Activation of Caspase-3 by Lysosomal Cysteine Proteases and Its Role in 2,2′-Azobis-(2-Amidinopropane)Dihydrochloride (AAPH)–Induced Apoptosis in HL-60 Cells[1]

Rumi Ishisaka,* Tomoko Kanno,† Jitsuo Akiyama,‡ Tamotsu Yoshioka,† Kozo Utsumi,† and Toshihiko Utsumi*,[2]

**Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515; †Institute of Medical Science, Kurashiki Medical Center, 250 Bakuro-cho, Kurashiki 710-8522; and ‡Doonan Institute of Medical Science, Hakodate 041-8502*

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We previously reported that in addition to mitochondrial cytochrome *c* dependent activation, lysosomal cysteine proteases were also involved in the activation of caspase-3. In this study, we have separately obtained the lysosomal and mitochondrial caspase-3 activating factors in a crude mitochondrial fraction and characterized their ability to activate pro-caspase-3 in the *in vitro* assay system. When a rat liver crude mitochondrial fraction containing lysosomes (ML) was treated with a low concentration of digitonin, lysosomal factors were selectively released without the release of a mitochondrial factor (cytochrome *c*, Cyt.c). Treatment of ML with Ca^{2+} in the presence of inorganic phosphate (P_i), in contrast, released mitochondrial Cyt.c without the release of lysosomal factors. The obtained lysosomal and mitochondrial factors activated caspase-3 in different manners; caspase-3 activation by lysosomal and mitochondrial factors was specifically suppressed by E-64, a cysteine protease inhibitor, and caspase-9 inhibitor, respectively. Thus, the activation of caspase-3 by lysosomal factors was found to be distinct from the activation by mitochondrial Cyt.c dependent formation of the Apaf-1/caspase-9 complex. To further determine whether or not the activation of caspase-3 by lysosomal cysteine proteases is involved in cellular apoptosis, the effect of E-64-d, a cell-permeable inhibitor of cysteine protease, on 2,2′-azobis-(2-amidinopropane)dihydrochloride (AAPH)–induced apoptosis in HL-60 cells was investigated. As a result, DNA fragmentation induced by AAPH was found to be remarkably (up to 50%) reduced by pretreatment with E-64-d, indicating the participation of lysosomal cysteine proteases in AAPH-induced apoptosis in HL-60 cells.

Key words: AAPH, apoptosis, caspase-3, cathepsin, cytochrome *c*.

Apoptosis, or programmed cell death, is a naturally occurring process of cell suicide that plays a crucial role in the development and maintenance of multicellular organisms (*1*). The biochemical basis for apoptotic cell death is constitutively present in most mammalian cells and can be activated by a wide variety of cellular signals (*2*). Although the number of studies on the mechanism of apoptosis have exponentially increased in the last few years, the precise molecular mechanism leading to apoptosis remains to be elucidated.

Several studies have suggested that cytochrome *c* (Cyt.c) released from mitochondria activates caspase-3 and plays an important role in the initiation of apoptosis (*3*, *4*). In the presence of Cyt.c and dATP, Apaf-1 (CED-4 like protein) interacts with pro-caspase-9 through its NH_2-terminal caspase recruitment domain (CARD), thereby activating caspase-9, which in turn activates caspase-3 (*5*, *6*). Thus, the mitochondrial Cyt.c dependent pathway has been postulated to play a critical role in caspase-3 activation.

We recently reported the activation of caspase-3 by lysosomal cysteine proteases released from a digitonin-treated crude mitochondrial fraction containing lysosomes (ML) (*7*). However, the differences in the mechanisms of activation of caspase-3 by lysosomal and mitochondrial factors were not characterized in detail. In addition, direct evidence indicating the involvement of lysosomal enzymes in the activation of caspase-3 during apoptosis in intact cells has not been reported so far.

In this study, we have separately obtained the lysosomal and mitochondrial caspase-3 activating factors in ML and characterized their ability to activate pro-caspase-3 in the

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[2] To whom correspondence should be addressed. Tel: +81-83-933-5856, Fax: +81-83-933-5820, E-mail: utsumi@agr.yamaguchi-u.ac.jp

Abbreviations: AAPH, 2,2′-azobis-(2-amidinopropane)dihydrochloride; AMC, 7-amino-4-methyl-coumarin; AO, acridine orange; Apaf, apoptotic protease activating factor; caspase, cysteinyl aspartate–specific protease; CsA, cyclospolin A; Cyt.c, cytochrome *c*; DTT, dithiothreitol; ML, crude mitochondrial fraction containing lysosomes; MPT, membrane permeability transition; P_i, inorganic phosphate; S-100, cytosolic fraction of rat liver; PAGE, polyacrylamide gel electrophoresis.

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in vitro assay system. Consequently, the activation of caspase-3 by lysosomal cysteine proteases was found to be distinct from the activation by mitochondrial Cyt.c dependent formation of the Apaf-1/caspase-9 complex. In order to further examine the involvement of lysosomal enzymes in the activation of caspase-3 during apoptosis in intact cells, the role of cysteine proteases in apoptosis in HL-60 cells induced by AAPH, a free radical–generating agent, was investigated. As a result, DNA fragmentation of HL-60 cells induced by AAPH was found to be remarkably (up to 50%) reduced by treatment with E-64-d, a cell-permeable inhibitor of cysteine protease, indicating the involvement of lysosomal cysteine proteases in AAPH-induced apoptosis in HL-60 cells. Thus, direct participation of lysosomal cysteine proteases in apoptosis was successfully demonstrated in both the *in vitro* and *in vivo* assay systems.

MATERIALS AND METHODS

Chemicals—Ac-DEVD-MCA, a fluorogenic tetrapeptide substrate for caspase-3, inhibitors of the caspase family (z-VAD-fmk, Ac-DEVD-CHO, and Ac-LEHD-CHO), and E-64-d were all obtained from the Peptide Institute (Osaka). E-64, anti-Cyt.c antibodies and anti–cathepsin B antibodies were obtained from Sigma (St. Louis, MO, USA), Pharmingen (San Diego, CA, USA), and Calbiochem, Ltd. (San Diego, USA), respectively. All other chemicals were of analytical grade and obtained from Nacalai Tesque (Kyoto).

Preparation of a Crude Mitochondrial Fraction Containing Lysosomes (ML) and S-100 Fractions—After overnight fasting, the liver of a Wistar rat weighing 200 g was homogenized in 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose. The homogenate was centrifuged at 750 $\times g$ for 10 min at 4°C. The supernatant was then collected and centrifuged at 10,000 $\times g$ for 10 min at 4°C to obtain ML. The ML was used after washing twice in the same buffer at 4°C (*8*). A rat liver cytosolic fraction (S-100) was obtained by centrifugation at 105,000 $\times g$ for 60 min at 4°C from a post-nuclear fraction of the liver homogenate in medium A [20 mM HEPES-KOH buffer (pH 7.5) containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT] containing 0.25 M sucrose. Cyt.c in S-100 was removed by immuno-precipitation with anti-Cyt.c antibodies. Cyt.c-free samples were stored at −80°C until use.

Treatment of ML with Digitonin, Triton X-100, or P_i plus Ca^{2+}—ML (1.5 mg protein/ml) in 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M KCl was treated with various concentrations of digitonin or Triton X-100 on ice for 10 min, or with 0.1 mM $CaCl_2$ in the presence of 2 mM P_i for 5 min at 25°C. After centrifugation at 20,000 $\times g$ for 10 min at 4°C, the supernatant was used for the caspase assay or Western blot analysis.

Assay for Caspase-3 Activation—Activation of caspase-3 was investigated using two different assay systems, [A] and [B]. In assay system [A], 80 μl of S-100 (100 μg protein) in medium A was incubated with 10 μl of the supernatant of the ML and 10 μl of 10 mM dATP in a final volume of 100 μl at 37°C for 120 min. In assay system [B], 100 μl of S-100 (100 μg protein) in 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M KCl was incubated with 200 μl of the ML supernatant in a final volume of 300 μl at 37°C for 120 min. After incubation, for both assay systems, caspase activity was measured in 20 mM HEPES buffer (pH 7.5) containing 10 μM Ac-DEVD-MCA, 0.1 M NaCl, and 5 mM DTT at 37°C for 60 min, as previously described (*9*). The activity of caspase-3 was expressed as the amount of Ac-DEVD-MCA cleaved within 1 min per 1 mg S-100 protein.

Gel Electrophoresis and Western Blot Analysis—The samples were dissolved in SDS-sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue] and then boiled at 100°C for 5 min. The samples were then subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon filter (Millipore), and incubated with either anti–cathepsin B (1:250 dilution) or anti-Cyt.c (1:1,000 dilution) antibodies. Subsequently, the filter was incubated with horseradish peroxidase–linked anti–rabbit IgG antibodies (1:4,000 dilution) or anti–mouse IgG monoclonal antibodies (1:2,000 dilution), and then analyzed with an ECL Western blot detection system (Amersham) (*9, 10*).

Cell Culture—Human leukemia HL-60 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C under a 5% CO_2/95% air atmosphere.

DNA Fragmentation Assay—The extent of DNA fragmentation in HL-60 cells was determined by means of a spectrophotometric assay using the diphenylamine method (*11*). After incubation with AAPH or H_2O_2, cells were lysed in 200 μl of lysis buffer [10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.5% Triton X-100] at 4°C for 30 min. The lysate was centrifuged at 13,000 $\times g$ at 4°C for 20 min to separate intact and fragmented chromatin. Both the pellet and supernatant were precipitated with 6% perchloric acid at 4°C for 30 min. The precipitates were sedimented at 13,000 $\times g$ at 4°C for 20 min. The DNA precipitates were heated at 70°C for 20 min in 50 μl of 6% perchloric acid, and then mixed with 100 μl of a diphenylamine solution containing 1.5% sulfuric acid and 0.01% acetaldehyde in acetic acid. After overnight incubation at 30°C in the dark, the absorbance at 600 nm was measured, and the percentage of DNA fragmentation was calculated as the ratio of DNA in the supernatant to total DNA.

Assay for Lysosomal Stability—HL-60 cells with or without treatment with AAPH were assessed for lysosomal stability by the acridine orange (AO) uptake method (*12*). Cells (2×10^6) treated with 0 or 5 mM AAPH for 24 h were fixed in 200 μl of 70% ethanol at 4°C for 4 h, and then resuspended in 40 μl of phosphate buffer saline (PBS), followed by incubation with 0.5 μg/ml AO at 37°C for 15 min. To determine the lysosomal stability, the fluorescence intensity of AO was determined with a FACS Caliber flow cytometer (Becton Dickinson) using CELL Quest software (Becton Dickinson).

RESULTS

Activation of Caspase-3 in Rat Liver Cytosol (S-100) Using the Supernatant of the Digitonin-Treated Crude Mitochondrial Fraction Containing Lysosomes (ML)—As previously observed, when the supernatant of digitonin (30 μM)-treated ML was incubated with rat liver cytosol (S-100) for 120 min at 37°C, remarkable cleavage of DEVD-MCA, a specific substrate for caspase-3, was observed (Fig. 1). This cleavage was almost completely suppressed by either a cysteine protease inhibitor (E-64) or a caspase-3 inhibitor (Ac-DEVD-CHO). The fact that the cleavage of

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DEVD-MCA was not observed in the absence of S-100 indicated that the DEVD-MCA cleavage was not due to direct cleavage by the supernatant of the digitonin (30 μM)-treated ML. These results indicated that some cysteine proteases that activate caspase-3 were released from ML on digitonin treatment.

Low Concentration of Digitonin Specifically Released Lysosomal Enzymes from the Crude Mitochondrial Fraction Containing Lysosomes (ML)—In order to determine whether or not the release of factors from ML with digitonin was specific for lysosomes, the release of cathepsin B, a lysosomal marker enzyme, and mitochondrial Cyt.c was monitored by Western blot analysis. As shown in Fig. 2A, the release of the two factors was strongly affected by the digitonin concentration; only cathepsin B was selectively released from ML at low concentration, whereas both factors were released at high concentration. In contrast, when Triton X-100 was used as the detergent, both factors were equally released as the concentration of Triton X-100 increased (Fig. 2B). Thus, a low concentration of digitonin specifically released lysosomal factors from ML without releasing mitochondrial factors.

Treatment of ML with Ca^{2+} in the Presence of P_i Specifically Released Cyt.c without Releasing Lysosomal Factors—

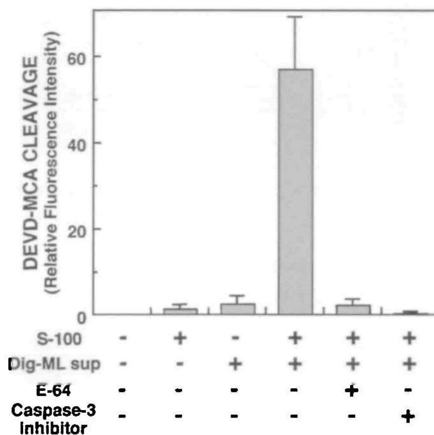

Fig. 1. **Activation of caspase-3 in rat liver cytosol (S-100) by the supernatant of a digitonin-treated crude mitochondrial fraction containing lysosomes (ML).** 200 μl of the supernatant of ML (1.5 mg protein/ml) treated with 30 μM digitonin was incubated with 100 μl of S-100 (1 mg protein/ml) in the presence or absence of 30 μM of E-64 or Ac-DEVD-CHO, and then caspase-3 activity was measured using assay system [B] as described under "MATERIALS AND METHODS." Data are expressed as means ± SD of three independent experiments.

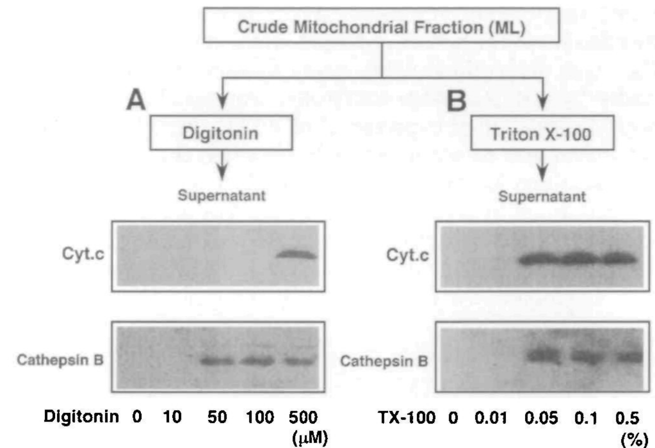

Fig. 2. **Effect of digitonin or Triton X-100 treatment on the release of mitochondrial and lysosomal factors from ML.** ML (1.5 mg protein/ml) was treated with various concentrations of either digitonin (A) or Triton X-100 (B) on ice for 10 min. After centrifugation at 20,000 ×*g* for 10 min at 4°C, the supernatant was subjected to Western blot analysis as described under "MATERIALS AND METHODS". The release of lysosomal and mitochondrial factors was monitored by measuring the release of cathepsin B, a lysosomal marker enzyme, and Cyt.c, respectively.

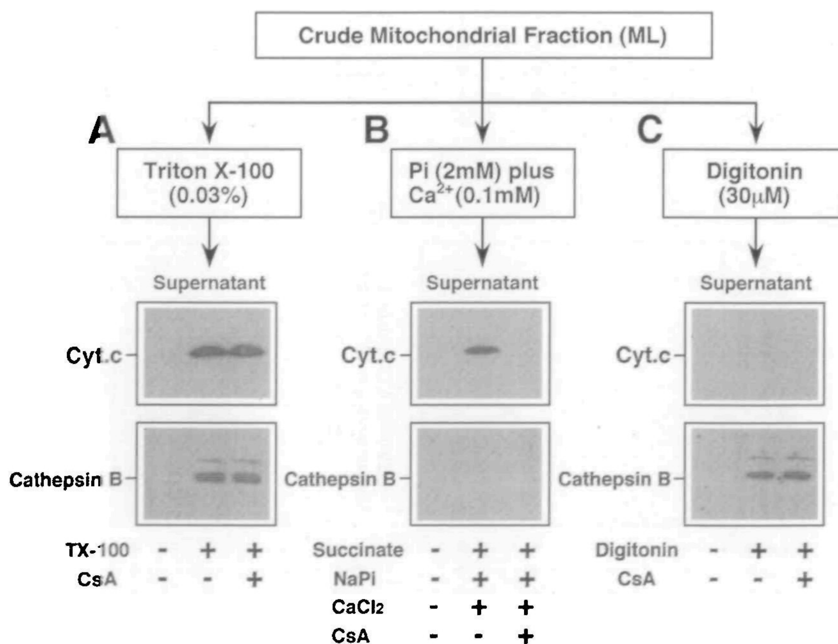

Fig. 3. **Effect of treatment with Ca^{2+} in the presence of P_i on the release of mitochondrial and lysosomal factors from ML.** ML (1.5 mg protein/ml) was treated with 2 mM P_i plus 0.1 mM Ca^{2+} for 5 min at 25°C in the presence or absence of 1 μM CsA (B). After centrifugation at 20,000 ×*g* for 10 min at 4°C, the supernatant was subjected to Western blot analysis as described in the legend of Fig. 1. The supernatants obtained on treatment with 0.03% Triton X-100 (A) and 30 μM digitonin (C) were analyzed using the same methods.

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In the presence of P_i, it was previously shown that Ca^{2+} decreases the membrane potential of mitochondria and releases Cyt.c (*13*, *14*). In accordance with these observations, when ML was treated with 0.1 mM Ca^{2+} in the presence of 2 mM P_i, Cyt.c was specifically released without the release of lysosomal factors (Fig. 3B). The release of Cyt.c was completely inhibited when the supernatant was obtained in the presence of 1 μM cyclosporin A (CsA), an inhibitor of membrane permeability transition (MPT), indicating that the release was mediated by opening of the MPT pore (Fig. 3B). In contrast, the release of mitochondrial or lysosomal factors from ML with Triton X-100 or digitonin was not affected by CsA (Fig. 3, A and C).

The Mitochondrial and Lysosomal Factors Activated Caspase-3 in Different Manners—When activation of caspase-3 by mitochondrial factors obtained on treatment with P_i plus Ca^{2+} was determined using assay system [A], as described under "MATERIALS AND METHODS", remarkable activation was observed in the presence of dATP (Fig. 4A, a). This activation was considerably reduced when the supernatant was obtained in the presence of CsA. The activation was also inhibited by a caspase-9 inhibitor. These results clearly indicated that the activation was dependent on Cyt.c and dATP dependent formation of the Apaf-1/caspase-9 complex. Lysosomal factors obtained by digitonin treatment, in contrast, did not show significant activation of caspase-3 in this assay system even in the presence of dATP (Fig. 4A, b).

However, when assay system [B] was used, remarkable activation of caspase-3 was observed with lysosomal factors obtained by digitonin treatment, and this activation was significantly reduced by E-64, a cysteine protease inhibitor

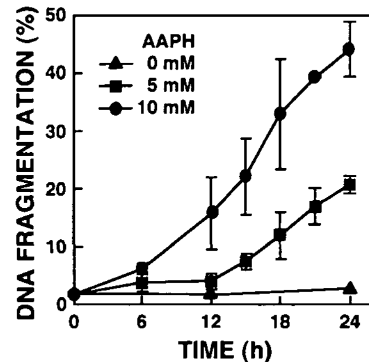

Fig. 5. **DNA fragmentation in HL-60 cells induced by AAPH.** Cells (4×10^5) were treated for various times with 0 mM (▲), 5 mM (■), or 10 mM (●) AAPH. The extent of DNA fragmentation was determined by the diphenylamine method. Data are expressed as means ± SD of three independent experiments.

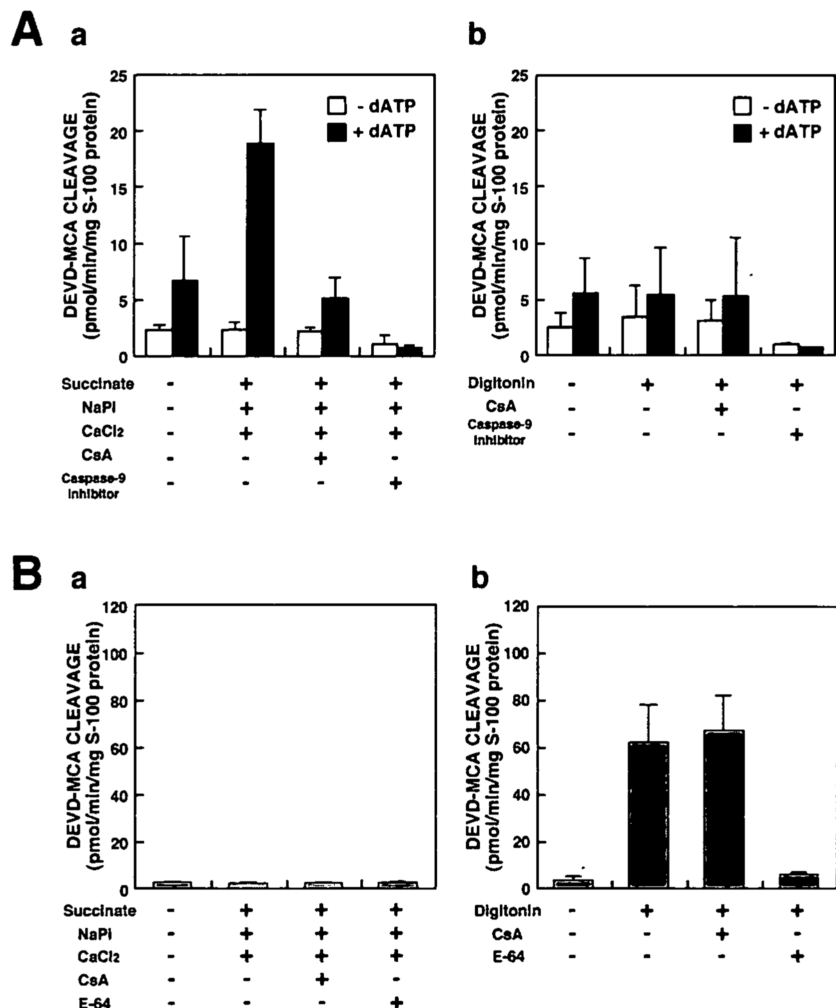

Fig. 4. **Caspase-3 activation by mitochondrial or lysosomal factors released from ML.** Activation of caspase-3 by mitochondrial and lysosomal factors was investigated using assay system [A] or [B] as described under "MATERIALS AND METHODS." (A) Activation of caspase-3 by mitochondrial factor released from ML. Caspase-3 in S-100 was incubated with a supernatant of P_i plus Ca^{2+}-treated ML (a) or a supernatant of digitonin-treated ML (b), and then caspase-3 activity was measured using assay system [A]. White boxes, without 1 mM dATP; Black boxes, with 1 mM dATP. (B) Activation of caspase-3 by lysosomal factors released from ML. Caspase-3 in S-100 was incubated with a supernatant of P_i plus Ca^{2+}-treated ML (a) or a supernatant of digitonin-treated ML (b), and then caspase-3 activity was measured using assay system [B]. The concentrations of succinate, NaP_i, $CaCl_2$, CsA, caspase-9 inhibitor, digitonin, and E-64 were 5 mM, 2 mM, 0.1 mM, 1 μM, 1 μM, 30 μM, and 30 μM, respectively. Data are expressed as means ± SD of three independent experiments.

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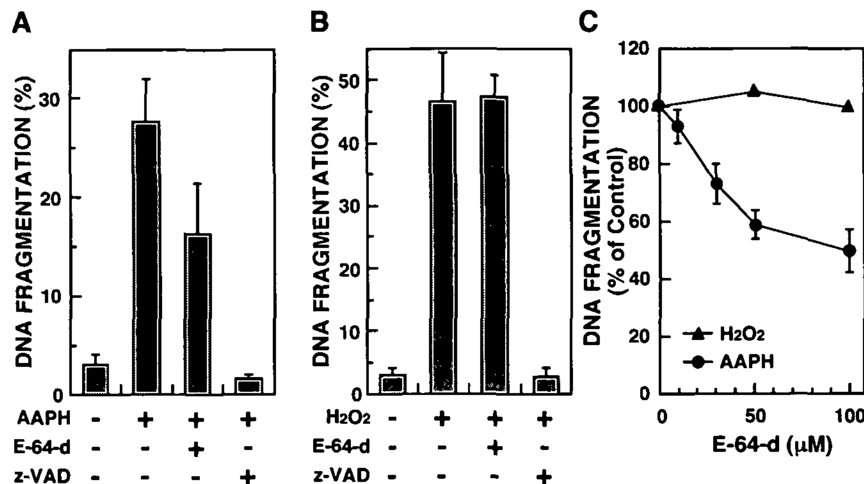

Fig. 6. **Effect of E-64-d on AAPH- or H_2O_2-induced DNA fragmentation in HL-60 cells.** (A) Effect of E-64-d on AAPH-induced DNA fragmentation in HL-60 cells. Cells (4 × 10^5) were pretreated with or without 50 μM E-64-d or 50 μM z-VAD-fmk for 3 h, followed by treatment with 0 or 5 mM AAPH for 24 h. (B) Effect of E-64-d on H_2O_2-induced DNA fragmentation in HL-60 cells. Cells (4 × 10^5) were pretreated as described in (A), followed by treatment with 0 or 10 μM H_2O_2 for 3 h. (C) Concentration-dependent inhibition of DNA fragmentation in HL-60 cells induced by E-64-d. Cells (4 × 10^5) were pretreated with various concentrations of E-64-d for 3 h, followed by treatment with 5 mM AAPH or 10 μM H_2O_2 as described in (A) and (B). The extent of DNA fragmentation was determined by the diphenylamine method. Data are expressed as means ± SD of three independent experiments.

(Fig. 4B, b). This activation was not affected when the supernatant of digitonin-treated ML was recovered in the presence of CsA. These results suggested that the caspase-3 activation was mediated by lysosomal cysteine protease(s). In fact, in this assay system, mitochondrial factors obtained on P_i plus Ca^{2+} treatment did not show any sign of activating caspase-3 (Fig. 4B, a).

Thus, the activation of caspase-3 by the supernatant of digitonin-treated ML was found to be distinct from the activation by mitochondrial Cyt.c dependent formation of the Apaf-1/caspase-9 complex.

AAPH-Induced Apoptosis in HL-60 Cells Is Significantly Inhibited by E-64-d, a Cell-Permeable Inhibitor of Cysteine Protease—To determine whether or not the activation of caspase-3 by lysosomal cysteine proteases is involved in cellular apoptosis, the effect of E-64-d, a cell-permeable inhibitor of cysteine protease, on AAPH-induced apoptosis in HL-60 cells was investigated.

As shown in Fig. 5, treatment of HL-60 cells with AAPH resulted in concentration- and time-dependent fragmentation of cellular DNA, as determined by means of the diphenylamine assay. This DNA fragmentation was completely inhibited by the pretreatment of cells with 50 μM z-VAD-fmk, an inhibitor for caspases, indicating that this reaction depends on the activation of caspase family proteases (Fig. 6A). Typical DNA ladder formation and characteristic morphological changes such as plasma membrane blebbing and cell shrinkage were also recognized in the AAPH-treated HL-60 cells with a comparable time course to that of DNA fragmentation (data not shown).

The DNA fragmentation after 24 h induced by 5 mM AAPH was remarkably (42%) suppressed by the pretreatment of cells with 50 μM E-64-d for 3 h (Fig. 6A). The dose dependence curve for this inhibition revealed that the inhibition was dependent on the E-64-d concentration, and was nearly saturated with 50 μM E-64-d (Fig. 6C). In contrast to the AAPH-induced apoptosis in HL-60 cells, E-64-d did not show any inhibitory activity against the apoptosis in HL-60 cells induced by a low concentration (10 μM) of hydrogen peroxide (H_2O_2) even with 100 μM E-64-d (Fig. 6, B and C). These results clearly indicate that lysosomal cysteine proteases are involved in the AAPH-induced apoptosis in HL-60 cells.

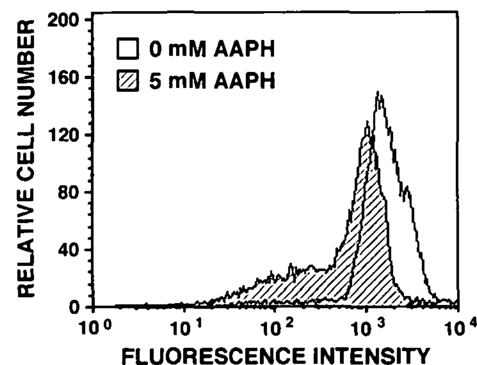

Fig. 7. **Flow cytometry analysis of lysosomal destabilization of HL-60 cells induced by AAPH.** Cells (2 × 10^6) were treated with 0 or 5 mM AAPH for 24 h and then fixed with 70% ethanol, followed by incubation with AO as described under "MATERIALS AND METHODS." After incubation, the fluorescence of AO incorporated in the cells was analyzed with a flow cytometer (Becton Dickinson). The shaded area represents cells treated with 5 mM AAPH.

Lysosomal Destabilization during AAPH-Induced Apoptosis—To determine whether or not lysosomal destabilization is observed during AAPH-induced apoptosis, HL-60 cells treated with 5 mM AAPH for 24 h were assessed for lysosomal stability using the acridine orange (AO) uptake method. As shown in Fig. 7, the number of cells showing lower fluorescence intensity was significantly increased by the treatment with AAPH, indicating the involvement of lysosomal destabilization in AAPH-induced apoptosis in HL-60 cells.

DISCUSSION

It has been reported that uncouplers and other compounds (including P_i plus Ca^{2+}) induce mitochondrial swelling following the opening of the MPT pore and release Cyt.c, thereby activating pro-caspase-9 (*13–16*). The activated caspase-9, in turn, activates pro-caspase-3 and the activated caspase-3 triggers apoptosis (*5, 6*). In addition to this

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mitochondrial Cyt.c dependent formation of the Apaf-1/caspase-9 complex, we recently reported that lysosomal enzymes also activate cytosolic pro-caspase-3 through an E-64–inhibitable mechanism (7). In this study, in order to characterize the differences in the mechanisms of activation of caspase-3 by lysosomal and mitochondrial factors, we have separately obtained the lysosomal and mitochondrial caspase-3 activating factors in ML and characterized their ability to activate pro-caspase-3 in the *in vitro* assay system.

The release of factors from lysosomes and mitochondria was determined by Western blot analysis using anti–cathepsin B (a lysosomal marker enzyme) and anti-Cyt.c antibodies, respectively. In the presence of physiological concentrations of P_i, Ca^{2+} selectively releases Cyt.c from mitochondria without releasing lysosomal enzymes. On the contrary, lysosomal enzymes can be released selectively with low concentrations of digitonin without the release of Cyt.c from mitochondria. The selective release of lysosomal factors from ML by a low concentration of digitonin seems to be based on the specific composition of the lysosomal membrane. The concentration of cholesterol in the lysosomal membrane (0.13 μmol/mg protein) is higher than in the mitochondrial outer (0.06 μmol/mg protein) and inner (0.02 μmol/mg protein) membrane (*17*). Hence, it is likely that at low concentration, digitonin specifically binds to cholesterol in the lysosomal membrane and forms insoluble complexes, thereby perturbing the lysosomal membrane without affecting the mitochondrial membrane.

The ability of the mitochondrial and lysosomal factors to activate caspase-3 was selectively determined using two distinct assay systems, [A] and [B]. A noticeable difference between the two assay systems is the amount of supernatant used in the assay. In assay system [A], activation of pro-caspase-3 in 80 μl of S-100 (100 μg protein) by the mitochondrial factor released from ML was strongly affected by the volume of the supernatant added to S-100, maximum activation being observed with 10 μl of the supernatant (data not shown). In assay system [B], in contrast, activation of pro-caspase-3 in 100 μl of S-100 (100 μg protein) by the lysosomal factors released from ML increased linearly as the volume of the added supernatant increased, and remarkable activation of pro-caspase-3 was observed when 200 μl of the supernatant was added to S-100 (data not shown). Therefore, to measure the activation of pro-caspase-3, 10 and 200 μl of the supernatant were used for assay systems [A] and [B], respectively. Since ML contains only a small portion of lysosomes, it is understandable that a relatively large amount of supernatant was required for the activation of caspase-3 in assay system [B]. Using these two assay systems, it was revealed that the activation of caspase-3 by lysosomal and mitochondrial factors was specifically inhibited by a cysteine protease inhibitor (E-64) and a caspase-9 inhibitor (Ac-LEHD-CHO), respectively. These results indicated that the activation of caspase-3 by lysosomal factors was distinct from the activation by mitochondrial Cyt.c dependent formation of the Apaf-1/caspase-9 complex.

To further determine whether or not the activation of caspase-3 by lysosomal cysteine proteases is involved in cellular apoptosis, the role of cysteine proteases in apoptosis in HL-60 cells induced by AAPH, a free radical–generating agent, was investigated. The exposure of HL-60 cells to AAPH led to concentration- and time-dependent fragmentation of cellular DNA, as determined by means of the diphenylamine assay. Typical DNA ladder formation and characteristic morphological changes were also recognized in the AAPH-treated HL-60 cells with a comparable time course to that of DNA fragmentation. It was further revealed that lysosomal destabilization, as determined by the AO-uptake method, was observed during AAPH-induced apoptosis in HL-60 cells.

The AAPH-induced DNA fragmentation was remarkably suppressed by the pretreatment of cells with E-64-d, a cell-permeable inhibitor of cysteine proteases. This inhibition was dependent on the E-64-d concentration, and was nearly saturated with 50 μM E-64-d. With this concentration, 42% of the DNA fragmentation induced by AAPH was inhibited. Thus, it seems likely that lysosomal cysteine proteases are partially involved in the AAPH-induced apoptosis. The rest of the DNA fragmentation seems to be mediated through a different pathway such as the mitochondrial Cyt.c dependent pathway. As it was reported that at concentrations higher than 50 μM, E-64-d inhibits not only lysosomal cysteine proteases but also calpine, a Ca^{2+}-dependent cysteine protease (*18*), the possibility of the involvement of calpine in the AAPH-induced apoptosis in HL-60 cells could not be excluded.

In contrast to the AAPH-induced apoptosis in HL-60 cells, H_2O_2-induced apoptosis in HL-60 cells was not inhibited by E-64-d. Since involvement of both the mitochondrial pathway and caspase-3 activation in the H_2O_2-induced apoptosis in HL-60 cells has been established (*19–22*), it was indicated that E-64-d did not directly inhibit the mitochondrial Cyt.c dependent pathway or caspase-3 activity in HL-60 cells. Overall, it is indicated that free radicals generated from AAPH induce destabilization of the lysosomal membrane and then the release of lysosomal cysteine proteases. The released proteases, in turn, might participate in the induction of apoptosis, probably through proteolytic activation of the caspase family of proteases.

The involvement of lysosomal destabilization in the induction of cellular apoptosis was recently reported for the apoptosis in Jurkat T cells induced by α-tocopheryl succinate (*12*). In this apoptosis, a decrease in the mitochondrial membrane potential was also noted, although this phenomenon occurred after the initiation of lysosomal rupture. Similar lysosomal destabilization was observed in apoptosis in Jurkat T cells induced by oxidative stress, growth factor starvation, and Fas activation (*23*). Cathepsins B and D are the proteases that have been shown to be relocalized upon lysosomal disruption during apoptosis (*24*, *25*). For example, during apoptosis in human foreskin fibroblasts induced by a free radical–generating quinone, the lysosomal release of cathepsin D was observed, and this phenomenon precedes relocation of Cyt.c and loss of the mitochondrial transmembrane potential (*25*). Thus, lysosomal destabilization and relocation of lysosomal proteases seem to be common phenomena in the early phase of these apoptosis. However, direct evidence indicating the activation of caspase-3 by lysosomal enzymes has not been reported. In our previous study (7) and the present one, we directly demonstrated that the lysosomal cysteine proteases are involved in the activation of caspase-3. In this case, as shown in the previous report, activation of caspase-3 by a supernatant of digitonin-treated ML was not inhibited by specific inhibi-

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tors of cathepsins B and D (CA-074 and pepstatin A, respectively), indicating the involvement of other lysosomal cysteine proteases (*7*).

In our recent study, we examined the effects of specific inhibitors of lysosomal cysteine proteases, such as cathepsin B, S, and L, on the activation of caspase-3, and revealed the possible involvement of cathepsin L-type protease in this activation (*26*). In this case, however, the activation of caspase-3 by purified cathepsin L was not observed, and the activation by caspase-3 was observed only in the presence of the supernatant of the ML, indicating that some unknown factor(s) in the ML-supernatant is involved in the activation of caspase-3 by cathepsin L. We are currently trying to identify this unknown factor(s) in the ML-supernatant.

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