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Properties of DNA fragmentation activity generated by ATP depletion

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Abstract

Internucleosomal DNA fragmentation is generally perceived as one of the characteristic features of apoptosis, most of which are driven by caspase activation dependent upon ATP. On the other hand, ATP depletion has been reported to induce apoptosis accompanying DNA fragmentation. To address this apparent paradox, we analyzed the DNA-fragmenting activity generated in ATP-depleted cells. In HL-60 promyelocytic leukemia cells cultured in glucose-free medium with oligomycin, internucleosomal DNA fragmentation occurred as an early event. The DNA fragmentation was blocked by serine protease inhibitors but not by caspase inhibitors. Consistently, ICAD/DFF45 could not inhibit the DNA-fragmenting activity of the ATP-depleted cytosol in a cell-free system. When ATP was supplied to the cell-free assay, 80% of the DNA-fragmenting activity was lost. The reduced activity was then restored by proteasome inhibitors, suggesting a role of proteasome to protect from a cellular insult derived from ATP-depletion. *Cell Death and Differentiation* (2000) **7,** 477–484.

Keywords: DNA fragmentation; endonuclease; serine protease; proteasome; ATP; caspase

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; DCI, 3,4-dichloroisocoumarin; DEVD, N-acetyl-Asp-Glu-Val-Asp-aldehyde; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; LLnL, N-acetyl-leucinyl-leucinyl-norleucinal; TLCK, N-tosyl-L-lysyl chloromethyl ketone; TPCK, tosyl-L-phenylalanyl chloromethyl ketone; VAD, carbobenzoxyl-Val-Ala-Asp-fluoromethyl ketone

Introduction

In multicellular organisms, a wide variety of stimuli induce organized cell death called apoptosis. The apoptotic process is characterized by an amplifying proteolytic cascade in which upstream (initiator) caspase mediate the activation of downstream (effector) caspases. Mitochondria intervene between these two classes of caspase, and play a decisive role in the death pathway by releasing apoptogenic molecules including cytochrome c.[1–7] Released cytochrome c in the cytosol forms a complex called apoptosome with Apaf-1 to catalyze the activation of caspase 9 in an ATP-dependent manner.[8–14] In functional apoptosome, hydrolysis of the high-energy bond of dATP/ATP is required for sequential conformational changes of Apaf-1 in the course of binding to cytochrome c and procaspase-9, resulting in recruitment of the substrates of caspase-9, procaspase-3 and possibly procaspase-7.[9,12,13] dATP/ATP hydrolytic energy is also used for overcoming inhibition of caspase activation by an anti-apoptotic protein, Bcl-2.[15] Also, in experiments using whole cells, ATP was required for the activation of caspase-3 in response to many apoptotic stimuli.[16,17] Activated caspases can initiate cleavage of other cellular substrates, producing the features of apoptosis characterized morphologically by condensation and fragmentation of nuclei and cells and biochemically by fragmentation of chromosomal DNA into nucleosomal units.[18] These studies substantiated the requirement of ATP for caspase activation in the apoptotic process.

In several studies, energy deficiency or ATP depletion induced apoptosis rather than necrosis.[19–24] For example, inhibition of glucose transport by cytochalasin B induced apoptosis in an interleukin 3 (IL-3)-dependent mast cell line, IC.DP cells.[20] Depletion of intracellular ATP by uncoupling the mitochondrial ATPase induced apoptotic cell death displaying DNA fragmentation in IL-3-dependent pro-B line cells.[19] More recently, energy deficiency due to hypoxia and hypoglycemia has been shown to cause apoptosis, which is possibly mediated by expression of hypoxia-inducible factor 1.[21] In light of the implication of ATP in apoptosome as described above, however, it seems unlikely that caspases are activated in ATP-depleted cells.

In the present study, promyelocytic leukemia HL-60 cells were cultured under ATP-depleted conditions. We focused on internucleosomal DNA fragmentation, and analyzed the properties of the molecular pathway, including proteases, leading to DNA fragmentation in a cell-free system. This study demonstrated that the process involves serine protease and that the downstream endonuclease is distinct from CAD/DFF40. Proteasome is also implicated in a possible cellular mechanism against this pathological event.

Results

DNA fragmentation induced by ATP depletion

Cellular ATP level decreases under glucose-free culture, and an inhibitor of F_0 unit of mitochondrial ATP synthase, oligomycin, promotes ATP depletion. In HL-60 cells cultured in glucose-free medium with 20 μM oligomycin, the ATP level

rapidly declined to less than 10% of the initial level of 2 mM in 1.5 h (Figure 1A). Agarose gel electrophoresis of the DNA extracted from the ATP-depleted cells revealed internucleosomal fragmentation, which was detected after 1 h of incubation and became obvious after 2 h (Figure 1B). Incubation over several hours induced non-segmental cleavage, yielding a smear pattern (data not shown), indicating that the DNA fragmentation observed herein was

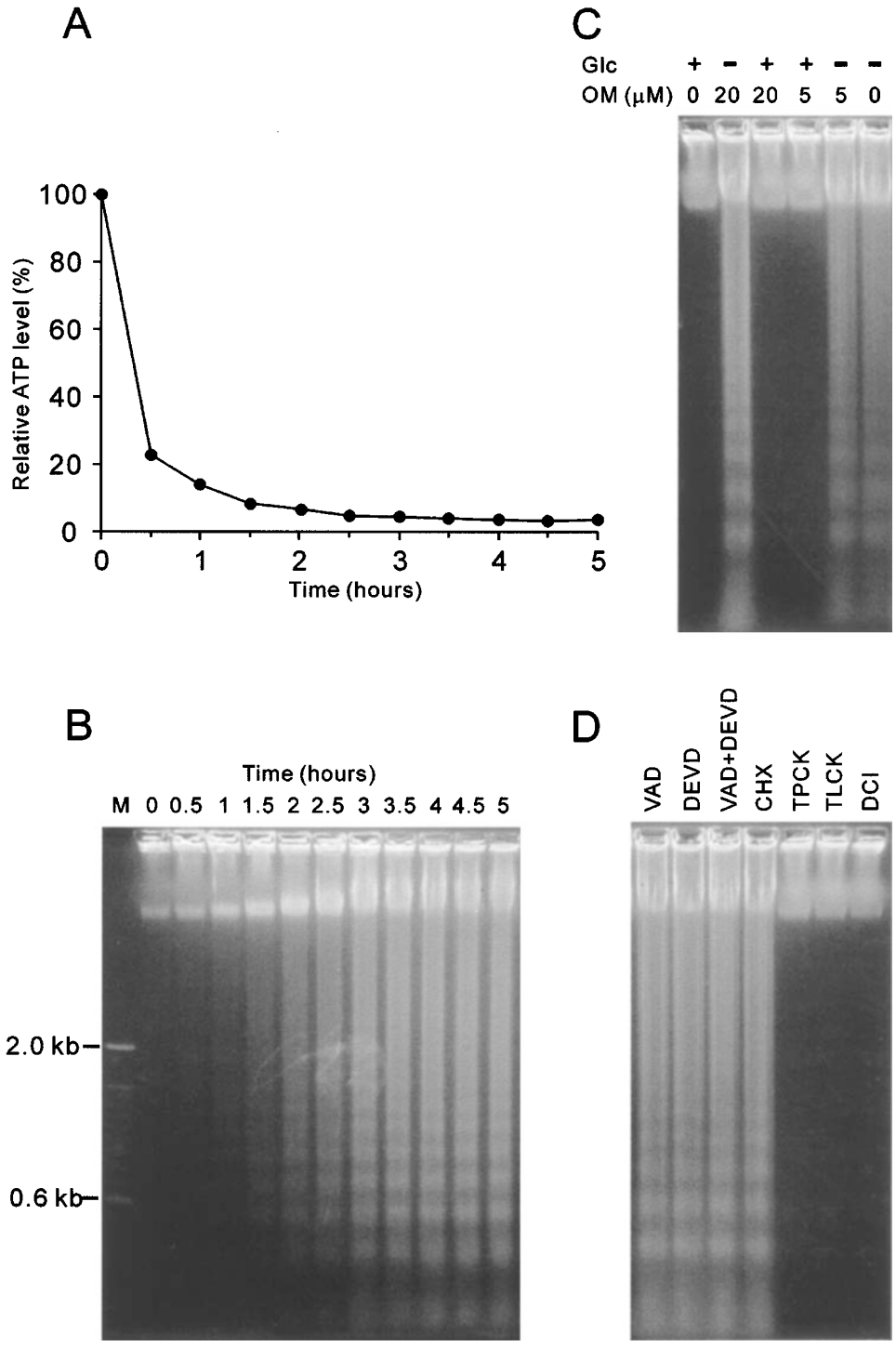

Figure 1 ATP depletion and internucleosomal DNA fragmentation. HL-60 cells were cultured in glucose-free medium with oligomycin. (**A**) Time-course of intracellular ATP level was determined by luciferin-luciferase method. (**B**) DNA fragmentation was analyzed by electrophoresis on 1.2% agarose gel followed by ethidium bromide staining. Each lane contains DNA from 1×10^7 treated or untreated cells. (**C**) Cells were cultured for 4 h under the indicated conditions. (Glc: glucose; OM: oligomycin) (**D**) Effect of protease inhibitors. Cells were cultured in glucose-free medium with 20 μM oligomycin for 4 h in the presence of 100 μM reagents. M, DNA size marker

an early phase of cell alterations under ATP depletion. DNA fragmentation was not observed in the presence of 11 mM glucose in the culture medium even if mitochondrial ATP generation was inhibited by oligomycin (Figure 1C). On the other hand, deprivation of glucose, without oligomycin, induced weak but apparent activity of DNA fragmentation.

Effects of protease inhibitors on the DNA fragmentation activity

Activity that induces internucleosomal fragmentation in response to various stimulation has been identified in different cells, and is blocked by inhibitors of caspases and/or other proteases.[26–33] In order to examine the possible involvement of proteases in inducing fragmentation, cell-permeable inhibitors were added to the culture medium of HL-60 cells. As shown in Figure 1D, a broad spectrum inhibitor of caspases, VAD, as well as an inhibitor, DEVD, specific to caspases-3 and 7 did not affect DNA fragmentation. In contrast, serine protease inhibitors, TPCK, TLCK and DCI, blocked the activity. The DNA fragmentation induced by ATP depletion did not require de novo synthesis of proteins, since DNA fragmentation was not affected by cycloheximide (Figure 1D).

Cell-free assay of DNA fragmentation

DNA fragmentation activity in the cell lysate was examined in a cell-free system. HL-60 cells were cultured in glucose-free medium containing 20 μM oligomycin for 4 h, and the cytosolic fraction was prepared from the cells and incubated with intact nuclei prepared from untreated cells. The activity was measured by ELISA of the fragmented DNA. DNA fragmentation was detectable with an up to 32-fold dilution of cytosol in this system (Figure 2A), and even an eightfold dilution of cytosol exhibited marked fragmentation after incubation for at least 10 min (Figure 2B). Dose-dependent inhibition of DNA fragmentation by TPCK was also demonstrated in the cell-free system (Figure 2C).

DNA fragmentation activity induced by ATP depletion was independent of CAD/DFF40

CAD/DFF40 is a well-characterized endonuclease responsible for the DNA fragmentation downstream of the caspase pathway. It is activated in the cytosol by the cleavage of an inhibitory molecule, ICAD/DFF45, that binds and stabilizes CAD/DFF40. Exogenous ICAD/DFF45 prevents CAD/DFF40 in a cell-free system.[25,34] As caspase inhibitors could not block DNA fragmentation in ATP-depleted cells (Figure 1), CAD/DFF40 was presumably not involved in the execution of observed DNA cleavage. To confirm this, we examined the effect of ICAD/DFF45 on DNA fragmentation. DNA fragmentation induced by FAS ligation or etoposide was abrogated by recombinant ICAD/DFF45 in a dose-dependent manner in Jurkat cells (Figure 3A). In contrast, the activity in ATP-depleted cytosol was resistant to ICAD/DFF45 in Jurkat cells and HL-60 (Figure 3A,B).

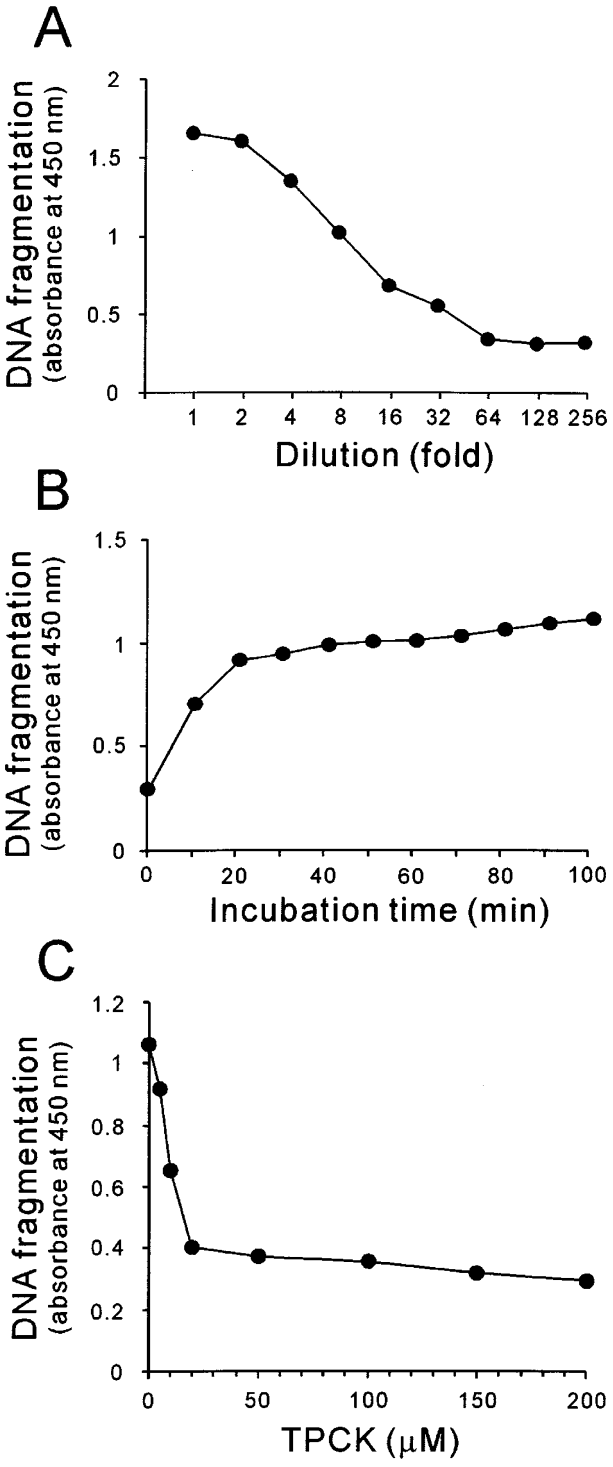

Figure 2 Cell-free assay of DNA-fragmenting activity of ATP-depleted cytosol. (**A**) ATP-depleted cytosol (2.5 mg protein) was serially diluted with nucleus buffer and incubated with 1×10^7 BrdU-labeled nuclei in 1 ml reaction volume at 37°C for 1 h. Fragmented DNA was measured by ELISA. (**B**) Time-course of DNA fragmentation generated by eightfold dilution of cytosol. (**C**) Effect of TPCK on the DNA-fragmenting activity of eightfold diluted cytosol

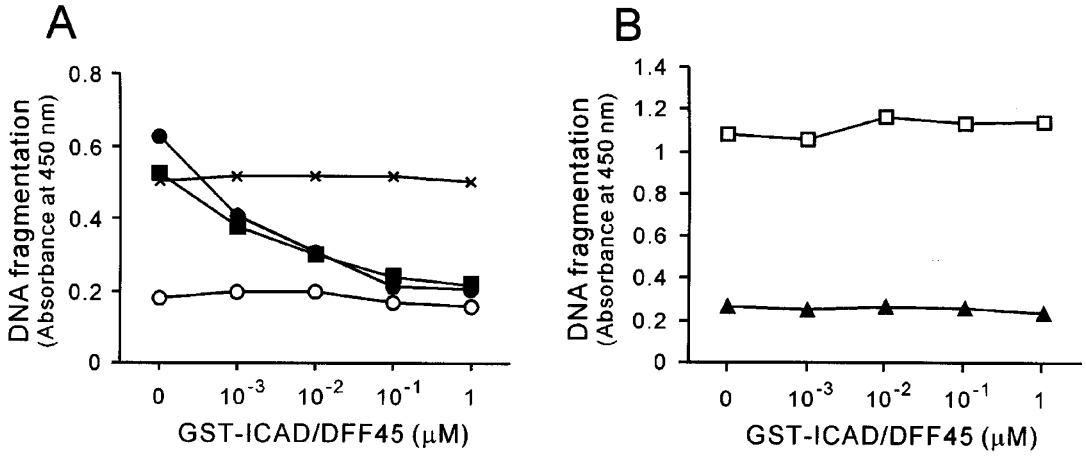

Figure 3 Effect of exogenous ICAD/DFF45 on DNA-fragmenting activity of ATP-depleted cytosol. (**A**) Jurkat cells were incubated with anti-FAS antibody (closed circles), control IgM (open circles) or etoposide (closed squares), or cultured in glucose-free medium with 20 μM oligomycin for 6 h (crosses). Cytosol (1 mg/ml) prepared from the cells was incubated with 10 μM DEVD followed by various concentrations of GST-ICAD/DFF45 in 20 μl reaction volume, each at 4°C for 30 min. The mixture was then incubated with 5×10^5 BrdU-labeled nuclei at 30°C for 2 h, and subjected to ELISA of fragmented DNA. (**B**) HL-60 cells were cultured under normal conditions (closed triangles) or in glucose-free medium with 20 μM oligomycin for 4 h (open squares). Cytosol of the cells was prepared and subjected to the same treatment as Jurkat cells except for incubation with BrdU-labeled nuclei at 37°C for 1 h before ELISA

Effect of ATP supply to ATP-depleted cytosol

To explore the ATP-dependent process that may relate to or antagonize DNA fragmentation, ATP-depleted cytosol at an eightfold dilution was incubated with 1 mM ATP prior to addition to intact nuclei. Approximately 80% of the DNA-cleaving activity was lost by this ATP supply (Figure 4), and the suppression reached a plateau after 40 min of preincubation with ATP addition (data not shown). Although it could be speculated that ATP-dependent DNA fragmentation, possibly mediated by caspases, was activated and contributed to the remaining 20% activity, caspase inhibitors did not change the fragmentation. On the other hand, TPCK eliminated the remaining activity (Figure 4).

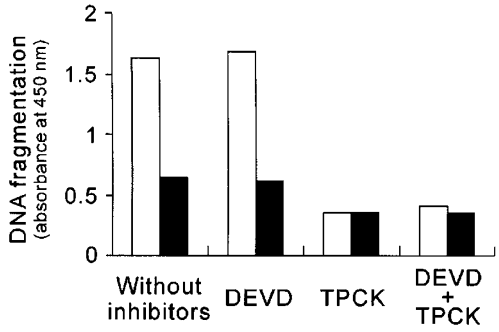

Figure 4 Effect of ATP addition to ATP-depleted cytosol. Cytosol (2.5 mg/ml) of ATP-depleted HL-60 cells was incubated with 0.2 mM DEVD, 0.2 mM TPCK or both at 37°C for 10 min, and then with (filled bars) or without 1 mM ATP (open bars) at 37°C for 1 h. Subsequently, the cytosol was incubated with 1×10^7 BrdU-labeled nuclei at 37°C for 1 h before ELISA of DNA-fragmenting activity

Involvement of proteasome in ATP-dependent suppression of DNA fragmentation

Since proteasome is the ATP-dependent machinery for protein degradation, it might contribute to the suppression of DNA fragmentation by ATP addition. To clarify this, we tested proteasome inhibitors, LLnL and lactacystin, in a cell-free assay. No detectable activity of DNA fragmentation was induced by up to 100 μM LLnL or lactacystin (Figure 5A), although induction of apoptosis in HL-60 cells by 50 μM LLnL has been reported previously.[33] On the contrary, proteasome inhibitors reversed the ATP-dependent suppression of DNA fragmentation in a dose-dependent manner (Figure 5A,B).

These results were further confirmed by agarose gel electrophoresis, which showed the fragmentation detected by ELISA to be internucleosomal in nature. As shown in Figure 5C, ATP addition prevented the DNA cleaving activity of ATP-depleted cytosol (Figure 5C, lane 2), and the effect was abrogated by LLnL (Figure 5C, lane 3) or lactacystin (data not shown). Although caspases are known to be degraded by proteasome, the restored activity was not caspase-dependent (Figure 5C, lanes 4 and 5). In contrast, TPCK completely inhibited the activity of ATP-depleted cytosol even in the presence of proteasome inhibitors (data not shown). Thus, these results indicate that the serine protease and/or its downstream factors responsible for DNA fragmentation can be degraded by proteasome.

Addition of ATP to normal cytosol in the presence of proteasome inhibitors slightly generated fragmented DNA (Figure 5A,B). This minimal cleavage was unrelated to proteasome inhibitors, since ATP addition alone could induce DNA fragmentation (Figure 5C, lane 10). This activity was abrogated by caspase inhibitors, suggesting slight caspase activation during sample preparation (Figure 5C, lanes 12 and 13).

Finally, the effect of proteasome inhibitors on cells cultured under normal conditions was examined. DNA fragmentation occurred in HL-60 cells incubated with LLnL

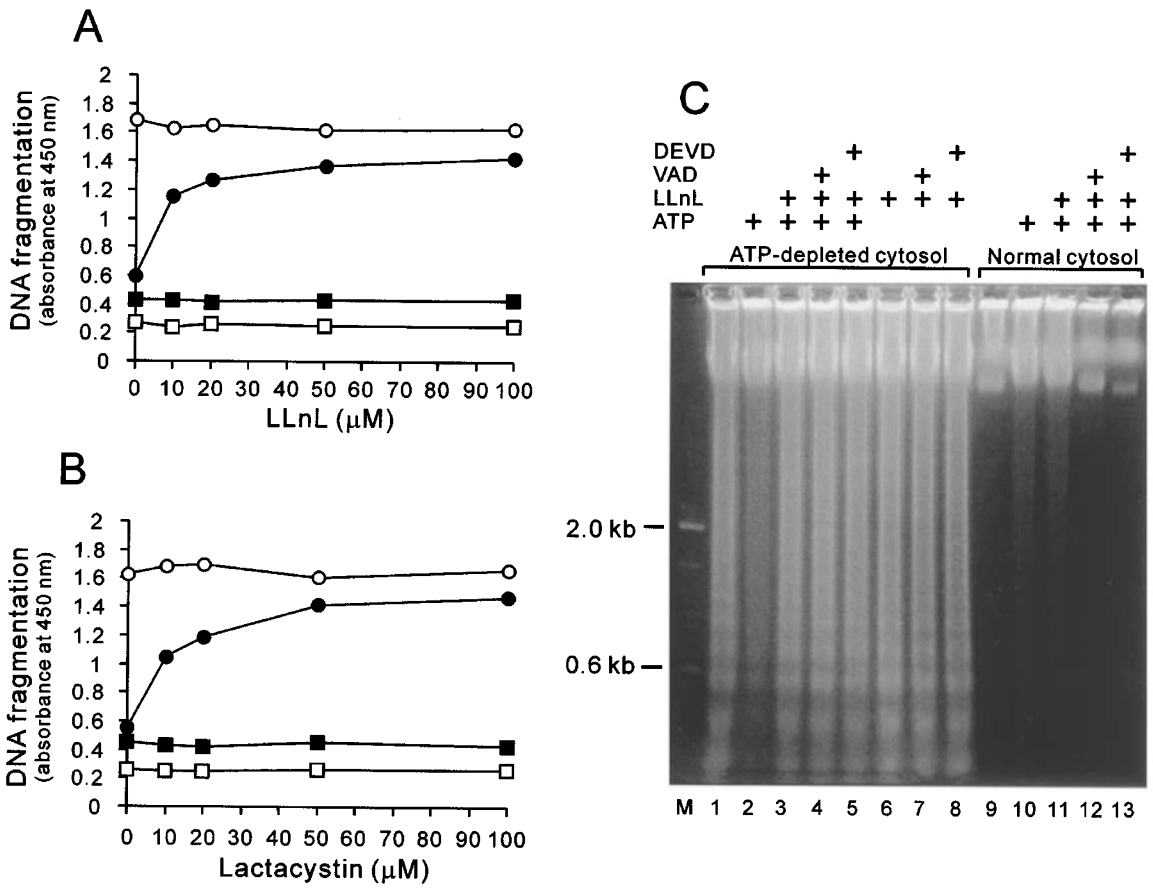

Figure 5 Effect of proteasome inhibitors on ATP-dependent suppression of DNA fragmentation. ATP-depleted cytosol (2.5 mg/ml) was incubated at 37°C for 10 min with various concentrations of LLnL (**A**) or lactacystin (**B**) in the presence of ATP, and DNA fragmenting activity was measured by ELISA. Closed circles, ATP-depleted cytosol with 1 mM ATP; open circles, ATP-depleted cytosol without ATP; closed squares, normal cytosol with 1 mM ATP; open squares, normal cytosol without ATP. (**C**) ATP-depleted cytosol (lanes 1–8) or normal cytosol (lanes 9–13) was incubated consecutively with 0.2 mM VAD or DEVD, 0.1 mM LLnL and 1 mM ATP, each at 37°C for 10 min. After incubation, the cytosol was incubated with intact nuclei, and DNA was extracted and analyzed by agarose gel electrophoresis

or lactacystin, and was not prevented by TPCK or caspase inhibitors (data not shown).

Discussion

Internucleosomal DNA cleavage occurred in the early phase of cellular alterations of HL-60 cells when cultured in glucose-free medium. This phenomenon was also observed in other cells including Jurkat T cells (Figure 3) and endothelial cells (data not shown). DNA fragmentation was augmented by additional treatment with oligomycin, which inhibits mitochondrial ATP generation and decreases intracellular ATP level to below 10%. In contrast, DNA fragmentation was not observed under glucose-supplied conditions, even in the presence of oligomycin, suggesting that glycolysis is sufficient to prevent this kind of DNA fragmentation.

In the present study, HL-60 cells in glucose-free culture did not display cell shrinkage, membrane blebbing or any other morphological features of apoptosis, probably because these alterations are brought about through ATP-dependent mechanisms.[16,17,35,36] This is consistent with the earlier studies of ATP depletion by Nicotera and Tsujimoto,[37–39] but apparent contradiction is the detection of internucleosomal DNA fragmentation, which may be dependent on the sensitivity of gel electrophoresis (see Materials and Methods). The DNA fragmentation induced by ATP depletion is detectable before the 'smear' pattern is evident, and it does not require caspase activation.

In a cell-free system, the activity responsible for internucleosomal endonucleolysis was demonstrated in the cytosol of ATP-depleted cells. DNA fragmentation activity was abolished by inhibition of serine proteases, but not of caspases. These results indicate that serine protease(s) play a key role upstream of endonuclease in DNA cleavage, and that caspases do not participate in the pathway. In addition, CAD/DFF40 is not the final executor endonuclease, because ICAD/DFF45 could not prevent the activity. To date, several studies have suggested involvement of serine protease(s) in the pathway leading to internucleosomal DNA cleavage in different cells and in response to various stimuli. However, the features of these activities seem to be distinct from that observed in the present study, especially relating to caspase-dependency. For example, in HL-60 cells treated with a topoisomerase inhibitor, camptothecin or etoposide, proteo-

lytic activity that is inhibited by a serine protease inhibitor and mediates endonuclease activation[29–31] has been reported, but this serine protease requires upstream caspase for activation. Wright *et al*. purified a 24-kDa serine protease that activates DNA fragmentation in UV light-treated U937 histiocytic lymphoma cells.[26,40–42] This protease was resistant to TLCK and was not activated in ATP-depleted HL-60 cells. Granzyme is a well-known serine protease that can activate apoptotic DNA fragmentation,[43–46] which is mediated by downstream caspase activation. In MOe7 myeloid progenitor cells, caspase-independent and TPCK-sensitive activity is generated in response to cytokine withdrawal, but it was resistant to TLCK.[47] The only exception is a serine protease activated by camptothecin, but not by etoposide, in a human hepatocellular carcinoma cell line Hep-3B, where the apoptotic pathway operates without caspase activation.[32] Isolation of the DNA fragmentation activity from the ATP-depleted cytosol is under way to clarify the relationship between these activities.

In the apoptotic pathway driven by caspase to internucleosomal DNA fragmentation, caspase-3 cleaves ICAD/DFF45 and activates CAD/DFF40 endonuclease.[25,34–36,48,49] In the present study, ICAD/DFF45 blocked the DNA fragmentation induced by etoposide as well as FAS ligation in Jurkat cells (Figure 3). This was an unexpected result because etoposide-induced DNA fragmentation was blocked by TPCK in an earlier study.[29,50] An additional pathway involving serine protease(s) may be present upstream of CAD/DFF40 activation.

Proteasome is a multicatalytic protease complex responsible for most non-lysosomal interactions.[51–53] Recent studies have revealed the involvement of proteasome in the apoptotic process by using proteasome inhibitors. The action is contradictory, though many studies have used proteasome inhibitors to demonstrate the induction of apoptosis. In the present study, the DNA-cleaving activity generated in ATP-depleted cytosol was decreased by incubation with ATP, and proteasome inhibitors restored the activity under this ATP-supplied conditions. These results suggest a function of proteasome to eliminate the incidental appearance of DNA-fragmenting activity and favor cells with a survival advantage in adverse environments, e.g. hypoxia and ischemia.

The proteasome inhibitor-mediated restoration of DNA fragmenting activity was suppressed by TPCK in the cell-free system. On the contrary, apoptotic DNA fragmentation was observed in HL-60 cells cultured under normal conditions with proteasome inhibitors, but it was not inhibited by TPCK or by caspase inhibitors. In the latter case where defective degradation of short-lived proteins relevant to cell cycle progression may trigger apoptosis,[33,54–56] serine proteases do not principally participate in this kind of DNA fragmentation pathway as well as caspases.[57]

Materials and Methods

Materials

Caspase inhibitors, carbobenzoxyl-Val-Ala-Asp-fluoromethyl ketone (VAD) and acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD), were from Bachem AG (Bubendorf, Switzerland) and Peptide Institute, Inc. (Osaka, Japan) respectively. Cycloheximide, N-tosyl-L-lysyl chloromethyl ketone (TLCK), tosyl-L-phenylalanyl chloromethyl ketone (TPCK), 3,4-dichloroisocoumarin (DCI), N-acetyl-leucinyl-leucinyl-norleucinal (LLnL) and oligomycin were purchased from Sigma (St. Louis, MO, USA). Lactacystin was from Calbiochem (La Jolla, CA, USA). ATP was from Oriental Yeast (Tokyo, Japan). Monoclonal anti-human Fas (cytocidal IgM clone 7C11) was from Immunotech (Marseille, France). Control IgM was from DAKO (Carpinteria, CA, USA). Etoposide was from Wako (Osaka, Japan). RPMI1640 was from Nikken (Kyoto, Japan). RNase I was purchased from Amersham-Pharmacia Biotech (Bucks, UK) and was prepared for use by boiling for 10 min to remove DNase activity.

Oligomycin, protease inhibitors and proteasome inhibitors were dissolved in dimethylsulfoxide (DMSO), and ATP was dissolved in distilled water.

Cells, culture and ATP depletion

HL-60 promyelocytic leukemia cells and Jurkat T cells were grown at 37°C under 5% CO_2, with humidified air in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. The logarithmically growing cells were used for experiments at a density less than 5×10^5 cells/ml. ATP depletion of the cells was carried out by replacing the medium with glucose-free medium containing oligomycin. Intracellular ATP levels were determined with a kit utilizing the luciferin-luciferase system (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions.

Gel electrophoresis

ATP-depleted cells were washed twice with ice-cold phosphate-buffered saline (PBS), incubated in lysis buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 10 mM EGTA, 0.5% SDS, 0.5 mg/ml RNase) at 37°C for 3 h and treated with 0.5 mg/ml proteinase K at 65°C for 3 h. After extraction with phenol, DNA samples were electrophoresed on 1.2% agarose gels. To secure sufficient sensitivity and resolution, DNA from 1×10^7 cells was loaded on a large gel (15 mm in thickness, 150 mm in length), and stained with 0.5 μg/ml ethidium bromide at 4°C overnight before fluorography.

Cell-free system

In a typical reaction system, 1×10^7 HL-60 cells were used for preparing isolated nuclei and test cytosol. Cells were washed three times with ice-cold PBS, and suspended in 1 ml ice-cold nucleus buffer (0.25 M sucrose, 50 mM HEPES, 50 mM KCl, 1 mM $MgCl_2$, pH 7.5) containing 0.1% NP-40. Following incubation for 10 min on ice, the cells were lyzed by vortex, and centrifuged at 2000 *g* for 5 min at 4°C. The supernatant (2.5 mg protein) was adjusted to 1 ml with nucleus buffer without NP-40 and used as test cytosol for subsequent analysis. The precipitated nuclei were suspended and washed twice with nucleus buffer and stored at −80°C until use. The 1×10^7 nuclei were incubated with the cytosol (2.5 mg protein) in 1 ml reaction volume at 37°C for 1 h in the presence of test reagents including protease inhibitors, and DNA fragmentation was analyzed by enzyme-linked immunosorbent assay (ELISA) as described below. For gel electrophoresis of fragmented DNA, the incubation mixture of nuclei and test cytosol was directly dissolved in lysis buffer, and then DNA was extracted for analysis.

ELISA of fragmented DNA

Fragmented DNA was measured using a Cellular DNA Fragmentation ELISA kit (Boehringer Mannheim GmbH, Mannheim, Germany), based on immunological measurement of 5-bromo-2′-deoxyuridine (BrdU)-labeled DNA as described previously.[58] Briefly, HL-60 cells were cultured for 16–20 h in the presence of 10 μM BrdU, and the nuclei were isolated. The 1×10^7 labeled nuclei were incubated with test cytosol (2.5 mg protein) in 1 ml reaction volume, and the mixture was centrifuged at 2000 *g* at 4°C for 5 min. The supernatant was subjected to ELISA of the cleaved DNA that leaked out from the nucleus. Values were expressed as the mean of triplicate measurements.

Recombinant ICAD/DFF45

A full length cDNA of ICAD/DFF45 was obtained by reverse-transcription polymerase chain reaction carried out on RNA from HL-60 cells using primers 5′-GGAGGTGACCGGGGAC-3′ (forward) and 5′-CTATGTGGGATCCTGTCTGGC-3′ (reverse). The amplified cDNA was inserted into pGEX-2T expression vector (Amersham-Pharmacia Biotech), and a glutathione S-transferase fusion protein GST-ICAD/DFF45 was purified by ion-exchange chromatography using a Mono-Q column (Amersham-Pharmacia Biotech).

To examine the effect of ICAD/DFF45 on the DNA-fragmenting activity of HL-60 cells in a cell-free system, the cytosol (2.5 mg protein/ml) from ATP-depleted cells was incubated with 10 μM DEVD to inactivate caspase-3 and then with GST-ICAD/DFF45, each at 4°C for 30 min.[25] Subsequently, the mixture was incubated with 1×10^7 BrdU-labeled nuclei at 37°C for 1 h, followed by ELISA of fragmented DNA.

For Jurkat cells, the effect of ICAD/DFF45 was examined similarly by the method for HL-60 cells described above, except for the cell-free assay in which 5×10^5 BrdU-labeled nuclei and 20 μg of test cytosol were incubated in a 20 μl solution at 30°C for 2 h. Test cytosol was prepared from the cells, which were incubated with 0.1 μg/ml anti-FAS 7C11 antibody or mouse control IgM at 37°C for 18 h, with 0.1 mM etoposide at 37°C for 18 h, or with 20 μM oligomycin in glucose-free medium at 37°C for 6 h beforehand.

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