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# Enhancement of $Ca^{2+}$-dependent Endonuclease Activity in L1210 Cells during Apoptosis Induced by 1-β-D-Arabinofuranosylcytosine: Possible Involvement of Activating Factor(s)

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**Internucleosomal DNA fragmentation and morphological changes in nuclei typical of apoptosis were observed in L1210 cells incubated with 1.0 μg/ml of 1-β-D-arabinofuranosylcytosine (ara-C). To investigate the mechanisms involved, we examined the activities of endogenous endonucleases in nuclei and cytoplasm. Both fractions of control cells contained $Ca^{2+}$-dependent endonuclease which was capable of mediating internucleosomal DNA fragmentation. The assay system using two kinds of target substrates, i.e., nuclear chromatin of CCRF-CEM cells and naked DNA purified from the same cells, revealed that the activity of $Ca^{2+}$-dependent endonuclease was enhanced in the crude nuclear extracts of cells treated with 1.0 μg/ml of ara-C for 24 h or 48 h. The activity was extracted more easily from ara-C-treated cells than control cells without sonication of the nuclear fraction. On the other hand, in the cytoplasmic fraction of the cells, the activity towards naked DNA was unchanged, whereas that towards nuclear chromatin was clearly enhanced. These results suggest that internucleosomal DNA fragmentation induced by ara-C treatment is associated with enhancement and activation of constitutively expressed $Ca^{2+}$-dependent endonuclease in L1210 cells.**

Key words: Apoptosis — 1-β-D-Arabinofuranosylcytosine — DNA fragmentation — $Ca^{2+}$-endonuclease

1-β-D-Arabinofuranosylcytosine (ara-C) is one of the most effective agents against acute myeloblastic leukemia. It is converted to an active metabolite, ara-C triphosphate (ara-CTP),[1,2] and is in part misincorporated into newly synthesized DNA of the cells in the S-phase[3,4] resulting in interference with chain elongation,[5] and causing inhibition of DNA synthesis,[6] dysfunction of DNA,[7] and a decrease in clonogenic survival.[3,5] Thus, the primary action mechanisms and cellular targets of ara-C have been extensively studied for years, but the precise pathway leading from these cellular changes to the final cell death remains unclear.

Recently, great interest has been focused on apoptosis as a mode of cell death both in physiological circumstances and in pathological situations induced by a variety of anti-cancer agents.[8–10] In human leukemic cell lines, ara-C has been reported to induce apoptosis with internucleosomal DNA fragmentation,[8,11–13] which is considered as a biological hallmark of apoptosis.[14,15] However, the precise mechanisms of internucleosomal DNA cleavage involved in apoptosis, as well as the endonucleases involved have not been clearly identified, though some candidate endonucleases have been reported.[16–25] Among them, $Ca^{2+}$- or $Ca^{2+}/Mg^{2+}$-dependent endonucleases have been reported to be responsible for nucleosomal DNA cleavage in thymocyte or lymphoid cell apoptosis induced by glucocorticoid treatment,[15,20–23] or by irradiation.[24,25] In the present study, we examined the activities of endogenous $Ca^{2+}$-dependent endonuclease in nuclei and cytoplasm during the process of ara-C-induced apoptosis in L1210 cells.

## MATERIALS AND METHODS

**Chemicals** ara-C and RNase A were purchased from Sigma (St. Louis, MO). Proteinase K was from Merck (Darmstadt, Germany). All the other chemicals were obtained from Nacalai Tesque (Kyoto), unless otherwise indicated.

**Cell culture** Murine leukemia L1210 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 μg/ml kanamycin (Meiji Seika, Tokyo) at 37°C under 5% $CO_2$ in humidified air. For ara-C treatment, exponentially growing cells were sedimented at 200*g* and resuspended in fresh medium at $1\times10^6$ cells/ml. Human T-lymphoblastic leukemia CCRF-CEM cell line was maintained similarly. The viable cells were counted by the trypan blue dye exclusion method.

**Clonogenic assay** After exposure to ara-C, cells were washed three times with the medium and plated in quadruplicate on 24-well plates (Falcon #3047, Becton Dickinson, Lincoln Park, NJ) at 200 cells/well in 0.4 ml

of the culture medium supplemented with 15% fetal calf serum, and 0.8% methylcellulose. Colonies (>50 cells) were counted after six days of culture.

**Extraction of DNA and agarose gel electrophoresis** After incubation with ara-C, cells were spun down, washed twice with phosphate-buffered saline (PBS) and incubated in the lysis buffer (10 m*M* Tris-HCl, pH 8.0, 20 m*M* EDTA, 1% sodium dodecyl sulfate and 0.5 mg/ml of proteinase K) at 42°C for 24 h. DNA was extracted with phenol/chloroform/isoamyl alcohol as described previously[26] with slight modifications, and treated with 100 $\mu$g/ml of RNase A for 1 h at 37°C. Precipitated DNA with ethanol at $-20$°C overnight was pelleted by centrifugation at 12,000*g* for 20 min and dissolved in TE buffer (10 m*M* Tris HCl, pH 8.0 and 1 m*M* EDTA). DNA concentrations were estimated by measuring the $A_{260}$. DNA (2.5 $\mu$g) was loaded onto 1.2% agarose gels (FMC BioProducts, Rockland, ME) and electrophoresis was performed in 0.5$\times$TAE buffer (20 m*M* Tris-acetate and 1 m*M* EDTA) for 75 min at 50 V. The gels were stained with 1 $\mu$g/ml of ethidium bromide and photographed under UV light.

**Preparation of cytoplasm and nuclei** The preparation was performed by the method of Kauffman[8] and Bertrand *et al.*[27] with slight modifications. Cells were harvested by centrifugation, washed twice with PBS, incubated on ice for 20 min at a density of $1.3\times10^7$ cells/ml in STKM buffer (250 m*M* sucrose, 50 m*M* Tris-HCl, pH 7.5, 25 m*M* KCl and 5 m*M* $MgCl_2$) containing 0.25% Triton X-100, and centrifuged at 1,800*g* for 8 min at 4°C. The supernatant was harvested as a cytoplasmic fraction and was either used immediately after preparation or stored for up to two weeks at $-80$°C. The pellet (nuclear fraction) was then resuspended at $5\times10^6$ nuclei/ml in STKM buffer and the Triton X-100 treatment was repeated. Finally sedimented nuclei were suspended in STKM buffer and examined under a microscope with a drop of 0.1% azur-C on a slide glass. Assay of glucose-6-phosphatase, a marker enzyme of microsomes, showed that there was less than 5% microsomal contamination in the nuclear fraction. In the cytoplasmic fraction, little contamination of DNA was detected by agarose gel electrophoresis.

**Preparation of crude nuclear extracts** Isolated L1210 cell nuclei were extracted in 0.1 ml/$10^7$ nuclei of extraction buffer (20 m*M* Tris HCl, pH 7.5, 1 m*M* EGTA, 0.35 *M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* phenylmethanesulfonyl fluoride and 1 m*M* 2-mercaptoethanol) at 4°C for 60 min, and centrifuged at 105,000*g* for 2 h. Occasionally, where indicated, they were sonicated (Ultrasonics, NY) for 5 s $\times$ 3 with 15-s cooling intervals before incubation at 4°C. The supernatants were collected as nuclear extracts and stored at $-80$°C for up to two weeks. Protein concentrations were determined by the method of Lowry.[28]

**Autodigestion of nuclei** L1210 cell nuclei were incubated at 37°C in STKM buffer in the presence of 1 m*M* EGTA or various concentrations of $CaCl_2$ for 60 min. The reaction was stopped by adding the lysis buffer and DNA was extracted as mentioned above except for 2-h treatment with proteinase K.

**Endonuclease assay** To examine how endonucleases work on DNA which sustains chromatin conformation and on naked DNA, two kinds of exogenous target substrates were employed. One is nuclei of CEM cells, which showed little endogeneous $Ca^{2+}$- or $Ca^{2+}/Mg^{2+}$-dependent endonuclease activity,[19] and the other is naked DNA purified from intact CEM cells. The assay mixture in a volume of 100 $\mu$l contained 5 m*M* $MgCl_2$, indicated concentrations of $CaCl_2$, enzyme-containing extracts (cytoplasm prepared from $10^6$ cells, or 40 $\mu$g proteins of nuclear extract), and target substrate ($10^6$ CEM nuclei suspended in STKM buffer or 10 $\mu$g of naked DNA). The reaction was performed at 37°C for the indicated time, and was stopped by adding the lysis buffer. The target DNA was isolated and fragmentation patterns were detected by agarose gel electrophoresis as described above. The quantification of fragmented DNA was done according to the method of Alnemri and Litwack[19] with slight modifications. Photographic negatives were scanned and the divided areas at the molecular weight marker position of 1.9 kilobase pairs were integrated by FAST SCAN, Personal Scanning Imager (Molecular Dynamics, Sunny Vale, CA). The percentage of fragmented DNA was determined by dividing the area in the region of under 1.9 kilobase pairs by the total area.

Each experiment was done at least three times and electrophoretic patterns were confirmed to be the same in a series of experiments.

## RESULTS

**Apoptosis induced by ara-C treatment** Table I shows that treatment with 1.0 $\mu$g/ml of ara-C slowly decreased the cell viability and it was effective in clonogenic assays. This treatment triggered apoptosis, as was confirmed by observation of nuclear morphology and the electrophoretic patterns of extracted DNA. Cells stained with the DNA-specific fluorochrome diamidino-2-phenylindole (DAPI) were observed by confocal laser scanning microscopy (LSM-GB200, Olympus, Tokyo) (Fig. 1). Chromatin condensation typical of apoptosis was observed as intensely fluorescent granules of different sizes in ara-C-treated cells. However, the nuclei of control cells kept an intact round form, and fine fluorescent grains were dispersed almost evenly in all parts of the nucleus. In electrophoretic analysis, isolated DNA of ara-C-treated cells was partly cleaved into 180–200 base pair units which showed mono- and oligonucleosomal fragments,

and this DNA fragmentation was prominent at 48 h (Fig. 2).

**$Ca^{2+}$-dependent endonuclease activities in nuclei and cytoplasm** Nuclei of L1210 cells were autodigested in STKM buffer with 1 m*M* EGTA or various concentrations of $CaCl_2$. Electrophoretic analysis revealed that in the presence of 5 m*M* $Mg^{2+}$, nuclear DNA was not degraded with 1 m*M* EGTA, while it was digested into nucleosomal units most prominently with 1 m*M* $Ca^{2+}$. The optimum concentration of $Ca^{2+}$ ranged from 1 m*M* to 2 m*M* in more precise experiments (data not shown), and it was inhibited by co-incubation with 50 $\mu M$ $Zn^{2+}$ (Fig. 3). $Zn^{2+}$ is known to inhibit endonuclease activity and the concentration for inhibition was at the same level as in the cases of thymocyte nuclei[20, 25] and human spleen cell nuclei.[29] On the other hand, when CEM nuclei were used as an exogenous target substrate in a nuclease assay, $Ca^{2+}$-dependent endonuclease activity was also detected in cytoplasm (5 m*M* $Mg^{2+}$ was included), and its character was similar to that of the activity in nuclei, i.e., the optimal concentration of $Ca^{2+}$ was 1 m*M*, and it was inhibited by 50 $\mu M$ $Zn^{2+}$ (Fig. 4). The requirement for $Mg^{2+}$ was not studied because it was necessary for preparation of the cytoplasm.

Table I. Effects of 1.0 $\mu$g/ml of ara-C Treatment in L1210 Cells

| Treatment time with ara-C (h) | Viability[a] (%) | Clonogenic survival[b] (%) |
|---|---|---|
| 0 | 95.8±0.8 | 99.7±6.3 |
| 24 | 91.0±4.4 | 3.8±0.6 |
| 48 | 83.2±3.5 | 3.6±0.8 |

*a*) Mean±SD of two separate experiments performed in triplicate.
*b*) Mean±SD of two separate experiments performed in quadruplicate.

**Effect of ara-C treatment on $Ca^{2+}$-dependent endonuclease activity in nuclear extract** To study the effect of ara-C treatment of cells on the $Ca^{2+}$-dependent endonuclease activity in nuclei, we extracted nuclear proteins with 0.35 *M* NaCl and examined its activity using both naked DNA and chromatin DNA of nuclei from CEM cells as target substrates. In control cells, this endonuclease could not be extracted effectively without sonication, since the substrates were not degraded (Fig. 5A, lane 1 of each panel). Increasing the protein amount of nuclear extracts did not induce any visible nucleosomal DNA fragmentation (data not shown). In contrast, nuclear extracts prepared from ara-C-treated cells digested naked DNA effectively, and chromatin DNA of CEM nuclei was degraded into nucleosomal units (Fig. 5A, lanes 2 and 3 of each panel). These results indicate that $Ca^{2+}$-dependent endonuclease is extractable from nuclei of ara-C-treated cells. When the sonication process was added in the preparation of nuclear extracts, this endonuclease was detected in control nuclear extracts (Fig. 5B, lane 1 of each panel), while its activity to digest both kinds of substrate DNA was prominent in the nuclear extracts from ara-C-treated cells (Fig. 5B, lanes 2 and 3 of each panel), and was inhibited by 50 $\mu M$ $Zn^{2+}$

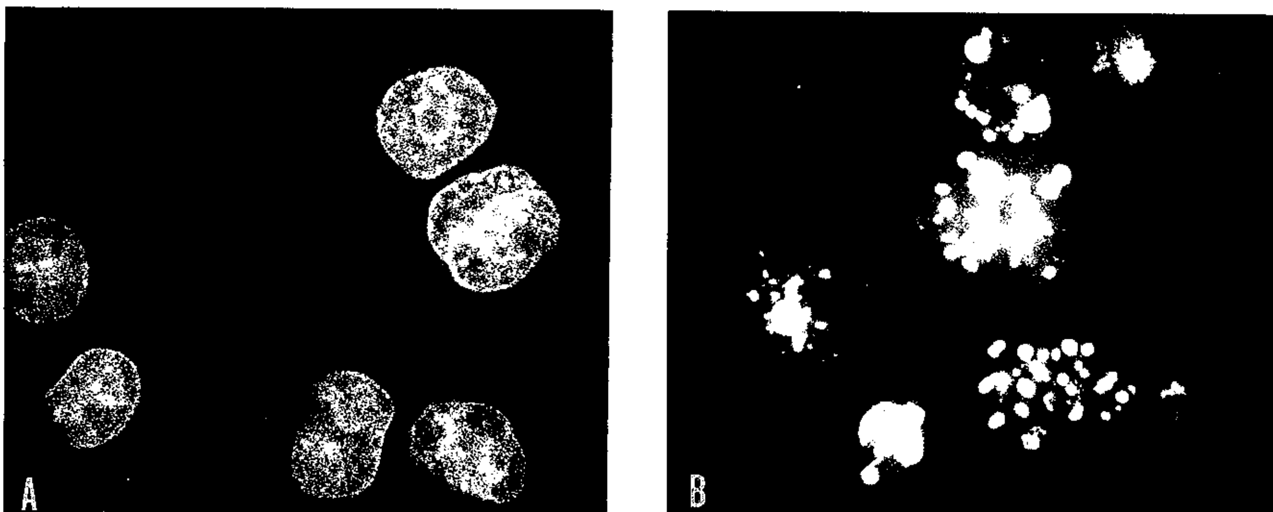


Fig. 1. UV fluorescence photographs of L1210 cells. (A) Control. (B) Apoptotic nuclear fragmentation of cells treated with 1 $\mu$g/ml of ara-C for 24 h. After drug treatment, cells were stained with 1 $\mu$g/ml of the DNA-specific fluorescent dye DAPI and were observed with a laser confocal scanning microscope. Original magnification was ×400.

(Fig. 5B, lane 4 of each panel). In addition, densitometric quantification demonstrated the enhancement of $Ca^{2+}$-dependent endonuclease activity in nuclear extracts of ara-C-treated cells (Fig. 5, C and D). In the extracts of ara-C-treated cells (24-h treatment), the percentage of fragmented chromatin DNA was 2.5 times the control in non-sonicated extracts, whereras it was 1.3 times the control in sonicated extracts.

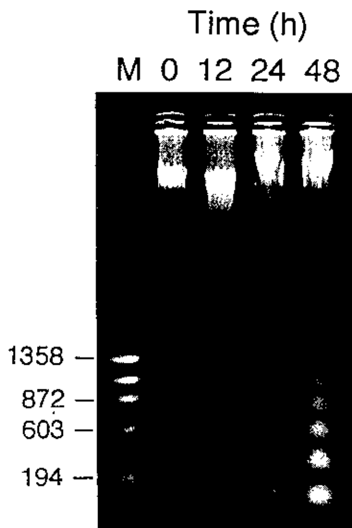


Fig. 2. Time course of ara-C-induced internucleosomal DNA fragmentation in L1210 cells. Cells were incubated with 1 μg/ml of ara-C for 0, 12, 24 or 48 h. Each DNA was isolated and 1.2% agarose gel electrophoresis was performed. M, molecular weight markers expressed in base pairs.

**Effect of ara-C treatment on $Ca^{2+}$-dependent endonuclease activity in cytoplasm** Activity of cytoplasmic $Ca^{2+}$-dependent endonuclease to digest naked DNA during reaction for 20 min was not changed by ara-C treatment of cells (Fig. 6A), and in the reaction for 60 min, each DNA was equally digested into the smallest size seen in the electrophoretic patterns (data not shown). In contrast, when CEM nuclei were used as target substrates in the assay, $Ca^{2+}$-dependent endonuclease activity to induce internucleosomal fragmentation was clearly enhanced depending on the incubation time with ara-C, and it was inhibited by 50 $\mu M$ $Zn^{2+}$ (Fig. 6B).

DISCUSSION

Cell death induced by anticancer agents had been considered to be passively brought about by cellular damage subsequent to the attack of the agents, each of which has its own targets, mechanisms of action, and primary effects.[30] However, common characteristics have been recognized in the process of cell death caused by a variety of anticancer agents, such as induction of *c-jun* expression which occurred almost immediately after drug treatment,[11, 12, 31, 32] involvement of p53[33] or *bcl-2*[9, 13] in the death response, and easily recognizable

Fig. 3. Autodigestions of nuclei of L1210 cells. Incubation of $10^6$ nuclei in 100 μl of STKM buffer with 1 m$M$ EGTA (lane 1), 0.1, 1.0, 10 m$M$ of $CaCl_2$ (lane 2, lane 3, and lane 4 respectively), or 1 m$M$ $CaCl_2$ and 50 $\mu M$ $ZnCl_2$ (lene 5) was conducted at 37°C for 60 min. Each DNA was isolated and 1.2% agarose gel electrophoresis was performed.

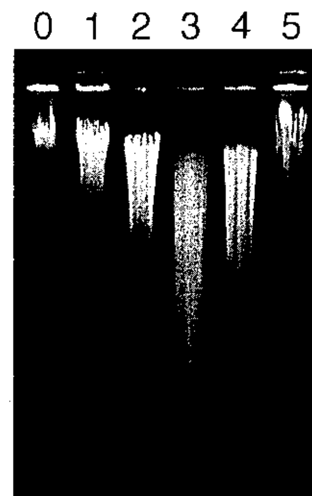


Fig. 4. Detection of endonuclease activity in cytoplasm of L1210 cells. Incubation of $10^6$ nuclei of CEM cells was done in STKM buffer with 1 m$M$ $CaCl_2$ (lane 0) or in a cytoplasmic fraction prepared from $10^6$ L1210 cells with 1 m$M$ EGTA (lane 1), 0.1, 1.0, 10 m$M$ $CaCl_2$ (lane 2, lane 3, and lane 4 respectively), or 1 m$M$ $CaCl_2$ and 50 $\mu M$ $ZnCl_2$ (lane 5) at 37°C for 120 min. Each DNA was isolated and 1.2% agarose gel electrophoresis was performed.

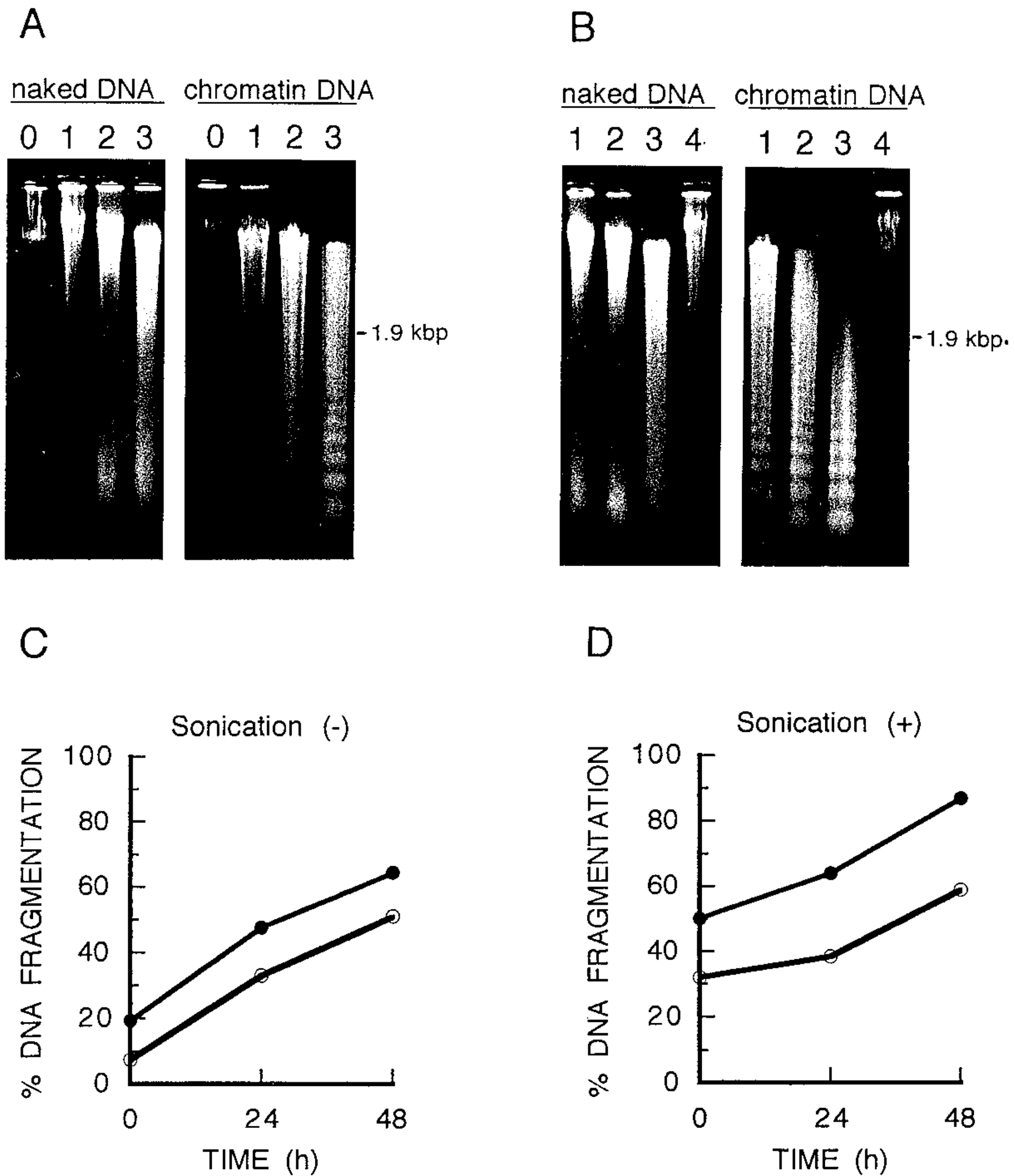


Fig. 5. (A) and (B). Effect of ara-C treatment on $Ca^{2+}$-dependent endonuclease activity in nuclear extracts prepared with (B) or without (A) sonication. The assay mixture contained 40 $\mu$g of proteins of each nuclear extract, 1.6 m*M* $CaCl_2$ and the target substrate (10 $\mu$g of naked DNA or $10^6$ nuclei of CEM cells), and was incubated at 37°C for 120 min. Each DNA was extracted and 1.2% agarose gel electrophoresis was performed. Lane 0 in (A) is the reaction blank (STKM buffer instead of nuclear extracts). Lanes 1–3 in each panel show the reactions in nuclear extracts from cells with ara-C-treatment for 0 h (control), 24 h, or 48 h respectively. Lane 4 in (B) has 50 $\mu M$ $ZnCl_2$ in addition to the same reaction components as in lane 3 of (B). (C) and (D). The percentage of fragmented DNA (smaller than 1.9 kilobase pairs) determined from densitometric scans. Nuclear extracts from cells after ara-C treatment for 0, 24 or 48 h were prepared with (D) or without (C) sonication, and fragmentation of naked DNA (open circles) and chromatin DNA in nuclei (closed circles) was examined. These data are representative of three experiments.

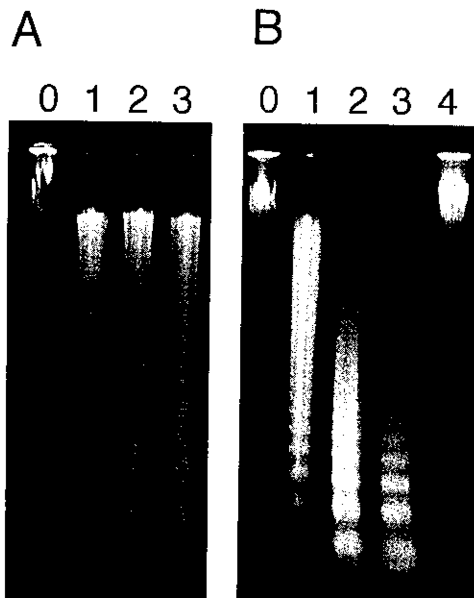


Fig. 6. Effect of ara-C treatment on $Ca^{2+}$-dependent endonuclease activity in cytoplasm. (A) Digestion of naked DNA. (B) Digestion of chromatin DNA in CEM cell nuclei. The assay mixture contained each cytoplasmic fraction prepared from $10^6$ cells as the enzyme source, 1 m*M* $CaCl_2$ and the target substrate, and it was incubated at 37°C for 20 min in (A), and for 120 min in (B). Each DNA was extracted and 1.2% agarose gel electrophoresis was performed. Lane 0 is the reaction blank (STKM buffer). Lanes 1–3 in each panel show reactions in cytoplasmic fractions of cells with ara-C-treatment for 0 h (control), 24 h, or 48 h respectively. Lane 4 in (B) has 50 $\mu M$ $ZnCl_2$ in addition to the same reaction components as in lane 3 of (B).

chromatin condensation and internucleosomal DNA fragmentation.[8–11, 34] Now it is accepted that many anticancer agents induce apoptosis within a therapeutic range of concentrations. Among apoptotic characteristics, internucleosomal DNA fragmentation is considered as one of the most useful hallmarks in spite of recent reports of its absence in apoptosis.[35–37]

Our results show that $Ca^{2+}$-dependent endonuclease may be responsible for the internucleosomal DNA fragmentation seen in the process of ara-C-induced apoptosis in L1210 cells. This cell line was found to possess $Ca^{2+}$-dependent endonuclease activity both in nuclei and in cytoplasm; the activity was inhibited by 50 $\mu M$ $Zn^{2+}$, and was enhanced in the nuclear extract of ara-C-treated cells. In addition, it was more readily extractable in nuclei of ara-C-treated cells than in control nuclei, from which it was not effectively extracted without sonication. While $Ca^{2+}$-dependent endonuclease is normally tightly bound to chromatin,[38, 39] in ara-C-treated cells the activation of this endonuclease might be accompanied with alteration in its conformation and/or in its interaction with chromatin, causing it to be extractable. Hashida *et al.*[39] reported that some DNA-binding proteins stimulated the activity of $Ca^{2+}/Mg^{2+}$-dependent endonuclease and stabilized the enzyme *in vitro*, so the interaction between the enzyme and chromatin may be modified by these proteins, which might be involved in the activation process of the enzyme. Endonucleases of the $Ca^{2+}/Mg^{2+}$-dependent class related to apoptosis were extracted from the nuclei of rat thymocytes[22, 24, 25] and human spleen,[29] and the activity was detected in nuclear extracts of lymphoid cells[23] and small-cell lung cancer cell lines.[40] Gaido and Cidlowski[22] purified a $Ca^{2+}$-dependent endonuclease (NUC18) from both control and glucocorticoid-treated rat thymocytes as a high-molecular-weight complex, and a low-molecular active form was found only in glucocorticoid-treated cells; they mentioned that there was difference in association with DNA between these two types of the nuclease. Nikonova *et al.*[24] reported that $Ca^{2+}/Mn^{2+}$- or $Ca^{2+}/Mg^{2+}$-dependent endonuclease was extracted from thymocyte nuclei of irradiated rats with 0.35 *M* NaCl, but from control nuclei with 0.5 *M* NaCl. It seems clear that there is a relationship between the extractability of the nuclease and its increased activity in apoptotic cells.

Regarding $Ca^{2+}$-dependent endonuclease activity in cytoplasm, ara-C treatment produced different results in the nuclease assays with the two kinds of target substrates. That is to say, unchanged activity to digest naked DNA and enhanced activity to cleave chromatin DNA into oligonucleosomes were observed. Similar results on cytoplasmic nuclease activities were obtained in apoptotic L1210 cells incubated with daunorubicin or camptothecin, but not in cytoplasm of control cells incubated with ara-CTP *in vitro* (data not shown), suggesting that there is no direct action of ara-C. The existence of activating factor(s) of $Ca^{2+}$-dependent endonuclease can explain these results. Although latent $Ca^{2+}$-dependent endonuclease may be constantly expressed in nucleus and cytoplasm, once a cell is switched into apoptosis by ara-C treatment, some factor(s) may be induced or be modulated, either in the nucleus or in the cytoplasm, to activate the endonuclease by releasing it from an inhibitor and/or by changing the interaction of chromatin DNA with the nuclease as mentioned above. In the latter case, it would presumably act on only chromatin DNA not on naked DNA in the endonuclease assay of cytoplasm of ara-C-treated cells. If the putative activating factor(s) is associated with a rigid structure of the nucleus as an inactive form, this factor(s) itself may be released during apoptosis and leak out into the cytoplasm. If such a factor(s) is induced or activated in the cytoplasm, it may be transferred to the nucleus and act

on a substrate there. Recently it has been reported that protease inhibitors blocked internucleosomal DNA degradation not only in apoptosis of thymocytes or HL-60 cells,[41,42] but also in autodigestion of liver nuclei with $Ca^{2+}$ and $Mg^{2+}$,[42] suggesting that internucleosomal DNA fragmentation is dependent upon proteolysis. A protease or its activator might be the putative activating factor(s) in our study. There have been a few reports about the induction of such activity by antineoplastic agents.[27]

In conclusion, enhancement and activation of $Ca^{2+}$-dependent endonuclease, constitutively expressed in a latent form in both nuclei and cytoplasm of L1210 cells, may be responsible for internucleosomal DNA fragmentation during apoptosis induced by ara-C treatment, and some factor(s) is expected to be involved in the activation process of the enzyme. A better understanding of the biochemical basis of drug-induced apoptosis could be valuable in chemotherapy.

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REFERENCES

1) Schrecker, A. W. and Urshel, M. J. Metabolism of 1-β-D-arabinofuranosylcytosine in leukemia L1210: studies with intact cells. *Cancer Res.*, **28**, 793–801 (1968).
2) Richel, D. J., Colly, L. P., Arentsen-Honders, M. W., Starrenburg, C. W. J. and Willemze, R. Deoxycytidine kinase, thymidine kinase and cytidine diaminase and the formation of ara-CTP in leukemic cells in different phases of the cell cycle. *Leuk. Res.*, **14**, 363–369 (1990).
3) Major, P. P., Egan, E. M., Beardsley, G. P., Minden, M. D. and Kufe, D. W. Lethality of human myeloblasts correlates with the incorporation of arabinofuranosylcytosine into DNA. *Proc. Natl. Acad. Sci. USA*, **78**, 3235–3239 (1981).
4) Takauji, R., Tohyama, K., Ueda, T. and Nakamura, T. Enhancement of cytosine arabinoside cytotoxicity by granulocyte/macrophage colony-stimulating factor and granulocyte colony-stimulating factor in a human myeloblastic leukemia cell line. *Jpn. J. Cancer Res.*, **84**, 445–450 (1993).
5) Kufe, D. W., Munroe, D., Herrick, D., Egan, E. and Spriggs, D. Effects of 1-β-D-arabinofuranosylcytosine incorporation on eukaryotic DNA template function. *Mol. Pharmacol.*, **26**, 128–134 (1984).
6) Major, P. P., Egan, E. M., Herrick, D. J. and Kufe, D. W. Effect of ara-C incorporation on deoxyribonucleic acid synthesis in cells. *Biochem. Pharmacol.*, **31**, 2937–2940 (1982).
7) Ohno, Y., Spriggs, D., Matsukage, A., Ohno, T. and Kufe, D. Effects of 1-β-D-arabinofuranosylcytosine incorporation on elongation of specific DNA sequences by DNA polymerase β. *Cancer Res.*, **48**, 1494–1498 (1988).
8) Kaufmann, S. H. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, campothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res.*, **49**, 5870–5878 (1989).
9) Sen, S. and D'Incalci, M. Apoptosis: biochemical events and relevance to cancer chemotherapy. *FEBS Lett.*, **307**, 122–127 (1992).
10) Yoshida, A., Ueda, T., Takauji, R., Liu, Y.-P., Fukushima, T., Inuzuka, M. and Nakamura, T. Role of calcium ion in induction of apoptosis by etoposide in human leukemia HL-60 cells. *Biochem. Biophys. Res. Commun.*, **196**, 927–934 (1993).
11) Gunji, H., Kharbanda, S. and Kufe, D. Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-arabinofuranosylcytosine. *Cancer Res.*, **51**, 741–743 (1991).
12) Bahlla, K., Tang, C., Ibrado, A. M.,Grant, S., Tourkina, E., Holladay, C., Hughes, M., Mahoney, M. E. and Huang, Y. Granulocyte-macrophage colony-stimulating factor/interleukin-3 fusion protein (pIXY321) enhances high-dose ara-C-induced programmed cell death or apoptosis in human myeloid leukemia cells. *Blood*, **80**, 2883–2890 (1992).
13) Miyashita, T. and Reed, J. C. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood*, **81**, 151–157 (1993).
14) Wyllie, A. H. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*, **284**, 555–556 (1980).
15) Arends, M. J., Morris, R. G. and Wyllie, A. H. Apoptosis: the role of the endonuclease. *Am. J. Pathol.*, **136**, 593–608 (1990).
16) Kyprianou, N., English, H. U. and Issacs, J. T. Activation of a $Ca^{2+}$-$Mg^{2+}$-dependent endonuclease as an early event in castration-induced prostatic cell death. *Prostate*, **13**, 103–117 (1988).
17) Peitsch, M. C., Polzar, B., Stephan, H., Crompton, T., MacDonald, H. R., Mannherz, H. G. and Tschopp, J. Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). *EMBO J.*, **12**, 371–377 (1993).
18) Barry, M. A. and Eastman, A. Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Arch. Biochem. Biophys.*, **300**, 440–450 (1993).
19) Alnemri, E. S. and Litwack, G. Activation of internucleosomal DNA cleavage in human CEM lymphocytes by glucocorticoid and novobiocin. *J. Biol. Chem.*, **265**, 17323–17333 (1990).
20) Cohen, J. J. and Duke, R. C. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.*, **132**, 38–42 (1984).
21) McConkey, D. J., Hartzell, P., Nicotera, P. and Orrenius,

S. Calcium-activated DNA fragmentation kills immature thymocytes. *FASEB J.*, **3**, 1843–1849 (1989).

22) Gaido, M. L. and Cidlowski, J. A. Identification, purification, and characterization of a calcium-dependent endonuclease (NUC18) from apoptotic rat thymocytes. *J. Biol. Chem.*, **266**, 18580–18585 (1991).

23) Caron-Leslie, L.-A. M., Schwartzman, R. A., Gaido, M. L., Compton, M. M. and Cidlowski, J. A. Identification and characterization of glucocorticoid-regulated nuclease(s) in lymphoid cells undergoing apoptosis. *J. Steroid Biochem. Mol. Biol.*, **40**, 661–671 (1991).

24) Nikonova, L. V., Beletsky, I. P. and Umansky, S. R. Properties of some nuclear nucleases of rat thymocytes and their changes in radiation-induced apoptosis. *Eur. J. Biochem.*, **215**, 893–901 (1993).

25) Tanuma, S. and Shiokawa, D. Multiple forms of nuclear deoxyribonuclease in rat thymocytes. *Biochem. Biophys. Res. Commun.*, **203**, 789–797 (1994).

26) Takano, Y. S., Harmon, B. V. and Kerr, J. F. R. Apoptosis induced by mild hyperthermia in human and murine tumour cell lines: a study using electron microscopy and DNA gel electrophoresis. *J. Pathol.*, **163**, 329–336 (1991).

27) Bertrand, R., Solary, E., O'Connor, P., Kohn, K. W. and Pommier, Y. Induction of a common pathway of apoptosis by staurosporine. *Exp. Cell Res.*, **211**, 314–321 (1994).

28) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265 (1951).

29) Ribeiro, J. M. and Carson, D. A. $Ca^{2+}/Mg^{2+}$-dependent endonuclease from human spleen: purification, properties, and role in apoptosis. *Biochemistry*, **32**, 9129–9136 (1993).

30) Nakamura, T. Action mechanism of antileukemic agents with special reference to nucleic acid metabolism of leukemic cells. *Acta Haematol. Jpn.*, **45**, 1203–1218 (1982).

31) Kharbanda, S., Gunji, H. and Kufe, D. Activation of the c-*jun* protooncogene in human myeloid leukemia cells treated with etoposide. *Am. Soc. Pharmacol. Exp. Ther.*, **39**, 697–701 (1991).

32) Kharbanda, S., Rubin, E., Gunji, H., Hinz, H., Giovanella, B., Pantazis, P. and Kufe, D. Camptothecin and its derivatives induce expression of the c-*jun* protooncogene in human myeloid leukemia cells. *Cancer Res.*, **51**, 6636–6642 (1991).

33) Lowe, S. W., Ruley, H. E., Jacks, T. and Housman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*, **74**, 957–967 (1993).

34) Kubota, M. Generation of DNA damage by anti-neoplastic agents. *Anti-Cancer Drugs*, **2**, 531–541 (1991).

35) Ucker, D. S., Obermiller, P. S., Eckhart, W., Apgar, J. R., Berger, N. A. and Meyers, J. Genome digestion is a dispensable consequence of physiological cell death mediated by cytotoxic T lymphocytes. *Mol. Cell. Biol.*, **12**, 3060–3069 (1992).

36) Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R. and Sikorska, M. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.*, **12**, 3679–3684 (1993).

37) Oberhammer, F., Bursch, W., Tiefenbacher, R., Fröschl, G., Pavelka, M., Purchio, T. and Schulte-Hermann, R. Apoptosis is induced by transforming growth factor-$\beta 1$ within 5 hours in regressing liver without significant fragmentation of the DNA. *Hepatology*, **18**, 1238–1246 (1993).

38) Nakamura, M., Sakaki, Y., Watanabe, N. and Takagi, Y. Purification and characterization of the $Ca^{2+}$ plus $Mg^{2+}$-dependent endodeoxyribonuclease from calf thymus chromatin. *J. Biochem.*, **89**, 143–152 (1981).

39) Hashida, T., Tanaka, Y., Matsunami, N., Yoshihara, K., Kamiya, T., Tanigawa, Y. and Koide, S. S. Purification and properties of bull seminal plasma $Ca^{2+}$, $Mg^{2+}$-dependent endonuclease. *J. Biol. Chem.*, **257**, 13114–13119 (1982).

40) Okamoto-Kubo, S., Nishio, K., Heike, Y., Yoshida, M., Ohmori, T. and Saijo, N. Apoptosis induced by etoposide in small-cell lung cancer cell lines. *Cancer Chemother. Pharmacol.*, **33**, 385–390 (1994).

41) Bruno, S., Bino, G. D., Lassota, P., Giaretti, W. and Darzynkiewicz, Z. Inhibitors of proteases prevent endonucleolysis accompanying apoptotic death of HL-60 leukemic cells and normal thymocytes. *Leukemia*, **6**, 1113–1120 (1992).

42) Walker, P. R., Weaver, V. M., Lach, B., LeBlanc, J. and Sikorska, M. Endonuclease activities associated with high molecular weight and internucleosomal DNA fragmentation in apoptosis. *Exp. Cell Res.*, **213**, 100–106 (1994).