after an acetaminophen overdose.[2] NAC is a glutathione (GSH) precursor and increases the intracellular GSH levels.[3] Accordingly, it provides protection against reperfused hepatic tissue injury and glomerular injury[4] by functioning as a scavenger of reactive oxygen species (ROS). NAC can also reportedly suppress the induction of human immunodeficiency virus (HIV) expression *in vitro* and may have some therapeutic value in HIV-infected patients.[5, 6] Recently, NAC was also suggested to prevent apoptosis.[7] However, the effect of NAC on cell damage induced by anticancer agents has not been fully established.

We previously reported that ROS play an important role in *cis*-dichlorodiammineplatinum (CDDP)-induced cytotoxicity in bladder cancer cells (KU7).[8] Our results suggested that intracellular GSH depletion enhances the CDDP-induced cytotoxicity in bladder cancer cells (KU7) through the enhancement of ROS generation. However, the relationship between ROS and CDDP is still unclear, though there are many reports on drug resistance involving CDDP and GSH. GSH is known to protect cells from ROS attack, improving both cellular metabolism and function.[5] Thus, NAC may have a significant impact on the CDDP-induced effects through ROS. The present study was, therefore, undertaken to investigate the effects of NAC on CDDP-induced ROS generation and cytotoxicity in bladder cancer cells.

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MATERIALS AND METHODS

Chemicals and drugs CDDP was obtained from Nippon Kayaku Co. (Tokyo). NAC and dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium, 0.25% trypsin and phosphate-buffered saline (PBS) were supplied by GIBCO (Grand Island, NY). DCFH-DA was dissolved in ethanol to prepare a 5 m*M* solution. 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB), glutathione reductase and NADPH were obtained from Boehringer-Mannheim Co. (Mannheim, Germany).

Cell line KU1 cells and KU7 cells, established bladder cancer cell lines, were maintained in monolayer culture in 75-cm^2 Corning culture flasks (Tokyo) containing RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and carbenicillin sodium in a humidified

atmosphere of 5% CO_2-95% air at 37°C. The culture medium was renewed every 3 days.

Cytotoxicity assay Cytotoxicity was determined as reported previously.[8] Cells (1×10^4/well) were plated in a total volume of 100 μl of medium, in a 96-well tissue culture plate (Corning), and the experiments were performed under the same conditions. Following a 24-h preincubation, the cells were exposed to CDDP in the concentration range of 0 to 53.3 μM for 24 h. To assess the effect of NAC on CDDP-induced cytotoxicity, the cells were preincubated with 1 m*M* NAC for 8 h. After the preincubation, the supernatant was discarded and cells were washed with PBS. Medium containing CDDP in the above concentration range was applied to each well. After 24-h incubation, cells were washed with PBS and cytotoxicity was determined by staining the cells with 0.2% crystal violet (in 2% ethanol). The absorbance value of each well was determined at 550 nm with a reference wavelangth of 405 nm by a Microplate reader (Bio-Rad, Tokyo). A linear relationship (R=0.953) was observed between the absorbance value and the cell number excluding trypan blue. Cell growth was expressed as percentage absorbance value compared with controls.

Measurement of intracellular GSH concentration Cells were seeded in culture dishes, and exposed to NAC (1 m*M*) for 8 h on the following day. After the exposure, the supernatant was removed and cells were collected after trypsinization. The cells were washed with PBS, homogenized in a Polytron homogenizer (Brinkmann, Westbury, NY) and centrifuged at 4°C (5000*g*, 30 min). The supernatant was harvested and the GSH concentration was determined by the GSH reductase-DTNB recycling method with a slight modification.[9] Briefly, the supernatant (1.5 ml) was incubated with 40 μl of DTNB at a concentration of 3.8 m*M* for 2 min. Then 200 μl of GSH reductase (6 units/ml) and 100 μl of NADPH (5.4 m*M*) were added to the mixture, and the concentration of GSH was estimated by using spectrophotometric measurement.

Determination of the net intracellular ROS generation To determine the net intracellular levels of ROS generated by CDDP, we used DCFH-DA, which permeates into cells and interacts with intracellular ROS to generate fluorescent dichlorofluorescein (DCF).[10] The amount of ROS was estimated from DCF production. Fluorescence intensity was determined in the cells at 590 nm with a reference wavelength of 490 nm by using a FluorImager (Molecular Dynamics, Tokyo). Cells were incubated at a density of 10^4 cells per well in a 96-well tissue culture plate for 24 h. After this preincubation period, the medium was discarded and attached cells were rinsed with PBS containing 5 m*M* glucose (PBSg). The cells were exposed to 5 μM DCFH-DA solution at 37°C. DCF production in each well was detected by using a FluorImager at 30-min intervals for 180 min after exposure to CDDP. In the experiment to determine the effect of NAC on CDDP-induced ROS generation, the attached cells were preincubated with 1 m*M* NAC for 8 h before the exposure to IC_{50} of CDDP (10 μM). DCF fluorescence was expressed as a percentage of the control intensity.

Detection of fragmented DNA in KU1 cells To assess the effect of NAC on CDDP-induced apoptosis, TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay was performed with an Apop Tag Kit (Oncor Inc., Gaithersburg, MD). After various treatments, cells were collected by trypsinization and rinsed with PBS. Until TUNEL assay, cells were suspended in 70% ethanol after fixation. Cellular fragmented DNA was detected by TUNEL end labeling utilizing the terminal deoxynucleotidyl transferase (Tdt) reaction.[11] To detect apoptosis, cells were incubated with Tdt and digoxigenin-deoxynucleotide triphosphate (dNTP) at 37°C for 1 h, then washed and exposed to fluorescein isothiocyanate (FITC)-labeled anti-digoxigenin for 30 min at room temperature. They were washed with PBS, and subjected to flow cytometry. During flow cytometric analysis, cells were analyzed on forward versus side scatter and apoptosis-associated fluorescence was measured using a log scale.

Statistical methods Data on IC_{50}, GSH concentration, the amount of intracellular ROS generation and apoptotic index were reported as mean values plus or minus the standard deviation (SD). Variables of different groups were compared by means of multiple comparison (ANOVA). A level of $P<0.05$ was accepted as being statistically significant.

RESULTS

Cytotoxicity assay To determine CDDP-induced cytotoxicity, spectrophotometric measurement was carried out by staining cells with crystal violet. For 24 h, CDDP induced cytotoxicity in KU1 cells in a dose-dependent manner (Fig. 1). The effect of NAC on CDDP-induced cytotoxicity was also assessed. NAC in a concentration range of 2 m*M* or higher exhibited cytotoxic effects in KU1 cells for 8 h. However, 1 m*M* NAC showed no cytotoxic effect on KU1 cells for 8 h. Thus, we used a maximum NAC concentration of 1 m*M* in the following procedures. The IC_{50} of CDDP in the presence of 1 m*M* NAC (20.3±1.6 μM, n=5) was significantly higher ($P<0.01$) than that of CDDP alone (10.2±1.2 μM, n=5) (Fig. 1). NAC at 0.5 m*M* showed modest but significant effects at lower CDDP concentration (3.3 or 6.7 μM). However, less than 1 m*M* NAC had no significant effect on the IC_{50} of CDDP.

Measurement of intracellular GSH concentration To determine the effect of NAC on intracellular GSH, we measured intracellular GSH concentration as described in "Materials and Methods." Intracellular GSH concentration

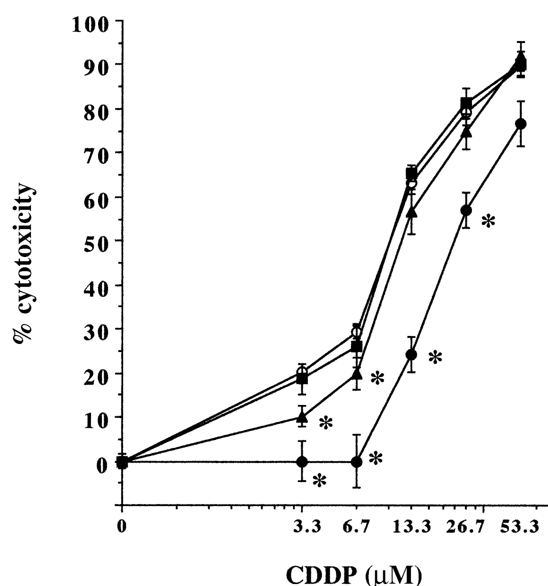

Fig. 1. Effects of NAC on CDDP-induced cytotoxicity in KU1 cells. NAC (1 m*M*) significantly reduced CDDP-induced cytotoxicity in KU1 cells. Each value represents the mean±SD of % cytotoxicity. * Significantly different from CDDP alone ($P<0.05$). Open circles, CDDP only; closed circles, CDDP with NAC 1 m*M*; closed triangles, CDDP with NAC 0.5 m*M*; closed squares, CDDP with NAC 0.25 m*M*.

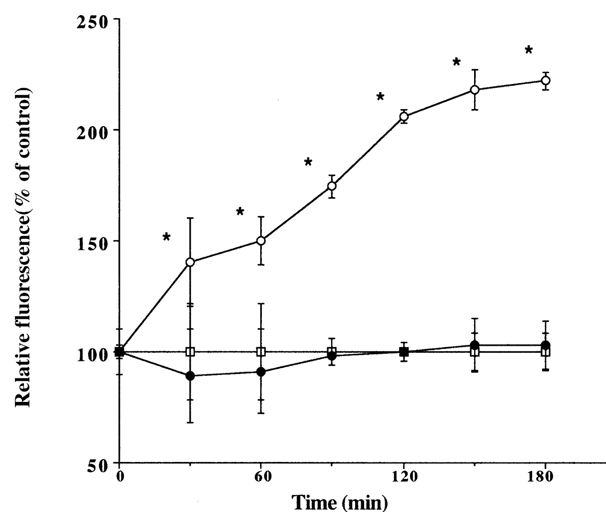

Fig. 2. Effects of NAC on CDDP-induced ROS generation in KU1 cells. CDDP stimulated ROS generation significantly, compared to controls. Pretreatment with NAC (1 m*M*) significantly blunted CDDP (10 μ*M*)-induced ROS generation. No significant difference was observed at each point between the control and CDDP with NAC. Each value represents the mean±SD of relative fluorescence. * Significantly different from the control or CDDP with NAC ($P<0.05$). Open circles, CDDP only; closed circles, CDDP with NAC; open squares, untreated controls.

in untreated KU1 cells was 15.9±7.6 nmol/10^6 cells (n=5). Intracellular GSH concentration in KU1 cells following 8 h exposure to NAC (1 m*M*) was 37.2±1.6 nmol/10^6 cells (n=5), which was significantly higher ($P<0.05$) than in untreated KU1 cells. On the other hand, intracellular GSH concentration in untreated KU7 cells was 34.0±7.3 nmol/10^6 cells (n=5), which was significantly higher than in untreated KU1 cells ($P<0.01$). Following 8 h exposure to NAC (1 m*M*), intracellular GSH concentration in KU7 cells was 38.2±2.6 nmol/10^6 cells (n=5), which was not significantly different from that in untreated KU7 cells. These results demonstrate that NAC functions as a GSH precursor in KU1 cells.

Measurement of intracellular ROS generation To determine the effect of NAC (1 m*M*) on CDDP-induced ROS generation, serial laser cytometry was performed by staining intracellular ROS with DCFH. CDDP produced a significant increase in DCF production in a time-dependent manner (Fig. 2). Within 30 min, intracellular DCF production induced by CDDP (10 μ*M*) increased to 140.0±10.0% ($P<0.05$) of the control, and was further elevated to 222.0±3.6% at 180 min. However, the CDDP-induced intracellular DCF production was significantly decreased to the baseline at each point by pretreatment with NAC (n=5, $P<0.05$). These data indicated that NAC scavenges CDDP-induced ROS, while CDDP stimulates ROS generation in KU1 cells.

Detection of CDDP-induced DNA fragmentation To determine if there was a change in apoptosis in KU1 cells after various treatments, cells were analyzed for apoptosis by flow cytometry with TUNEL assay. The mean index of CDDP (10 μ*M*)-induced apoptosis was 31.1±3.8% ($P<0.01$, n=5), which was significantly higher than the control (3.7±1.1%; Fig. 3a). Pretreatment with NAC (1 m*M*, 8 h) significantly blunted CDDP-induced apoptosis (11.2±2.6%; Fig. 3b). In addition, CDDP (5 μ*M*)-induced apoptosis (10.9±1.8%) was also significantly inhibited by pretreatment with NAC (1.1±0.7%, $P<0.01$, n=5). These results demonstrated that NAC ameliorates CDDP-induced apoptosis in KU1 cells.

DISCUSSION

ROS have been reported to play an important role in the cytotoxicity of several chemotherapeutic agents[12)] and tumor necrosis factor (TNF).[13)] In addition, we found that pretreatment with GSH depletors, either buthionin sulfoximine (BSO) or diethyl maleate (DEM), decreased the level of intracellular GSH and enhanced the CDDP-induced cytotoxicity through a significant increase in ROS generation in KU7 cells.[8)] Increased production of ROS

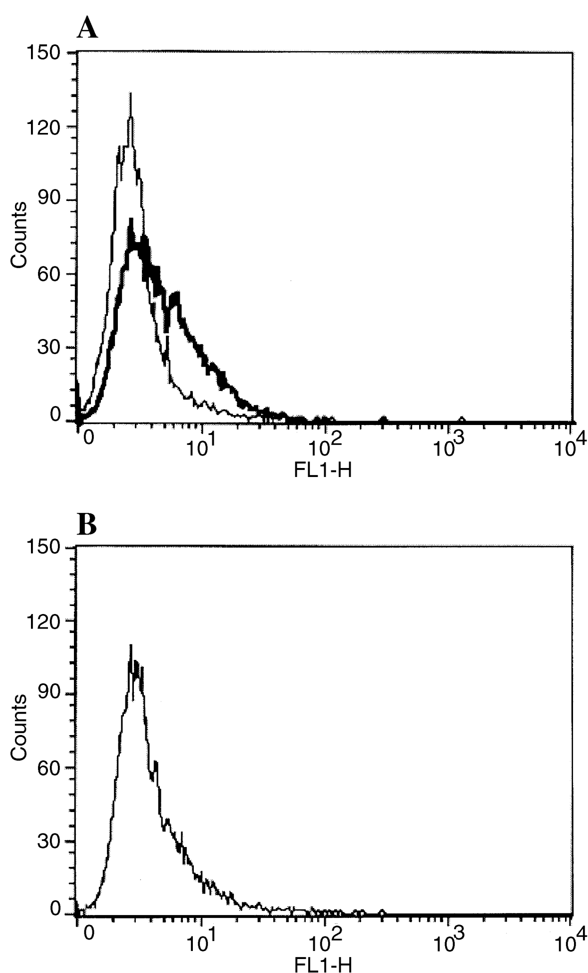

Fig. 3. Flow cytometric detection of fragmented DNA in KU1 cells. Cells were stimulated and isolated for flow cytometry as described in "Materials and Methods." The horizontal axis shows the intensity of fragmented DNA, and the vertical axis shows cell number. A shows that CDDP induces apoptosis in KU1 cells. Thin line, control; thick line, CDDP only. B shows that pretreatment with NAC inhibits CDDP-induced apoptosis in KU1 cells.

was also observed inside the mitochondria in CDDP-induced acute renal failure and TNF α-induced cell damage,[14, 15] suggesting that the mitochondria are a source of ROS. Thus, ROS appear to be a mediator of cytotoxicity. However, whether CDDP-induced cytotoxicity can be modified by ROS is unclear.

There are two major known modes of antioxidant action of NAC: 1) it acts as a scavenger of ROS[16] and 2) it augments the intracellular levels of GSH.[7] NAC has been shown to act as an ROS scavenger in adult respiratory distress syndrome by increasing the intracellular levels of GSH.[3] Moreover, pretreatment with NAC is reported to modify the mitochondrial structure, rendering the cells less susceptible to TNF-mediated injury.[17] Nevertheless, the mechanism of NAC's protective effects on cell damage is complex and the effects of NAC on the CDDP-induced cytotoxicity have not yet been elucidated. We therefore examined whether NAC modulates CDDP-induced effects, including ROS generation and cytotoxicity.

The mechanisms of drug resistance to CDDP have been quite well characterized. CDDP is inactivated by direct binding to GSH[18] and then actively exported from cells as a GSH-platinum complex via a GS-X pump.[19] Furthermore, GSH is reported to scavenge ROS and to play a role in the reduction of hydrogen peroxide and organic peroxides.[20] Thus, GSH is considered to be involved in multi-drug resistance in cancer cells. The present study indicated that NAC increases intracellular GSH levels and inhibits the CDDP-induced cytotoxicity through a decrease in ROS generation in bladder cancer cells (KU1). These results are consistent with major roles of NAC as a GSH precursor and a scavenger. We also examined the effects of NAC on another bladder cancer cell line (KU7), which contains a significantly higher intracellular GSH level than KU1. In KU7 cells, NAC neither increased intracellular GSH concentration nor modified CDDP-induced cytotoxicity (data not shown) under the same conditions as used for KU1 cells. How does this inconsistency arise? Since NAC affected not cells containing a higher GSH concentration, but cells containing a lower GSH concentration in the present study, it is presumed that the effect of NAC depends on intracellular GSH. In addition, the concentration of NAC used throughout this study was 1 m*M*, which is a high dose. A lower concentration of NAC may be effective with a longer preincubation time, or may produce a significant effect at a lower concentration of CDDP. On the other hand, Lerza *et al.*[21] described the protective effects of NAC on CDDP-induced hematopoietic toxicity in mice. In their study, NAC was not effective when administered at the time of CDDP exposure. They suggested that NAC may be effective if administered well before CDDP exposure. Our results appear to support their speculation, because our pretreatment with NAC was effective in KU1 cells. Moreover, Gogu and Agrawal[6] postulated a scavenging action of NAC on the zidovudine (AZT)-induced myelosuppression in mice, whereas NAC did not protect against CDDP-induced myelosuppression.[21] There are conflicting results as to whether NAC protects against drug-induced toxicity through scavenging ROS. Accordingly, further investigation is still required regarding methods of NAC administration and the effectiveness of NAC.

CDDP causes apoptosis that is associated with CDDP-induced cytotoxicity.[22] Apoptosis in many cell systems is

suppressed by antioxidants, suggesting a direct role of ROS in apoptosis.[22] In contrast, antioxidant preloading with substances such as NAC prevents intracytoplasmic thiol imbalance and contributes to an impairment of apoptosis.[23] In the present study, TUNEL assay showed that CDDP induces apoptosis in KU1 cells. Of particular interest is the finding that the pretreatment with NAC significantly reduced CDDP-induced apoptosis. This strongly suggests that ROS mediate the CDDP-induced apoptosis in KU1 cells. But the CDDP-induced cytotoxicity might include not only apoptosis, but also necrosis in this system.

The current study demonstrated that NAC, a GSH precursor, significantly inhibits CDDP-induced cytotoxicity through scavenging ROS while GSH depletors enhance CDDP-induced cytotoxicity through enhancing ROS. These results support the view that ROS mediate CDDP-induced cytotoxicity including apoptosis.

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