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Original Contribution

MONOCHLORAMINE INHIBITS ETOPOSIDE-INDUCED APOPTOSIS WITH AN INCREASE IN DNA ABERRATION

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Abstract—Monochloramine (NH_2Cl) is a physiological oxidant produced by activated neutrophils, and it affects apoptosis signaling. We studied the effects of NH_2Cl on the cell death induced by etoposide, a widely used anticancer agent that is directed to DNA topoisomerase II. Jurkat T cells, a human acute T cell leukemia cell line, were pretreated with 70 μM of NH_2Cl for 10 min. After 24 h, 5–30 μM of etoposide was added to the NH_2Cl pretreated and control cells, and their apoptosis, caspase activity, cell morphology, and cellular DNA contents were measured. NH_2Cl pretreatment significantly inhibited apoptosis and caspase activation induced by etoposide or camptothecin, a DNA topoisomerase I poison, but not by staurosporine or Fas stimulation. The apoptosis inhibition actually resulted in the proliferation of the survived cells and, notably, the survived cells showed more aberrant morphology, such as variation in nuclear size, nuclear fragments, and multinucleated cells. DNA content analysis of the survived cells showed an increase in aneuploid nuclei. Cell cycle analysis after 24 h of NH_2Cl treatment showed a significant decrease in S phase cells with a concurrent increase in G_0/G_1 phase cells, which suggested that NH_2Cl induced G_1 arrest. Using synchronized Jurkat cells, etoposide and camptothecin were found to be particularly cytotoxic to S phase cells, whereas staurosporine and Fas stimulation were not. Thus NH_2Cl-induced G_1 arrest was a likely cause of the observed resistance to etoposide. These observations suggested that inflammation-derived oxidants may make the tumor cells more resistant to etoposide and increase the risk of tumor progression and the development of secondary tumors by increasing the survival of DNA damage-bearing cells. © 2001 Elsevier Science Inc.

Keywords—Reactive oxygen species, Apoptosis, DNA damage, Cell cycle, Drug resistance, Free radicals

INTRODUCTION

Etoposide is a widely used anticancer agent against a variety of neoplasm such as small cell lung cancer, non-Hodgkin's lymphoma, leukemia, and germ cell tumors [1]. Despite its excellent anticancer activity, occasional development of secondary malignancy, mostly leukemia, is a serious problem [2]. Etoposide is a DNA topoisomerase II-directed agent. This enzyme relieves DNA windings and tangles by making transient DNA breaks and religations [3]. Etoposide stabilizes topoisomerase II-DNA complex, which is then converted to frank DNA strand breaks [4]. As a result, etoposide-treated cells show chromosomal aberrations and mutations, which includes large deletions, insertions, and DNA rearrangements [5]. These DNA damages are, on one hand, essential for the anticancer activity of etoposide, but on the other, they contribute to the development of secondary malignancy.

Cell death induced by etoposide often shows morphological and biochemical features of apoptosis [6]. The cell death signaling pathway includes c-Jun NH_2-terminal kinase activation [7], cytochrome *c* release from mitochondria, and caspase activation [8,9]. Thus, factors that affect apoptosis signaling may alter etoposide-induced cell death and tumor development. Among them, reactive oxygen species derived from inflammation are particularly interesting because inflammatory reaction often associates with neoplasm and some reactive oxygen species affect apoptosis signaling [10]. We have reported that monochloramine (NH_2Cl), a physiologically relevant oxidant derived from activated neutrophils, enhanced Fas-induced apoptosis, but, paradoxically, inhibited etoposide-induced apoptosis in Jurkat T cells [11].

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Chloramines are neutrophil-derived oxidants that are formed spontaneously in the reaction of hypochlorite with amines [12]. Hypochlorite is produced from H_2O_2 and Cl^- in the reaction catalyzed by myeloperoxidase [13]. Chloramines preferentially oxidize thiols and thioethers, and would modify cellular redox state and affect cell signaling. Chloramines can inhibit DNA repair [14], generation of inflammatory mediators by macrophages [15], and protein kinase C-mediated cellular responses [16]. In addition, substantial amounts of chloramines can be produced at the site of inflammation. In vitro study showed that neutrophil suspension at a concentration found in blood easily produces about 100 μM of chloramine in a short-term culture [14]. These data suggest that chloramines are important oxidants in inflammation with signal-modulating functions. In this paper we report the effects of NH_2Cl, a membrane-permeable derivative of chloramines, on the etoposide-induced cell death and DNA aberrations, and its mechanisms.

MATERIALS AND METHODS

Reagents

Etoposide and camptothecin were obtained from Calbiochem (La Jolla, CA, USA). Staurosporine was from Sigma Chemical Co. (St. Louis, MO, USA). Apoptosis-inducing antihuman Fas antibody (clone CH-11) was from MBL (Nagoya, Japan). Apoptosis detection kit using FITC-labeled annexin V was from Trevigen Inc. (Gaithersburg, MD, USA). Fluorogenic peptide substrates (Ac-Asp-Glu-Val-Asp (DEVD) -α-(4-methyl-coumaryl-7-amide) (MCA)) was from Peptide Institute Inc. (Osaka, Japan). RNase A was from Nakarai Tesque (Kyoto, Japan). Monochloramine (NH_2Cl) was prepared just before experiments as described previously [17]. All other reagents were of analytical grade or better.

Cell preparation and NH_2Cl pretreatment

Jurkat T cell, a human acute T cell leukemia cell line, was obtained from Hayashibara Biochemical Laboratories Inc. (Fujisaki Cell Center; Okayama, Japan). The cell culture medium was RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 110 mg/l sodium pyruvate (from Life Technologies, Inc.; Gaithersburg, MD, USA). Cells were grown in a CO_2 incubator containing 5% CO_2 at 37°C.

Monochloramine pretreatment was performed 24 h prior to the apoptosis induction as follows: Jurkat cells were suspended in the fresh medium at 1×10^6 cells/ml, then 70 μM of NH_2Cl were added and incubated for 10 min at 37°C. As NH_2Cl rapidly reacted with cells and medium components, virtually all NH_2Cl disappeared during the 10 min incubation period. The treated cells were separated from the medium by centrifugation at $500 \times g$ for 5 min, resuspended in a fresh medium at 0.5×10^6 cells/ml, and cultured for 24 h. Although NH_2Cl treatment alone induced slight apoptosis as described before [11], the dead cells were less than 10% at 24 h.

In addition to NH_2Cl, the effects of various oxidants, namely H_2O_2, HOCl, and taurine chloramine, were also studied using the same procedure. The concentrations of these oxidants were measured by their UV absorption spectra as described previously [17–19].

Apoptosis induction

Control and NH_2Cl-pretreated cells were collected and resuspended in a fresh medium at 1×10^6 cells/ml. Apoptosis-inducing reagents were added at the following final concentrations: etoposide, 5–30 μM; camptothecin, 1 μM; staurosporine, 100 nM; anti-Fas antibody, 100 ng/ml. Subsequent to the addition of each chemical, the cells were incubated for the indicated times in a CO_2 incubator at 37°C with 5% CO_2. For the Fas-stimulated cells, fetal bovine serum was omitted from the medium because it contains some factor that inhibits apoptosis [20].

Detection of apoptosis and necrosis

Detection of apoptosis and necrosis was performed as described previously [11] by FITC-labeled annexin V and propidium iodide (PI) dual-staining method, using a flow cytometer (FACSCalibur; Becton Dickinson; Mountain View, CA, USA). Cells stained with FITC-annexin V but not with PI were considered as apoptotic cells, and PI-positive cells were considered as necrotic cells. Data were collected from morphometrically homogeneous cell population, which typically contained about 80% of the cells. In some experiments, apoptosis was also measured using terminal deoxynucleotidyl transferase-mediated FITC-dUTP nick end labeling (TUNEL) method (MEBSTAIN apoptosis kit direct; MBL).

Measurement of caspase activity

Caspase activity in the cell lysate was studied by measuring the 7-amino-4-methyl-coumarine (AMC) liberation from the synthetic peptide substrate, Ac-DEVD-MCA, as described previously [11]. The released AMC was measured by a fluorescent spectrophotometer at ex-

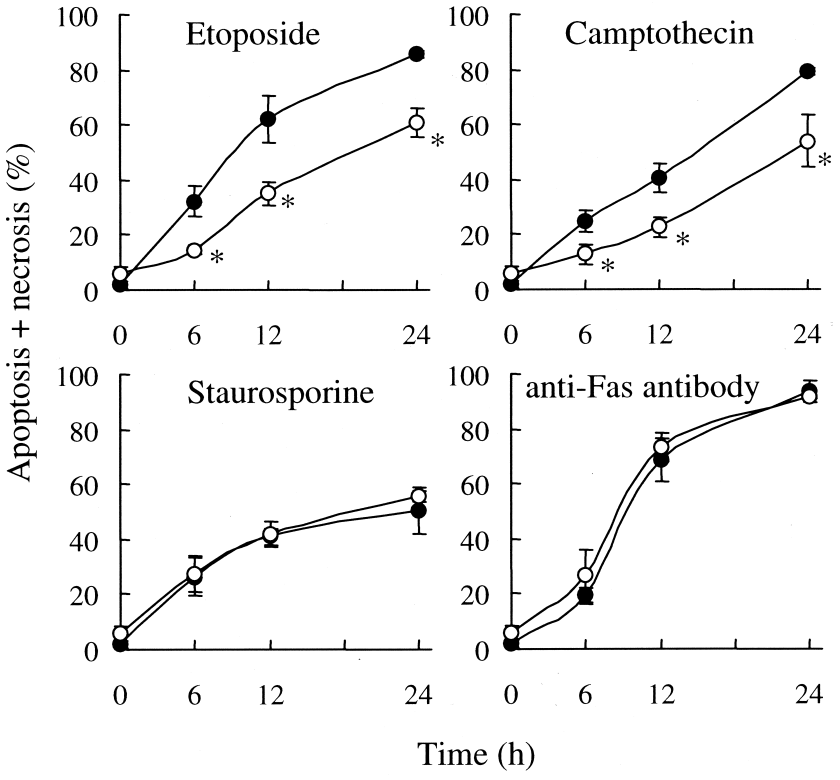

Fig. 1. Inhibition of etoposide- or camptothecin-induced apoptosis in NH_2Cl-pretreated Jurkat cells. Control and NH_2Cl-pretreated cells (70 μM NH_2Cl for 10 min, 24 h prior to the apoptosis induction) were added with apoptosis-inducing chemicals (etoposide: 30 μM, camptothecin: 1 μM, staurosporine: 100 nM, anti-Fas antibody: 100 ng/ml). Apoptosis and necrosis were studied by FITC-annexin-V and PI dual-staining method. Closed circle: no pretreatment, open circle: NH_2Cl-pretreated cells. *Significantly lower than the corresponding "no pretreatment" samples ($p < .05$). Mean ± SD for three independent experiments.

citation 380 nm and emission 460 nm. This method preferentially detects the activity of execution caspases, such as caspase 3.

Analysis of cell survival, DNA ploidy, and cell morphology after NH_2Cl and/or etoposide treatment

Control and NH_2Cl-pretreated cells were incubated with 5 μM etoposide for 6 h, then washed three times, and resuspended in a fresh medium at 1×10^6 cells/ml. Cells were cultured for 6 d and the viable cells were counted at the indicated times by a trypan-blue exclusion method.

On day 6, cell samples were analyzed for their DNA content by PI staining method as described previously [21], except that the samples were supplemented with RNase A (200 μg/ml). Cell smears were also prepared on the day 6, stained with May-Grünwald-Giemsa stain, and observed with a light microscope.

Cell cycle analysis and synchronous culture

Cell cycle was studied by measuring the DNA content as described above. Jurkat cells were synchronized by a double thymidine block method. Briefly, nonsynchronized cells were added with 2 mM thymidine and incubated for 11 h, when most of the G_2/M population disappeared. Then the cells were washed twice and resuspended in a fresh medium. After 23 h, when most of the cells passed through S phase, 2 mM thymidine was added again and incubated for 11 h. After confirming that most of the cells were in G_0/G_1 phase, cells were washed twice and resuspended in a fresh medium. After 15 h, when 78% of the cells were in S phase, samples were collected as S phase-enriched cells, and apoptosis-inducing chemicals were added. Some cells were continued culturing without apoptosis induction for up to 26 h, when 67% of the cells returned to G_0/G_1 phase (G_0/G_1 phase-enriched cells), and then apoptosis-inducing chemicals were added.

Statistical analysis

Results were tabulated for the indicated number of experimental samples. Group means were compared using Student's *t*-test for unpaired or paired samples with a two-tailed distribution.

Table 1. Inhibition of Etoposide and Camptothecin-Induced Apoptosis by NH_2Cl

	TUNEL-positive cells (%)	
	No pretreatment	NH_2Cl (70 μM)
None	11.2 ± 0.1	13.5 ± 0.5
Etoposide	56.4 ± 1.9*	21.5 ± 2.4†*
Camptothecin	39.8 ± 1.4*	15.5 ± 1.3†

Cells were treated as described in Fig. 1 and their apoptosis was evaluated by DNA fragmentation using TUNEL method 24 h after apoptosis induction. Cell samples were processed according to the manufacturer's instruction and analyzed by a flow cytometer. Mean ± SD for three independent experiments.

* Significantly increased from "None" sample ($p < .05$).

† Significantly decreased from "No pretreatment" samples ($p < .05$).

Table 2. Effects of Various Oxidants on Etoposide-Induced Apoptosis

Pretreatment	Apoptosis (%)
None	38.5 ± 1.0
H_2O_2 (70 μM)	31.6 ± 1.9*
Taurine chloramine (70 μM)	40.6 ± 2.0
HOCl (70 μM)	39.8 ± 1.4
HOCl (700 μM)	20.1 ± 1.0*
NH_4^+ (100 μM)	38.2 ± 1.0
NH_4^+ (100 μM) + HOCl (700 μM)	19.7 ± 0.9*
NH_2Cl (70 μM)	14.8 ± 1.0*

Jurkat cells in fresh medium (1×10^6 cells/ml) were pretreated with the indicated chemicals for 10 min, then the medium was changed and incubated for 24 h before etoposide addition (30 μM). Apoptosis was measured 6 h after etoposide treatment using annexin V method. Mean ± SD for three independent experiments.

* Significantly decreased from "None" sample ($p < .05$).

RESULTS

Inhibition of etoposide or camptothecin-induced apoptosis in NH_2Cl-treated Jurkat cells

Monochloramine treatment (70 μM for 10 min) of the Jurkat cells 24 h prior to the addition of etoposide (30 μM) resulted in a significant decrease in apoptotic cell death (Fig. 1). The dead cell population in NH_2Cl + etoposide-treated group was significantly decreased from that of etoposide-alone group at 6 h and later. Cell death induced by camptothecin, a DNA topoisomerase I poison, was also inhibited significantly by NH_2Cl pretreatment. In contrast, staurosporine- or Fas-induced apoptosis was neither inhibited nor enhanced by NH_2Cl treatment. In all cases, cell death began with apoptotic characteristics, i.e., externalization of phosphatidylserine with PI exclusion. Propidium iodide-positive cells increased substantially only after 24 h of apoptosis induction (data not shown). Apoptosis was also evaluated by DNA fragmentation using TUNEL method (Table 1). Consistent with the results described above, addition of etoposide or camptothecin resulted in the emergence of TUNEL-positive cells, which was inhibited significantly by the NH_2Cl pretreatment.

Table 2 shows the effects of other neutrophil-derived oxidants on etoposide-induced apoptosis. Taurine chloramine and HOCl had no effects at 70 μM. H_2O_2 (70 μM) slightly inhibited the apoptosis at 6 h, but this inhibitory effect was not observed at later time points (data not shown). Higher dose of HOCl (700 μM) significantly inhibited the etoposide-induced apoptosis, and this effect was not altered significantly by the presence of NH_4^+ (100 μM).

Cell survival, morphology, and DNA content after etoposide treatment

To compare cell survival, control and NH_2Cl-pretreated cells were incubated with 5 μM of etoposide for 6 h, then washed three times, and the culture was continued for 6 d. Among the NH_2Cl + etoposide-treated cells, viable cells decreased on day 1, then gradually increased by proliferation, and, on day 5, viable cells increased significantly from day 0 (Fig. 2). This is in good contrast to the etoposide-alone samples, in which viable cells decreased steadily. The amount of viable cells in the NH_2Cl + etoposide-treated group was significantly higher than that of the etoposide-alone group at day 1 and later.

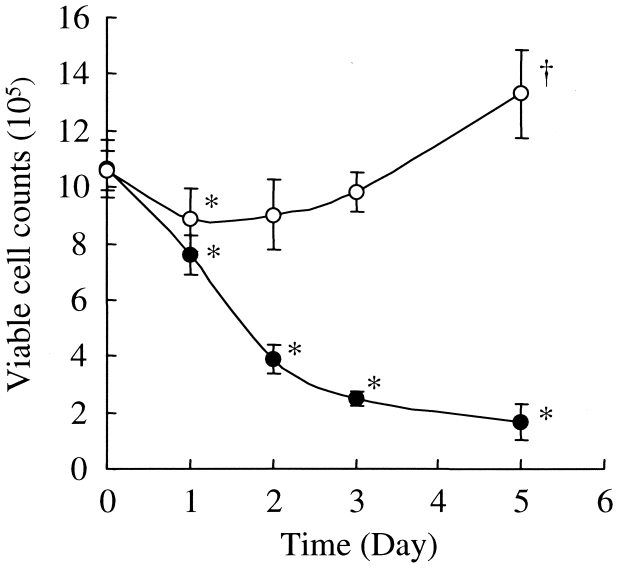

Fig. 2. Cell survival after etoposide treatment in the cells with or without NH_2Cl pretreatment. Control and NH_2Cl-pretreated cells (70 μM NH_2Cl for 10 min, 24 h prior to the etoposide addition) were incubated with 5 μM etoposide for 6 h, then washed three times, and resuspended in a fresh medium at 1×10^6 cells/ml. Cell culture was continued for 5 d and the viable cells were counted by a trypan-blue exclusion method. Closed circle: cells without pretreatment. Open circle: cells pretreated with NH_2Cl. *Significantly decreased from the day 0 samples. †Significantly increased from the day 0 sample ($p < .05$). Mean ± SD for three independent experiments.

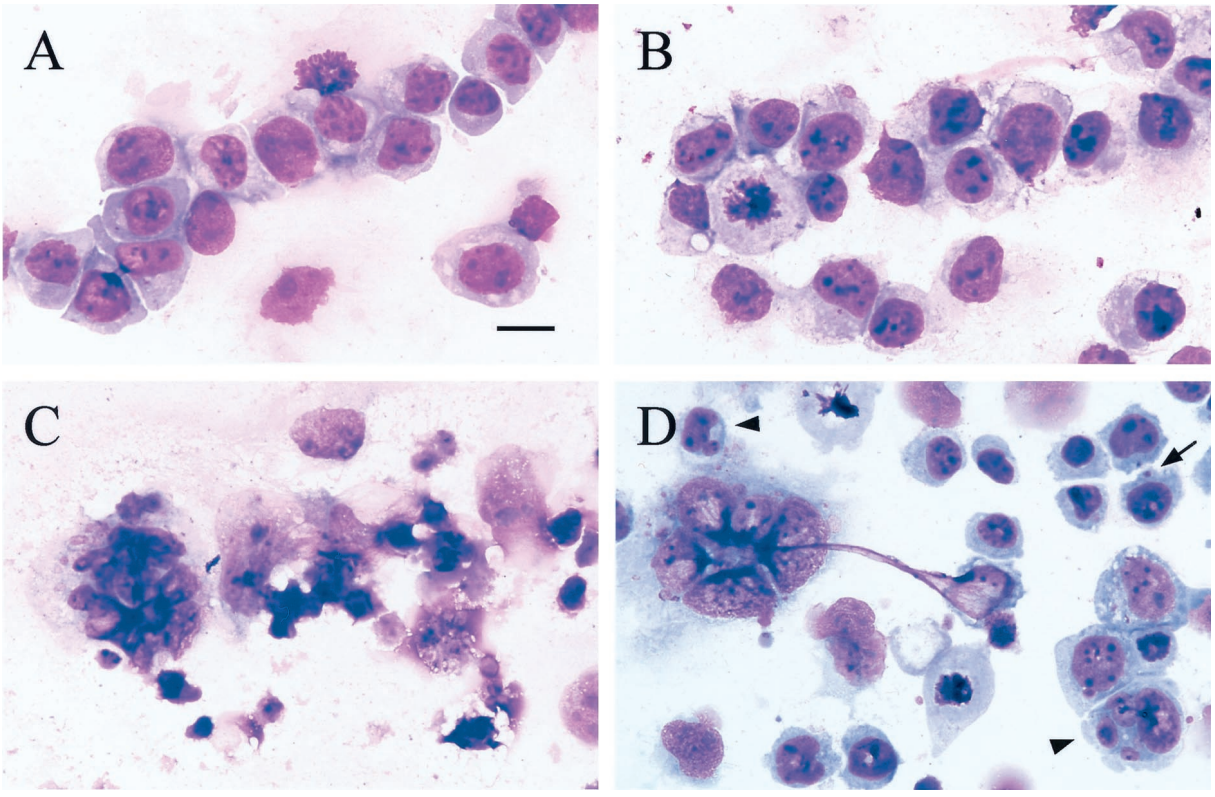

Fig. 3. Microscopic appearance of the Jurkat cells treated with NH_2Cl and/or etoposide (5μM). Cells were treated as described in Fig. 2 and cultured for 6 d. Cell smears were prepared and stained with May-Grünwald Giemsa stain. (A) Nontreated control cells; (B) NH_2Cl treatment alone; (C) etoposide treatment alone; (D) NH_2Cl + etoposide-treated cells. Note nuclear size variation, multinucleated cells (arrowhead), nuclear fragments (arrow), and incompletely divided cells that are connected with thin strand of chromatin. The scale bar indicates 20 μm, and is applicable to all figures. Representative data from three independent experiments.

The survived cells were studied microscopically 6 d after the etoposide treatment. Etoposide treatment alone resulted in marked cell death (Fig. 3C). Viable cells were scanty and most of the cells had condensed, pyknotic nuclei. A few large cells were also seen. Among NH_2Cl + etoposide-treated cells, viable cells were more numerous (Fig. 3D). Notably, greater variation in the nuclear size, multinucleated cells with unequal-sized nuclei, cells with nuclear fragments, and incompletely divided cells that were connected with a thin strand of chromatin were also observed (Fig. 3D). Monochloramine treatment alone (Fig. 3B) showed no apparent change from the nontreated controls (Fig. 3A), and both of the cells were relatively regular in size.

DNA content of the survived cells were also measured using the same 6 d samples. In Fig. 4, area 2 contains G_0/G_1, S, and G_2/M phases of the diploid cells. Most of the cells in control (Fig. 4A) and NH_2Cl-alone group (Fig. 4B) localized in this area. Area 1 represents the hypodiploid nuclei, which are considered to be dead cells [21]. Most of the etoposide-treated cells (Fig. 4C) were in area 1. Area 3 indicates hypertetraploid nuclei. Notably, NH_2Cl + etoposide-treated group (Fig. 4D) showed a significant increase in area 3 (3.0 $\pm$ 0.7%, $p < .05$) when compared with the other groups, in which area 3 comprised less than 1%. Area 4 includes duplicated events, in which two or more cells were counted as a single event by mistake, and was excluded from the analysis.

Caspase activation

Caspase activity was measured 6 h after apoptosis induction. Caspase activation, as measured by DEVD cleavage activity, was significantly inhibited by NH_2Cl pretreatment when etoposide or camptothecin was used as a stimulant (Fig. 5). In contrast, Fas-induced caspase activation was enhanced slightly by NH_2Cl pretreatment. Staurosporine-induced caspase activation was not affected significantly.

Cell cycle analysis of the NH_2Cl-pretreated and control cells

As etoposide and camptothecin are reported to be particularly cytotoxic to S phase cells, we studied the cell

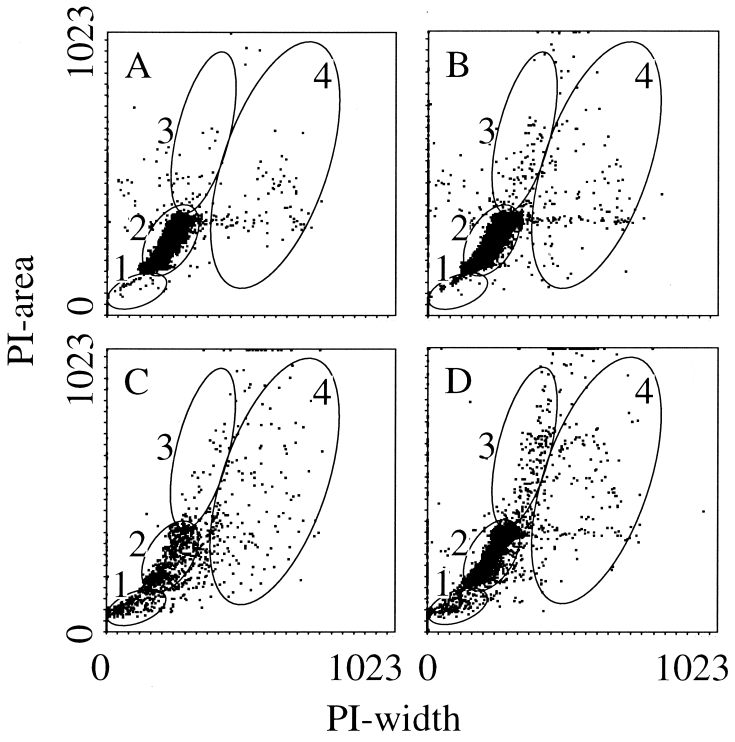

Fig. 4. DNA content analysis in the Jurkat cells treated with NH_2Cl and/or etoposide. Cells were treated as described in Fig. 2 and cultured for 6 d. Cells were lysed in a hypotonic buffer containing 0.1% Triton X-100 and 200 μg/ml of RNase A, stained with propidium iodide (PI), and analyzed by a flow cytometer. PI area indicates the DNA amount. PI width was simultaneously measured to exclude duplicated events, in which two or more cells were counted as a single event by mistake. (A) Control cells; (B) NH_2Cl treatment alone; (C) etoposide treatment alone; (D) NH_2Cl + etoposide-treated cells. 10,000 events of each were shown. Area 1: hypodiploid cells; area 2: diploid to tetraploid cells; area 3: hypertetraploid cells; area 4 duplicated events (excluded from the analysis). Representative data from three independent experiments.

cycle changes after NH_2Cl treatment. After 24 h of NH_2Cl treatment (70 μM, 10 min), S phase population (17.9 $\pm$ 1.0%) decreased significantly from that of controls (30.9 $\pm$ 1.4%), with a concurrent increase in the G_0/G_1 phase population (Fig. 6). G_2/M phase population did not change significantly.

Cell cycle dependence of the apoptosis

Cell cycle dependence of the apoptosis induced by each chemical was studied using synchronized Jurkat cells. As expected, etoposide- and camptothecin-induced apoptosis were definitely cell cycle dependent, in which S phase-enriched cells were more susceptible to apoptosis than G_0/G_1 phase-enriched cells (Fig. 7). In contrast, staurosporine- or Fas-induced apoptosis was not different significantly between S phase and G_0/G_1 phase.

DISCUSSION

In this paper, we reported that NH_2Cl pretreatment made the Jurkat cells more resistant to etoposide-induced apoptosis. This inhibition was probably attributed to the G_1 arrest by the NH_2Cl pretreatment. At the time of etoposide addition, NH_2Cl-pretreated cells had a significant decrease in S phase cells with a concurrent increase in G_0/G_1 phase cells. Our data, as well as previous reports [9,22], showed that etoposide- and camptothecin-induced apoptosis were more pronounced in S phase cells than G_0/G_1 cells. In contrast, staurosporine- and Fas-induced apoptosis were relatively independent of cell cycle. Thus, the observed inhibition of apoptosis by etoposide and camptothecin, but not by staurosporine and Fas stimulation, can be explained by the G_1 arrest by the NH_2Cl pretreatment.

In this experiment, we added etoposide 24 h after the NH_2Cl treatment because the S phase population was minimal at this time point. The significant decrease in S phase population was observed at 12 h after the NH_2Cl treatment, reached a minimum at 24 h, and partially recovered at 48 h (data not shown). By contrast, the inhibition of etoposide-induced apoptosis was observed even when etoposide was added immediately after NH_2Cl treatment [11], reached a maximum at 12–24 h, and declined at 48 h (data not shown). This discrepancy can be explained by the lag

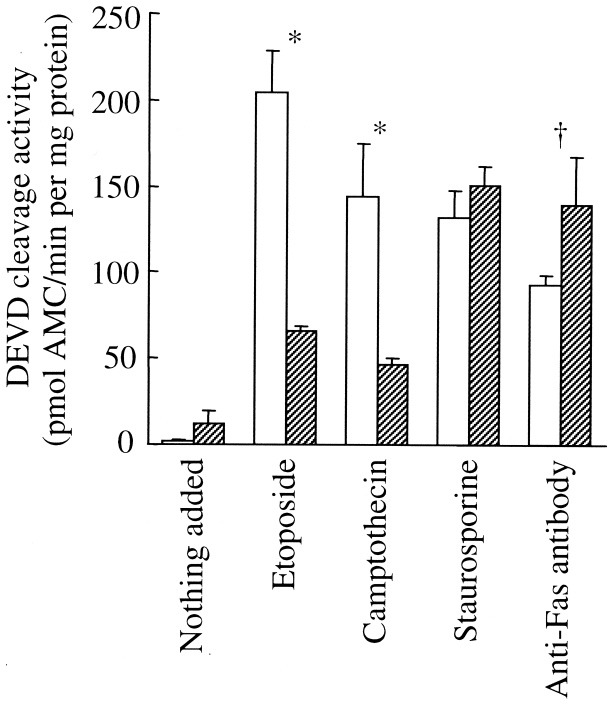

Fig. 5. Caspase activation by various apoptosis-inducing chemicals in the cells pretreated with or without NH_2Cl. Cells were pretreated with or without NH_2Cl and apoptosis-inducing chemicals were added as described in Fig. 1. Caspase activities were measured 6 h after the induction of apoptosis. Open bars: cells without pretreatment. Hatched bars: NH_2Cl-pretreated cells. *Significantly decreased in NH_2Cl-pretreated cells ($p < .05$). †Significantly increased in NH_2Cl-pretreated cells ($p < .05$). Mean ± SD for three independent experiments.

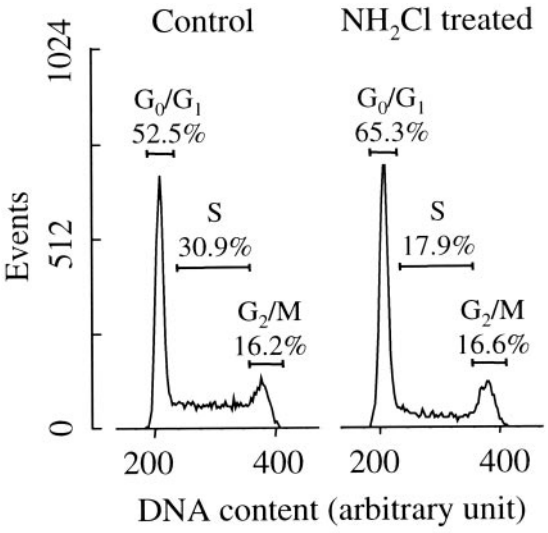

Fig. 6. Cell cycle analysis in control and NH_2Cl-treated cells. Cells were treated with 70μM NH_2Cl for 10 min and the medium was changed and cultured for 24 h. DNA content was measured by PI. G_0/G_1, S, and G_2/M phase populations (% of viable cells) were shown. Representative data of three independent analysis.

between the actual cell proliferation status and the cell cycle distribution.

NH_2Cl + etoposide-treated cells probably received less DNA damage when compared with the cells treated with etoposide alone. DNA topoisomerase IIα is reported to be the major target of etoposide [23], and this enzyme is required for the formation of DNA strand breaks [5]. The expression of DNA topoisomerase IIα increases in late S phase [24]. It is also reported that the amount of DNA topoisomerase II parallels with the cytotoxicity of etoposide [25]. Thus, NH_2Cl-pretreated cells, as they had less S phase cells, probably received less DNA damage. Consistently, etoposide-induced caspase activation was significantly lower in NH_2Cl-pretreated cells. As staurosporine- or Fas stimulation-induced caspase activation was not inhibited by NH_2Cl pretreatment, execution caspases seem to be intact. Fas-induced caspase activation was enhanced in NH_2Cl-treated cells, which is consistent with our previous report [11].

Another point to be noted is that the NH_2Cl-pretreated cells survived the etoposide treatment with damages in the DNA, which would increase the risk of neoplastic transformation and tumor progression. Microscopic observation showed nuclear fragments and variation in nuclear size. Flow cytometric measurement of DNA content showed an increase in hypertetraploid cells. These findings suggest an increase in the aneuploid cells in the survived population. Aneuploidy causes a massive imbalance in the gene dose, which would result in the metabolic imbalance through uncoordinated expression of proteins and enzymes. Recent analysis showed that aneuploidy produces qualitative changes in metabolism and genetic instability, which are the bases for tumor-specific phenotype, such as abnormal morphology, invasiveness, and progression to malignancy [26]. Indeed, tumor progression in human neoplasm often accompanies aberrant nuclear morphology.

The increase in aneuploid cells in our samples was probably caused by the genotoxic effects of etoposide, which is known to cause large deletions, insertions, and DNA rearrangements [27]. When G_0/G_1 phase-enriched cells (prepared by the thymidine block method) were treated with etoposide for 6 h and cultured for 6 d, cells with aberrant morphology that was similar to NH_2Cl + etoposide-treated cells were observed (data not shown). Thus, etoposide was primarily important for the emergence of aberrant morphology in this experimental condition. Secondary leukemia by etoposide treatment often shows rearrangement of *MLL* gene in chromosome 11 [2]. Such DNA rearrangements might also increase when cells survived the etoposide treatment. Thus, the cell survival with damages in the DNA after etoposide treatment may increase the risk of neoplastic transformation and tumor progression.

Chloramine derivatives are physiologically relevant

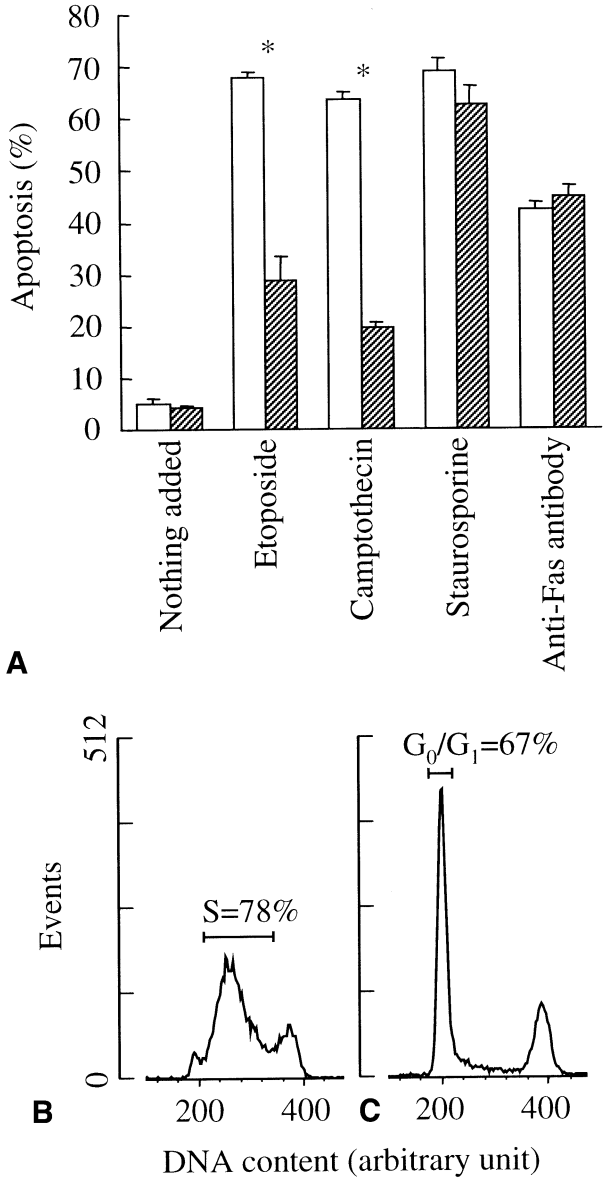

Fig. 7. Cell cycle dependence of the apoptosis induced by various chemicals. Jurkat cells were synchronized by a double thymidine block method, and S phase-enriched cells (B) and G_0/G_1 phase-enriched cells (C) were obtained. (A) Cell death was studied 6 h after the induction of apoptosis (etoposide: 30 μM, camptothecin: 1 μM, staurosporine: 100 nM, anti-Fas antibody: 100 ng/ml). Open bars: S phase-enriched cells. Hatched bars: G_0/G_1 phase-enriched cells. *Significantly higher in S phase-enriched cells ($p < .05$). Mean ± SD for three independent experiments.

oxidants that would be produced especially in an acute inflammation. Membrane-permeable chloramines, such as NH_2Cl, are also likely to be produced, because NH_4^+ exists at 20–40 μM in normal serum. Among various oxidants derived from activated neutrophils, NH_2Cl was most effective in inhibiting the etoposide-induced apoptosis, when compared with H_2O_2, HOCl, and taurine chloramine. As taurine chloramine, a membrane-impermeable chloramine, was not effective, the target of chloramine was probably located inside the cell. HOCl was less effective in inhibiting the etoposide-induced apoptosis. Because of its high reactivity, a substantial amount of added HOCl probably reacted with medium and membrane components, and minor fraction of HOCl went into the cell. Supplementation of NH_4^+ (100 μM) did not enhance the inhibitory effect of HOCl, which suggested that the conversion of HOCl to NH_2Cl outside the cell was not required, although it is still possible that HOCl is converted to chloramine derivatives inside the cell.

Although we should be careful in extrapolating these in vitro results to the in vivo condition, our data indicate that inflammation-derived oxidants can attenuate etoposide cytotoxicity through the cell cycle arrest. Besides etoposide, other therapeutic methods, such as radiation and other drug therapeutics, may also be affected by NH_2Cl, because their cytotoxic effects depend on the cell cycle [28,29]. In addition, our data also indicated that inflammation-derived oxidants may enhance tumor progression by facilitating the survival of DNA damage-bearing cells. As various degrees of inflammation are usually associated with neoplasm, and recent evidence suggests that inflammation-derived active oxygen species accelerate tumor progression [30], this possibility also needs to be studied further.

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REFERENCES

[1] Hande, K. R. Clinical applications of anticancer drugs targeted to topoisomerase II. *Biochim. Biophys. Acta* **1400:**173–184; 1998.
[2] Felix, C. A. Secondary leukemias induced by topoisomerase-targeted drugs. *Biochim. Biophys. Acta* **1400:**233–255; 1998.
[3] Burden, D. A.; Osheroff, N. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochim. Biophys. Acta* **1400:**139–154; 1998.
[4] Howard, M. T.; Neece, S. H.; Matson, S. W.; Kreuzer, K. N. Disruption of a topoisomerase-DNA cleavage complex by a DNA helicase. *Proc. Natl. Acad. Sci. USA* **91:**12031–12035; 1994.
[5] Berger, N. A.; Chatterjee, S.; Schmotzer, J. A.; Helms, S. R. Etoposide (VP-16-213)-induced gene alterations: potential contribution to cell death. *Proc. Natl. Acad. Sci. USA* **88:**8740–8743; 1991.
[6] Mesner, P. W. J.; Budihardjo, I. I.; Kaufmann, S. H. Chemotherapy-induced apoptosis. *Adv. Pharmacol.* **41:**461–499; 1997.
[7] Seimiya, H.; Mashima, T.; Toho, M.; Tsuruo, T. C-Jun NH2-terminal kinase-mediated activation of interleukin-1β-converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis. *J. Biol. Chem.* **272:**4631–4636; 1997.
[8] Martins, L. M.; Mesner, P. W.; Kottke, T. J.; Basi, G. S.; Sinha, S.; Tung, J. S.; Svingen, P. A.; Madden, B. J.; Takahashi, A.; McCormick, D. J.; Earnshaw, W. C.; Kaufmann, S. H. Comparison of caspase activation and subcellular localization in HL-60 and K562 cells undergoing etoposide-induced apoptosis. *Blood* **90:**4283–4296; 1997.
[9] Kaufmann, S. H. Cell death induced by topoisomerase-targeted drugs: more questions than answers. *Biochim. Biophys. Acta* **1400:**195–211; 1998.

[10] Jabs, T. Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem. Pharmacol.* **57:**231–245; 1999.
[11] Ogino, T.; Ma, Y.; Than, T. A.; Omori, M.; Okada, S. Monochloramine enhances Fas (APO-1/CD95)-induced apoptosis in Jurkat T cells. *J. Leukoc. Biol.* **67:**46–52; 2000.
[12] Test, S. T.; Lampert, M. B.; Ossanna, P. J.; Thoene, J. G.; Weiss, S. J. Generation of nitrogen-chlorine oxidants by human phagocytes. *J. Clin. Invest.* **74:**1341–1349; 1984.
[13] Weiss, S. J.; Klein, R.; Slivka, A.; Wei, M. Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. *J. Clin. Invest.* **70:**598–607; 1982.
[14] Pero, R. W.; Sheng, Y.; Olsson, A.; Bryngelsson, C.; Lund-Pero, M. Hypochlorous acid/N-chloramines are naturally produced DNA repair inhibitors. *Carcinogenesis* **17:**13–18; 1996.
[15] Marcinkiewicz, J.; Grabowska, A.; Bereta, J.; Stelmaszynska, T. Taurine chloramine, a product of activated neutrophils, inhibits in vitro the generation of nitric oxide and other macrophage inflammatory mediators. *J. Leukoc. Biol.* **58:**667–674; 1995.
[16] Ogino, T.; Kobuchi, H.; Sen, C. K.; Roy, S.; Packer, L.; Maguire, J. J. Monochloramine inhibits phorbol ester-inducible neutrophil respiratory burst activation and T cell interleukin-2 receptor expression by inhibiting inducible protein kinase C activity. *J. Biol. Chem.* **272:**26247–26252; 1997.
[17] Thomas, E. L.; Grisham, M. B.; Jefferson, M. M. Preparation and characterization of chloramines. *Meth. Enzymol.* **132:**569–585; 1986.
[18] Nelson, D. P.; Kiesow, L. A. Enthalpy of decomposition of hydrogen peroxide by catalase at 25°C (with molar extinction coefficients of H_2O_2 solutions in the UV). *Anal. Biochem.* **49:** 474–478; 1972.
[19] Arnhold, J.; Wiegel, D.; Richter, O.; Hammerschmidt, S.; Arnold, K.; Krumbiegel, M. Modification of low-density lipoproteins by sodium hypochlorite. *Biomed. Biochim. Acta* **50:**967–973; 1991.
[20] Kikuchi, H.; Sugiyama, S.; Imajoh-Ohmi, S. A novel antiapoptosis serum factor that downregulates fas-mediated apoptosis. *J. Biochem.* **117:**936–939; 1995.
[21] Nicoletti, I.; Migliorati, G.; Pagliacci, M. C.; Grignani, F.; Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Meth.* **139:**271–279; 1991.
[22] D'Arpa, P.; Beardmore, C.; Liu, L. F. Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. *Cancer Res.* **50:**6919–6924; 1990.
[23] Drake, F. H.; Hofmann, G. A.; Bartus, H. F.; Mattern, M. R.; Crooke, S. T.; Mirabelli, C. K. Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry* **28:**8154–8160; 1989.
[24] Isaacs, R. J.; Davies, S. L.; Sandri, M. I.; Redwood, C.; Wells, N. J.; Hickson, I. D. Physiological regulation of eukaryotic topoisomerase II. *Biochim. Biophys. Acta* **1400:**121–137; 1998.
[25] Sullivan, D. M.; Latham, M. D.; Ross, W. E. Proliferation-dependent topoisomerase II content as a determinant of antineoplastic drug action in human, mouse, and Chinese hamster ovary cells. *Cancer Res.* **47:**3973–3979; 1987.
[26] Rasnick, D.; Duesberg, P. H. How aneuploidy affects metabolic control and causes cancer. *Biochem. J.* **340:**621–630; 1999.
[27] Baguley, B. C.; Ferguson, L. R. Mutagenic properties of topoisomerase-targeted drugs. *Biochim. Biophys. Acta* **1400:**213–222; 1998.
[28] Denekamp, J. Cell kinetics and radiation biology. *Int. J. Radiat. Biol.* **49:**357–380; 1986.
[29] Mauro, F.; Gohde, W.; Schumann, J.; Teodori, L.; Spano, M. Considerations in the design of possible cell cycle effective drugs. *Int. J. Radiat. Biol.* **49:**307–333; 1986.
[30] Okada, F.; Nakai, K.; Kobayashi, T.; Shibata, T.; Tagami, S.; Kawakami, Y.; Kitazawa, T.; Kominami, R.; Yoshimura, S.; Suzuki, K.; Taniguchi, N.; Inanami, O.; Kuwabara, M.; Kishida, H.; Nakae, D.; Konishi, Y.; Moriuchi, T.; Hosokawa, M. Inflammatory cell-mediated tumor progression and minisatellite mutation correlate with the decrease of antioxidative enzymes in murine fibrosarcoma cells. *Br. J. Cancer* **79:**377–385; 1999.

ABBREVIATIONS

Ac-DEVD-MCA—acetyl-L-Asp-L-Glu-L-Val-L-Asp-α-(4-methyl-coumaryl-7-amide)
AMC—7-amino-4-methyl-coumarin
FITC—fluorescein isothiocyanate
PI—propidium iodide
TUNEL—terminal deoxynucleotidyl transferase-mediated FITC-dUTP nick end labeling