Apoptosis induction resulting from proteasome inhibition

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Proteases are known to be involved in the apoptotic pathway. We report here that benzyloxycarbonyl(Z)-Leu-Leu-leucinal (ZLLLal), a leupeptin analogue, can induce apoptosis in MOLT-4 and L5178Y cells. ZLLLal is a cell-permeant inhibitor of proteasome. Among the protease inhibitors tested, only calpain inhibitor I (acetyl-Leu-Leu-norleucinal) and ZLLLal caused a marked induction of apoptosis in MOLT-4 cells. In contrast, Z-Leu-leucinal, a specific inhibitor of calpain, did not induce apoptosis. When MOLT-4 cells were incubated in the presence of ZLLLal, p53 accumulated in the cells. These results strongly suggest that inhibition of proteasome induces p53-dependent apoptosis and that proteasome can protect cells from apoptosis.

INTRODUCTION

Apoptosis, a physiologically controlled cell death [1,2], is a complex process which includes the recognition of a signal, signal transduction, and the degradation of cellular DNA. There apparently are a number of factors involved in the apoptosis process. One of the factors may be protease activity, since suppression of protease activity often blocks the development of apoptosis induced by various agents [3–10].

Although little is known about the protease(s) targeted by the inhibitors used, the one best understood is the interleukin-1β converting enzyme (ICE) homologue, apopain, which is thought to induce apoptosis via the degradation of poly(ADP-ribose) polymerase [11–13]. In addition to apopain, two papers also suggest the involvement of calpain, a calcium-dependent protease, in apoptosis [5,14]. The nature of their involvement is controversial. In one report, inhibitors of calpain blocked apoptosis, showing a positive function of calpain in the induction of apoptosis [5]. In contrast, the other work showed that calpain inhibitors accelerated apoptosis [14], suggesting a negative role. Additional work is necessary to determine the role of calpain in apoptosis, since the so-called calpain inhibitors used in these reports are not specific to calpain; the inhibitors are also effective on proteasome, a multicatalytic protease, and are sometimes used as proteasome inhibitors at the cellular level [15,16].

Proteasome is a unique high-molecular-mass protease complex of 22–31 kDa subunits, and possesses several distinct catalytic activities. Since gene disruption of proteasome subunits is lethal for yeast, proteasome is essential for cell proliferation [17]. In mammalian cells, proteasome is abundantly expressed in malignant leukaemia cells. Its expression increases greatly during blastogenic transformation of normal blood mononuclear cells [18]. These results, together with our finding that the inhibition of proteasome activity induces the differentiation of PC12 cells [19], suggest that proteasome is a positive regulator of cell proliferation, and that its inhibition results in differentiation in some types of cells and possibly in apoptosis in other types, depending on their potentialities.

We have examined the possibility mentioned above, i.e. the involvement of calpain or proteasome in apoptosis, using MOLT-4 cells. MOLT-4 cells are derived from a human T-cell leukaemia line and die via apoptosis after X-irradiation [20,21], probably due to the induction of p53 [22,23]. In the present work, the effects of various protease inhibitors on the induction of apoptosis were studied. We have found that only inhibitors of proteasome induced apoptosis in MOLT-4 cells, and that this effect was accompanied by an increase in the level of p53.

MATERIALS AND METHODS

Chemicals

Benzyloxycarbonyl(Z)-Leu-Leu-leucinal (ZLLLal) and Z-Leu-leucinal (ZLLal) were synthesized following the method of Ito et al. [24]. Acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (Ac-YVAD-cmk) and calpain inhibitor I were obtained from Bachem (Bubendorf, Switzerland) and Boehringer Mannheim respectively. All other protease inhibitors were purchased from Sigma. All other reagents were of analytical grade. Chemicals were dissolved in DMSO. Aliquots of the solutions were added to cell cultures. The final concentration of DMSO in the medium was less than 1 % (v/v).

Cell line and culture

MOLT-4 cells derived from a human T-cell leukaemia were grown in RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum and antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin). L5178Y cells derived from mouse lymphocytic leukaemia were cultured in Fischer's medium supplemented with 10 % horse serum and antibiotics as for MOLT-4 cells. Cells were maintained at 37 °C in a humidified 5 % CO_2/95 % air atmosphere. The population doubling times were 18 h for MOLT-4 and 8 h for L5178Y cells.

X-irradiation

X-irradiation was performed using a 150 kV X-ray generator unit operating at 5 mA and equipped with an external filter of 0.1 mm Cu and 0.5 mm Al at a dose rate of 0.75–0.78 Gy/min;

Abbreviations used: ZLLLal, benzyloxycarbonyl(Z)-Leu-Leu-leucinal; ZLLal, Z-Leu-leucinal; E64c, (2*S*,3*S*)-*trans*-epoxysuccinyl-leucylamido-3-methyl-butane; E64d, (2*S*,3*S*)-*trans*-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester; Ac-YVAD-cmk, acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone; TLCK, *N*-α-tosyl-lysyl chloromethyl ketone; ICE, interleukin-1β converting enzyme.

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effective energy was 48 keV. Exponentially growing cells (5×10^5 cells/ml) were irradiated in a plastic tissue-culture flask (25 cm^2).

Morphological analysis

Cells were fixed in a solution of methyl alcohol/acetic acid (3:1, v/v). Fixed cells were placed on a glass slide, stained with 1% Giemsa solution, and observed under an optical microscope. Apoptotic cells were scored from morphological changes (nuclear fragmentation) as reported previously [20]. The number of cells counted per sample was more than 1000. The results are shown as an average of three independent experiments.

DNA isolation and agarose-gel electrophoresis

DNA was isolated from 2×10^6 cells using a DNA extraction kit, Sepa Gene® (Sanko Junyaku Co. Ltd., Tokyo, Japan). The extracted DNA was treated with RNase A (0.32 mg/ml) for 1 h at 37 °C, and applied to an agarose gel (2%) for electrophoresis. The gel was stained with an ethidium bromide solution (1.0 μg/ml) and observed with a UV light illuminator.

Western blot analysis

Cells were lysed by sonication in an SDS buffer [1% (w/v) SDS/0.04 M Tris/HCl, pH 6.8/7.5% (w/v) glycerol/0.05 M dithiothreitol] containing PMSF (1 mM), leupeptin (10 μg/ml), and pepstatin A (1 μg/ml). Equal amounts of protein (10 μg) were loaded into each lane of a 10%-polyacrylamide gel, electrophoresed, and blotted onto a nitrocellulose membrane (Hybond®-C extra, Amersham, Little Chalfont, Bucks., U.K.) following the method of Towbin et al. [25]. A monoclonal antibody against human p53 (pAb1801; Novocastra Laboratories, U.K.) was used as the primary antibody. The signal was then developed with the enhanced chemiluminescence Western blot detection system (ECL; Amersham).

RESULTS AND DISCUSSION

To study the involvement of proteases in apoptosis, we examined the effects of a protease inhibitor ZLLLal on X-ray-induced apoptosis in MOLT-4 cells. Table 1 shows the effects of ZLLLal on the induction of apoptosis in X-irradiated MOLT-4 cells. The results indicate that ZLLLal did not inhibit X-ray-induced apoptosis, but that ZLLLal itself induced apoptosis in unirradiated cells in a dose-dependent manner. These results were unexpected in the light of current knowledge of the involvement of proteases in apoptosis (i.e. proteases are involved in the progression of apoptosis). In addition, the effects of ZLLLal and X-rays on the induction of apoptosis were additive,

Table 1 Effects of ZLLLal on X-ray-induced apoptosis in MOLT-4 cells

MOLT-4 cells exposed to 18 Gy of X-rays (+) or unirradiated (−) were cultured at 37 °C for 6 h in RPMI medium (5×10^5 cells/ml) in the absence or presence of 2 or 5 μM ZLLLal. Apoptotic cells were scored from morphological changes (nuclear fragmentation) as reported previously [20]. Results are the means ± S.E.M. of three independent experiments.

Concentration of ZLLLal (μM)	Percentage of apoptotic cells	
	X-rays (−)	X-rays (+)
0	2.3 ± 0.8	36.5 ± 5.5
2	45.8 ± 2.0	76.0 ± 2.8
5	51.1 ± 2.0	76.9 ± 4.1

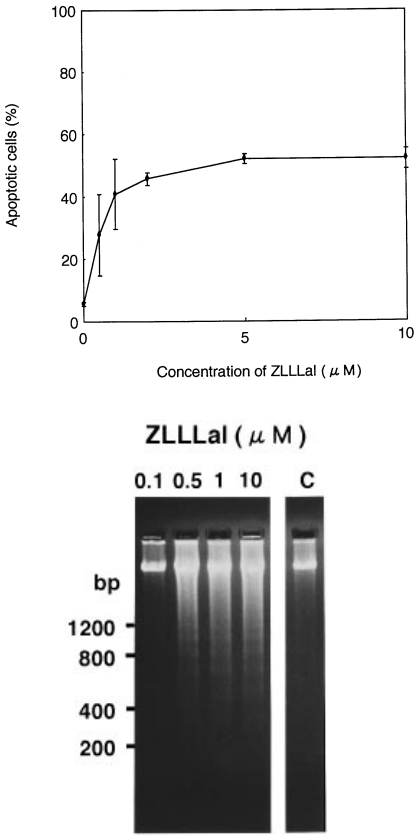

Figure 1 Induction of apoptosis in MOLT-4 cells by ZLLLal

MOLT-4 cells were incubated at 37 °C for 6 h in the presence of various concentrations of ZLLLal. Top: morphological changes. Bottom: DNA fragmentation into nucleosomes and nucleosome multiples (DNA ladder formation). C, control.

suggesting that apoptosis is induced by both agents via a common pathway.

The evidence that the protease inhibitor alone induced apoptosis was confirmed with morphological (Figure 1, top) and biochemical studies (Figure 1, bottom). These results strongly suggest that proteases play an important role in the induction, and also in the suppression, of apoptosis. The induction of apoptosis by ZLLLal was also observed in L5178Y mouse cells (derived from mouse lymphocytic leukaemia cells) when measured morphologically and biochemically (Figure 2). This indicates that the effects of ZLLLal are not MOLT-4 cell specific.

ZLLLal is known to inhibit both calpain [26] and proteasome [27]. We have studied various protease inhibitors (Table 2) to define the role and specificity of ZLLLal in the induction of apoptosis. These included leupeptin for serine proteases and cysteine proteases; (2*S*,3*S*)-*trans*-epoxysuccinyl-leucylamido-3-methyl-butane (E64c) and (2*S*,3*S*)-*trans*-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester (E64d) for cysteine proteases; *N*-α-tosyl-lysylchloromethyl ketone (TLCK) for trypsin-like proteases; PMSF for serine proteases; pepstatin A for aspartic proteases; phosphoramidon for metalloproteases; Ac-YVAD-cmk for ICE, a cysteine protease; calpain inhibitor I for calpain and proteasome [15]; and ZLLal for calpain [28], in addition to ZLLLal. Among the inhibitors examined, only ZLLLal and calpain inhibitor I strongly induced apoptosis (Table 2). We and others have previously reported that ZLLLal and calpain inhibitor I both inhibit protein degradation caused

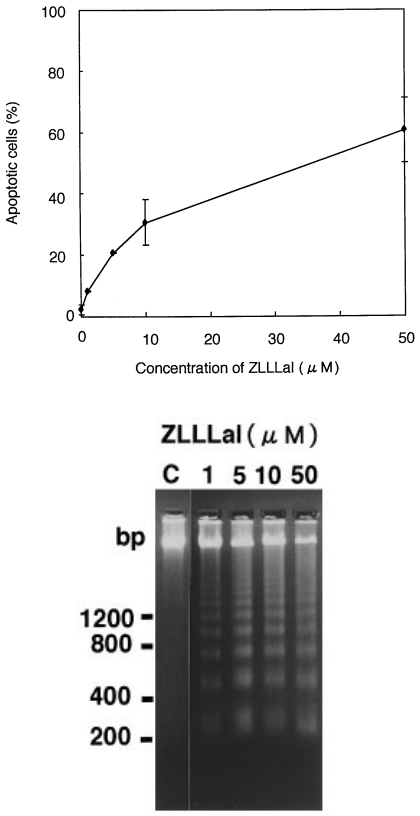

Figure 2 Induction of apoptosis in L5178Y cells by ZLLLal

L5178Y cells were incubated at 37 °C for 6 h in the presence of various concentrations of ZLLLal. Top: morphological changes. Bottom: DNA fragmentation into nucleosomes and nucleosome multiples (DNA ladder formation). C, control.

Table 2 Effects of protease inhibitors on the induction of apoptosis in MOLT-4 cells

Cells were incubated for 6 h in the presence of various protease inhibitors at a 100 μM concentration. Controls were MOLT-4 cells incubated with 1% (v/v) DMSO instead of an inhibitor. Apoptotic cells were scored from morphological changes (nuclear fragmentation) as reported previously [20]. Results are the means ± S.E.M. of three independent experiments. The effect of ZLLLal at 1 μM concentration is also shown for comparison. No morphological changes other than apoptosis were detected in these experiments with the concentrations of inhibitors used.

Protease inhibitors (100 μM)	Percentage of apoptotic cells
Control	1.9 ± 0.2
Leupeptin	2.2 ± 0.5
E64c	1.6 ± 0.4
E64d	4.3 ± 2.8
TLCK	8.4 ± 2.7
PMSF	2.7 ± 0.8
Pepstatin A	2.2 ± 0.3
Phosphoramidon	2.2 ± 0.9
Ac-YVAD-cmk	3.0 ± 1.4
Calpain inhibitor I	52.2 ± 6.5
ZLLal	5.4 ± 1.4
ZLLLal (1 μM)	42.7 ± 5.2

by purified proteasomes or calpain [15,27,28]. These results strongly suggest the possibility that inhibition of the proteasome or calpain activity was involved in the induction of apoptosis by these drugs. In addition, ZLLal, which is a strong inhibitor of calpain but a poor one of proteasome [28], did not induce apoptosis (Table 2). Moreover, we have previously reported that ZLLLal induced neuronal differentiation of PC12 cells [19], as did lactacystin (a proteasome-specific inhibitor) [29], suggesting that ZLLLal can also inhibit proteasome activity intracellularly. Taken together, the present observations strongly suggest that ZLLLal and calpain inhibitor I affect cell viability by inhibiting proteasome itself. Some workers have recently argued that the inhibition of calpain-like activity induced the apoptosis observed in their reports [14,30]. They did not consider the possibility of the involvement of proteasome to explain their results. These results also lend support to the idea that cell death caused by ZLLLal or calpain inhibitor I results from the inhibition of proteasome.

Figure 3 Accumulation of p53 in MOLT-4 cells treated with ZLLLal

Cell lysates from MOLT-4 cells cultured in the presence of 1 μM ZLLLal or 100 μM ZLLal were subjected to Western blot analysis. MOLT-4 cells were treated with ZLLLal or ZLLal for various time intervals (h).

It has been demonstrated that p53 is hydrolysed through the ubiquitin-dependent proteolytic pathway catalysed by proteasome [31,32]. We have examined the level of p53 in MOLT-4 cells when incubated in the presence of ZLLLal or ZLLal (Figure 3). The level of p53 increased with time in cells incubated with ZLLLal but not with ZLLal. These results suggest that MOLT-4 cells died through apoptosis due to the accumulation of p53 as a consequence of the inhibition of proteasome. These results are in agreement with evidence that proteasome is involved in the ubiquitin-dependent proteolytic pathway through which p53 is degraded [32], and that overexpression of p53 induces apoptosis [33]. X-irradiated MOLT-4 cells died via apoptosis, which was preceded by an increase in p53 levels (H. Nakano and K. Shinohara, unpublished work). This lends support to the idea that the cause of induction of apoptosis by ZLLLal is an increase in p53, resulting from inhibition of proteasome. This increase in p53 may result primarily from the inhibition of the ubiquitin-dependent proteolytic pathway catalysed by proteasome, which degrades p53, and not from an increase in p53 production, since it has been demonstrated that an increase in p53 protein is not accompanied by an increase in p53 mRNA [34,35]. In conclusion, the present results suggest that inhibition of proteasome induces apoptosis in the cell through the accumulation of p53. Presumably proteasome plays an important role as a regulatory factor influencing physiological levels of p53.

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