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Apoptosis induced by etoposide in small-cell lung cancer cell lines

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Abstract. The DNA fragmentation, a parameter of apoptosis, in non-small (NSCLC) and small (SCLC) cell lung cancer cell lines (N231 and PC-9) was evaluated. The DNA fragmentation in SCLC lines, but not in NSCLC lines, was observed in overgrown cells without exposure to anticancer drugs. In etoposide (VP-16)-treated N231 but not PC-9 cells, DNA fragmentation continued to increase up to 42 h, and the increase was dependent on the concentration of VP-16. The endonuclease activity of VP-16-treated N231, but not PC-9, cells required both Ca^{2+} and Mg^{2+} for full activity. It was elevated in a time- and concentration-dependent manner. As this activity was not affected by addition of cycloheximide, the activation of the endonuclease activity without protein synthesis may be involved in VP-16-induced cytotoxicity in N231.

Introduction

There are two types of cell death, apoptosis and necrosis [1, 2]. Apoptosis is considered to be an active process of gene-directed cellular self-destruction, whereas necrosis is essentially an accidental occurrence, being the outcome of severe injurious changes in the environment of the affected cells [3, 4]. Recently, it was reported that apoptosis was induced not only by physiological events but also by non-physiological events, such as irradiation [5, 6], hyperthermia [5, 7] or stimulation of hormones [8, 9], toxins [5, 10], cytokines [11], or ceramide [12]. Anticancer drugs such as antimetabolites [13, 14], topoisomerase inhibitors [15, 16], alkylating agents [17], tubulin-interacting agents [18, 19] or CDDP [20] also induced apoptosis of several cancer cells. However, few data are available on apoptosis in lung cancer cell lines induced by anticancer drugs. Clinically, small cell lung cancer (SCLC) is more sensitive than non-small cell lung cancer (NSCLC) to every anticancer drug tested so far. The radiosensitivity of SCLC is also higher than that of NSCLC. The reasons for these differences have so far remained largely unknown.

During apoptosis, a series of well-defined degenerative changes occur within the cell [2–4], which ultimately result in the degradation of the nuclear DNA into oligonucleosome chains. In many cases, Ca^{2+}/Mg^{2+}-dependent endonuclease acts as a key molecule for the inter-nucleosomal cleavage followed by the cellular condensation or fragmentation distinguishable as ladder formations in the electrophoresis gel [21].

In this paper, we report that DNA fragmentation was induced in four SCLC cell lines but not in four NSCLC cell lines under conditions of confluent growth and under treatment with VP-16. The endonuclease activity of the SCLC cell lines was higher than that of the NSCLC cell lines and was enhanced by the treatment with VP-16.

Materials and methods

Chemicals. Etoposide (VP-16) was obtained from Bristol Myers Squibb Japan (Tokyo, Japan), [^{14}C]Thymidine ([^{14}C]-Thd) was purchased from Amersham Japan (Tokyo, Japan), and all other chemicals used were purchased from Sigma (St. Louis, Mo.) unless stated otherwise.

Cell lines and culture. Four human SCLC cell lines (SBC-1, SBC-3, SBC-5 and N231), and four NSCLC cell lines (PC-7, PC-9, PC-13 and PC-14) were used in the experiments. N231 established at the National Cancer Institute, Bethesda, Md. was obtained from Dr. Y. Shimosato, National Cancer Center Research Institute, Tokyo. SBC-1, SBC-3 and

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SBC-5, established at Okayama University, were obtained from ATCC. PC7, PC-9, PC-13 and PC-14, kindly provided by Professor Y. Hayata, Tokyo Medical College, were derived from previously untreated patients. The cells were grown in RPMI 1640 medium supplemented with 10% w/v heat-inactivated fetal bovine serum (Immunobiological Laboratories, Fujioka, Japan), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37° C. "Overgrown" condition was defined as that in which 20% of dead cells were present. In this condition, the pH of the medium was about 7.2.

Cell viability assay. Cells were assayed for viability using the 3-(4, *S*-dimethylthazol-2-yl)-2, *S*-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann [22]. After VP-16 treatment, the cells were cultured in drug-free medium for the number of hours indicated. MTT was added to the cultures (500 mg/ml) and incubated at 37° C for 4 h. The intracellular formazan crystals formed were solubilyzed with dimethyl sulfoxide (DMSO) and the absorbance of the solution at 560 nm was measured using a spectrophotometer.

Determination of internucleosomal DNA cleavage. After VP-16 treatment, 2×10^6 cells were harvested and treated as described by Smith et al. [23]. The pellets were resuspended in lysis buffer (20 μl) containing 0.5% (w/v) sodium lauryl sarkosinate and 0.5 mg/ml proteinase K and incubated at 50° C for 1 h, after which 10 μl of 0.5 mg/ml RNase A was added, followed by further incubation at 50° C for 1 h. Conventional agarose gel electrophoresis was carried out using 2% (w/v) agarose gel containing 0.1 μg/ml ethidium bromide in 0.045 M Tris-borate and 1 mM ethylene-diamine tetraacetic acid (EDTA) at 40 V for 18 h, after which the gels were photographed under UV light with a Polaroid camera using Polaroid positive-negative film 667.

Preparation of nuclear extracts. Nuclear extracts were prepared as described by Lee et al. [24] with several modifications. Cells treated with VP-16 or control vehicle were harvested and washed in ice-cold phosphate-buffered saline (PBS). The packed cells were suspended in one packed-cell volume of buffer A [10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethansulfonic acid) (HEPES)] containing 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM dithiothreitol, pH 7.9, at 4° C and kept on ice for 15 min. Then, the cells were aspirated through 25-gauge needles and the crude nuclei were obtained by centrifugation for 20 min at 25,000 *g*. The crude nuclear pellet was resuspended in two-thirds of one packed-cell volume of buffer C [0.35 M NaCl, 20 mM HEPES, 2.5% (v/v) glycerol, 1 mM EDTA, 1 mM glutathione, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol, pH 7.9] followed by stirring for 30 min at 4° C (TOMY micro tube mixer MT-360). The resulting supernatant was desalted and replaced with buffer D (25 mM Tris-HCl, 20% glycerol, 0.1 mM EDTA, 1 mM glutathione, 0.5 mM PMSF, 1 mM dithiothreitol by filtration, pH 7.9) [Ultrafree-MC with PTGC (10,000 NMWL) membrane, Millipore]. The nuclear extracts that remained on the filters were collected, aliquots were frozen in dry ice and ethanol and stored at –80° C until required for use. The protein content of each extract was determined using the method of Bradford [25].

DNA substrate preparation. ^{14}C-Thd-labeled DNA was prepared as follows: 2×10^7 cells were incubated in 10 ml culture medium containing 0.5 μCi/ml of [^{14}C]-Thd (specific activity, 58 mCi/mmol, Amersham) for 24 h. The cells were harvested by low-speed centrifugation and the DNA was extracted using the detergent-salt method. The cell pellet was suspended in 10 mM-10 mM Tris-EDTA (pH 7.5) and incubated with 100 μg/ml proteinase K and 0.5% (w/v) SDS at 70° C for 15 min, after which more proteinase K was added and incubated at 37° C for 18 h. The sample was treated with 100 μg/ml of RNase A at 37° C for 1 h, and the DNA was precipitated with absolute ethanol, left overnight at 4° C in 70% (v/v) ethanol, then dried and dissolved in 1 mM-1 mM Tris-EDTA (pH 7.5).

Nuclease activity assay. The nuclease assay was carried out as described by Hashida et al. [26] with several modifications. The enzyme activity was determined by measuring the formation of acid-soluble DNA fragments. Aliquots (100 μg) of the nuclear extracts were incubated in 0.5 ml reaction mixture (50 mM Tris, 5 mM NaCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol and ^{14}C-Thd-labeled DNA [approximately 7×10^4 cpm/min per 5 μg DNA], pH 7.4) at 37° C for 20 min for N231 and 40 min for PC-9 cells, after which the reaction was terminated by adding 0.5 ml ice-cold 10% (w/v) trichloroacetic acid. Undigested DNA was precipitated by standing on ice for 15 min and centrifugation, and the radioactivity in the supernatant was counted using a scintillation counter (Beckman). The optimal reaction time was determined after confirmation of a linear relationship between the amounts of ^{14}C-DNA fragmentation and nuclear extract added.

Inhibition by $ZnSO_4$ of VP-16-induced endonuclease activity. Various concentration of $ZnSO_4$ was added to the reaction mixture to determine the effects on endonuclease activity as described previously [27]. N231 cells were exposed to 100 μg/ml of VP-16 for 3 h followed by incubation in drug-free medium for 16 h. The nuclease activity of the cells incubated with or without $ZnSO_4$ was determined as described previously. The results were expressed as percentages of the control activity obtained under the standard assay conditions.

Results

Comparison of DNA fragmentation in human lung cancer cell lines

The exponential growth and overgrown phases of NSCLC and SCLC lines were examined and compared for DNA fragmentation. In overgrown SCLC cell lines DNA fragmentation was observed, while no DNA fragmentation was observed in NSCLC cell lines either in exponential growth or in overgrown phases (Fig. 1 a, b).

Cell viability of N231 and PC-9 cells after exposure to VP-16

The N231 and PC-9 cells were exposed to 100 μg/ml VP-16 for 3 h. VP-16 was washed away with fresh culture medium, and the cells were incubated in drug-free medium. The time-dependent changes in optical density were evaluated using MTT assays (Fig. 2 a, b). Both cell lines grew in a time-dependent manner within 45 h in the control cultures, whereas there was no increase in either cell line after VP-16 treatment. Growth inhibition in N231 cells was calculated from the formula: 1–(optimal density of the treated cells/optimal density of control cells) and was 17.7%, 49.0%, and 63.3% at 16 h, 24 h and 48 h, respectively. Growth inhibition in PC-9 cells was 20.2% and 48% at 21 h and 45 h, respectively.

DNA fragmentation in N231 and PC-9 cells after VP-16 treatment

The N231 and PC-9 cells were incubated with 100 μg/ml VP-16 for 3 h, after which the remaining VP-16 was washed away with culture medium and the cells were cultured in drug-free medium for various times. In the N231 cells, DNA fragmentation was observed after 16 h and increased in proportion to the incubation time (Fig. 3 a),

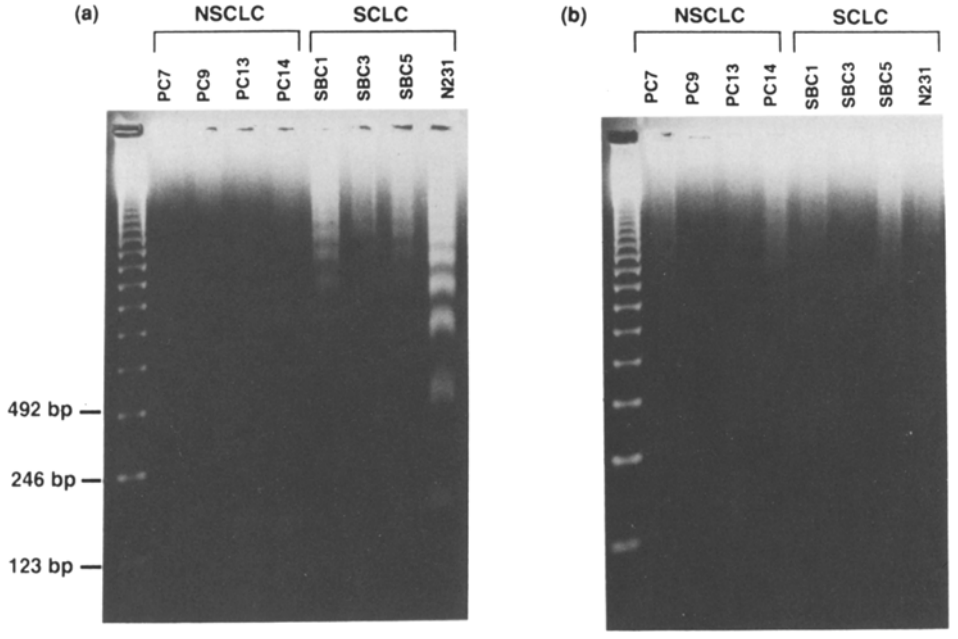

Fig. 1a, b. Comparison of DNA fragmentation in human lung cancer cell lines. Overgrown (**a**) and exponential (**b**) phases of NSCLC and SCLC lines were examined. 'Overgrown' cells was defined as meaning that cells included 20% of dead cells in culture medium

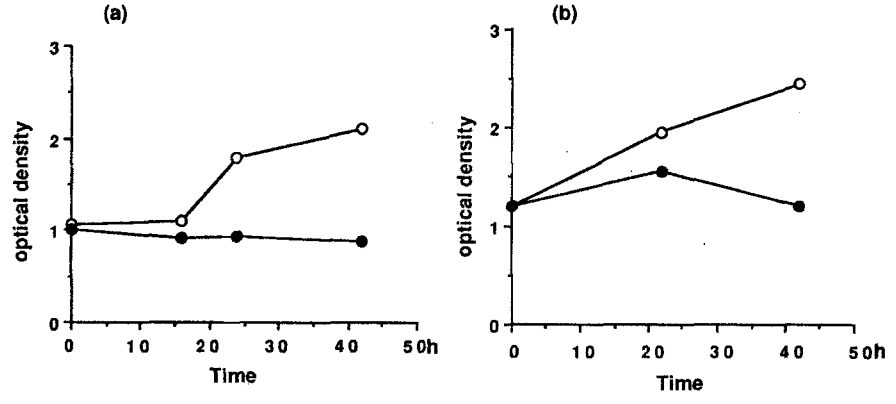

Fig. 2a, b. The optical densities of **a** N231 and **b** PC-9 cells with (●) or without (○) 100 μg/ml of VP-16, according to the incubation time. Cells were analyzed at the times indicated (h), and viability was determined by MTT assays as described in "*Materials and methods*"

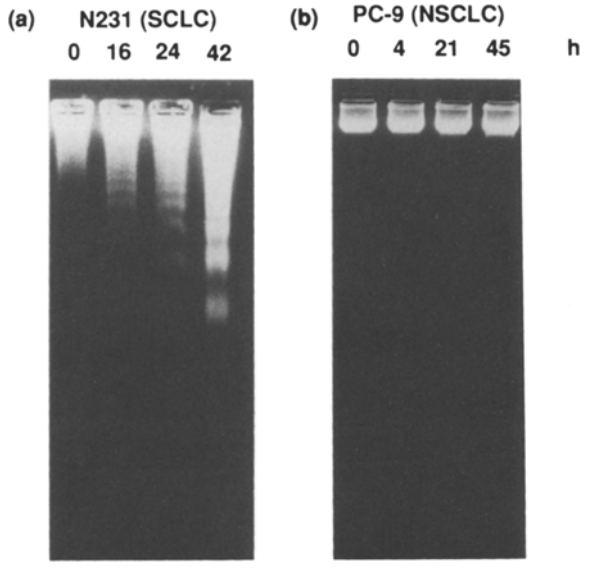

Fig. 3a, b. DNA fragmentation in **a** N231 and **b** PC-9 cell lines exposed to VP-16 (100 μg/ml), visualized by separating the DNA fragments on agarose gel. The N231 and PC-9 cells were harvested at the times indicated (h) after exposure to VP-16 or control vehicle for 3 h

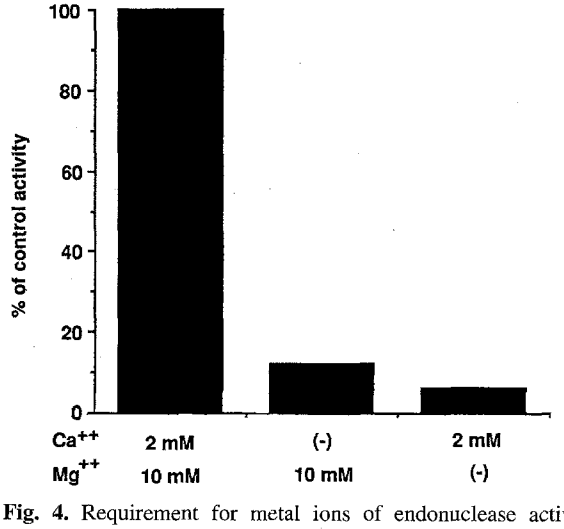

Fig. 4. Requirement for metal ions of endonuclease activity. The endonuclease activity of N231 cell was measured as described in "Materials and methods." The concentrations of Ca^{2+} and Mg^{2+} in the reaction mixture were 2 mM and 10 mM respectively. Results are expressed as precentages of the control activity obtained under the standard assay conditions

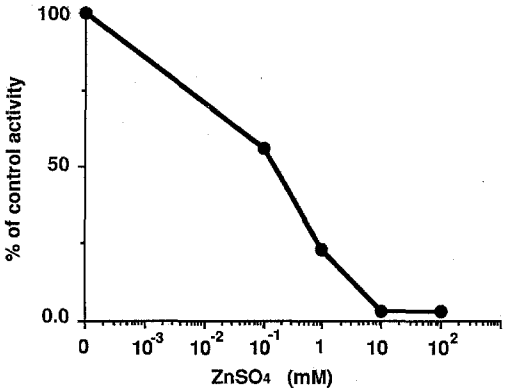

Fig. 5. Effect of $ZnSO_4$ on endonuclease activity of the N231 nuclear extract. The indicated concentrations of $ZnSO_4$ were added to the reaction mixture to determine the effects on endonuclease activity. Results are expressed as percentages of the control activity obtained under the standard assay conditions.

whereas no DNA fragmentation was detected in the PC-9 cells after incubation for 45 h (Fig. 3b), when the cell damage in PC-9 cells was equal to that in N231 cells at 24 h.

Changes in endonuclease activities of N231 and PC-9 cells after treatment with VP-16

The endonuclease from N231 cells required Mg^{2+} and/or Ca^{2+} for full activity (Fig. 4), and if either or both of Mg^{2+} and Ca^{2+} was removed from the assay medium, the endonuclease activity was markedly reduced. The endonuclease activity of N231 cells was suppressed by addition of $ZnSO_4$ (Fig. 5). The enzyme activity was higher in N231 than PC-9 cells even before incubation with VP-16 and increased in proportion with the concentration of VP-16 in N231, but not in PC-9 cells (Table 1).

Table 1. Endonuclease activity of N231 and PC-9 cell lines

Cell lines	DNA solubilized (%)		
	Concentration of VP-16 (μg/ml)		
	0	100	200
N231	26	47	65
PC-9	12	11	13

Enzyme activities were expressed as percentage counts of VP-16-exposed cells relative to those of the [^{14}C]-Thd-labeled DNA (approximately 7×10^4 cpm/min per 5 μg DNA) added per assay, as described in "*Materials and methods.*"

Discussion

In this report, we have shown the apoptosis induced by one of major anticancer drugs, VP-16, in SCLC and NSCLC cell lines.

In Fig. 1, it is seen that DNA fragmentation could be induced in four SCLC cell lines, but not in four NSCLC cell lines, under conditions of confluent growth without exposure to an anticancer drug. A typical SCLC cell line, N231, showed VP-16-induced concentration-dependent DNA fragmentation, whereas PC-9, an NSCLC, showed no DNA fragmentation after exposure to higher concentrations (200 μg/ml) with VP-16 (data not shown). DNA fragmentation in PC-9 cells treated with 100 μg/ml VP-16 was not observed at 48 h (Fig. 3), although the condition is equitoxic to N231 cultured for 24 h (Fig. 2). This difference might mean that DNA fragmentation was more easily induced in N231 than in PC-9 and was important for initiation of cancer cell death induced by VP-16 in N231 cells but not in PC-9 cells.

Recently, many reports have demonstrated that major anti-cancer agents, such as topoisomerase inhibitors [15, 16] or tubulin-interacting agents [18, 19], induce apoptosis of cancer cells. The mechanisms of apoptosis induced by these drugs, especially by topoisomerase II inhibitors, have been studied by many investigators [28–30]. One of the mechanisms followed by apoptosis was the activation of Ca^{2+}/Mg^{2+}-dependent endonuclease [27, 31]. Our data indicated that the Mg^{2+}/Ca^{2+}-dependent endonuclease activity in the nucleus of N231 cells was augmented by the treatment of VP-16 (Table 1). As the inhibition of protein synthesis by cycloheximide failed to affect the DNA fragmentation of N231 (data not shown), the protein synthesis, including endonuclease, was not involved in the activation of endonuclease.

We have already reported that the activity of poly(ADP-ribose)polymerase is higher in SCLC cell lines than in NSCLC cell lines [32]. Rice et al. have shown that Ca^{2+}/Mg^{2+}-dependent endonuclease is activated by poly(ADP-ribose)polymerase [31], the difference in content of this enzyme might be the reason for the easier induction of apoptosis in the N231 cell line.

Kaufman et al. have shown that the etoposide-induced endonuclease activity in HL-60 cell is not inhibited at the concentration of cycloheximide that inhibits protein synthesis [33]. These workers also showed that nuclei from HL-60 cells did not give rise to the characteristic patterns of DNA cleavage when incubated with the drugs but that the reaction of a cytoplasmic extract from treated cells initiated DNA cleavage. They suggested that endonuclease might normally be limited to the extranuclear material of the cell until it is activated. Even though we have no data on the endonuclease activity of extranuclear materials, this hypothesis is one of the candidates considered for explanation of our results.

Rubin et al. reported the transient expression of the c-*jun* and c-*fos* genes in HL-60 and U-937 cells after treatment with VP-16 [34]. They suggested that together with the induction of c-*fos*, the synthesis of the transcriptional activation complex may activate the genes responsible for a programmed cell death. But our data show that the inhibition of protein synthesis by cycloheximide failed to affect the DNA fragmentation induced by VP-16. The mechanism shown by Rubin et al. might not be involved in DNA fragmentation in N231 cells.

Other candidates were the phosphorylation of cell cycle control protein p34 [35, 36], the expression of the c-*myc* oncogene [37–40] or a mammalian homologue of the RAD9 gene of yeast [41, 42]. However, we have no data on expression of these gene products.

Clinically, SCLC is more sensitive than NSCLC to every anti-cancer drug tested so far. This is the first study to demonstrate that apoptosis can be more readily induced in SCLC than in NSCLC lines and that endonuclease activity is closely related to the extent of DNA fragmentation with anticancer drugs. Our data also suggest that it might be possible to use DNA fragmentation and/or the endonuclease activity of cancer cell lines as indicators in sensitivity tests for anticancer drugs. Further studies using other cancer cell lines and other anticancer agents should be carried out to establish whether this is a generalized phenomenon and such would be feasible.

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