Inhibition of etoposide-induced apoptosis with peptide aldehyde inhibitors of proteasome

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Recent investigations have indicated the involvement of proteasome in programmed cell death. The present studies show that although peptide aldehyde inhibitors of proteasome are by themselves weak inducers of apoptosis, they inhibit the apoptotic effect of the anticancer drug etoposide in rat thymocytes. Acetyl-Leu-Leu-norvalinal (LLnV-al) and other related peptide aldehydes inhibited the increase in caspase activity and DNA fragmentation that followed treatment with etoposide and their effect was related to their potency as proteasome inhibitors. To inhibit etoposide-induced apoptosis, LLnV-al must be present

within 3 h of treatment with etoposide, in the same way as the inhibitor of protein synthesis cycloheximide must be. Etoposide caused a rapid accumulation of p53 protein that was not inhibited by LLnV-al, which was also a strong inducer of p53. Peptide aldehydes were also weak activators of caspase activity, suggesting that the same mechanism, i.e. the blocking of proteasome function, both triggers apoptosis and inhibits the effect of etoposide. These results are consistent with a model in which proteasome is selectively involved in the pathway used by etoposide to induce cell suicide.

INTRODUCTION

The 26 S proteasome is a conserved proteolytic complex composed of a 20 S catalytic core and associated regulatory proteins [1,2]. The most common mechanism for marking a protein for degradation via proteasome is ubiquitination [3]; however, other mechanisms exist [1]. Proteasome is involved in cell cycle regulation and cell proliferation [4], and various cyclins and proto-oncogenic proteins are proteolytically degraded by proteasome. The availability of specific inhibitors [5] has revealed the role of proteasome in other cellular events such as antigen presentation [5], the regulation of gene expression [6] and also apoptosis [7,8].

One event characteristic of apoptosis is the activation of a proteolytic cascade involving several caspases [9,10]; some reports have suggested that proteasome might be an upstream regulator of these proteases [7,8]. A role of proteasome in cell death is also supported by the up-regulation of ubiquitin gene expression during apoptosis [11,12], the activation of proteasome during the programmed cell death of muscles in *Manduca sexta* [13,14] and its selective cellular localization in apoptosis [15,16].

Although proteasome inhibitors attenuated some forms of apoptosis in primary cultures of untransformed cells [7,8], the blocking of proteasome function in tumour cell lines has been implicated in the induction of apoptosis [17–20]. This suggests a different response to proteasome inhibition linked to the proliferative status of the cell [20]. Consequently, proteasome inhibitors can be considered as potential antineoplastic agents. In addition, specific proteasome inhibitors might be useful tools to investigate the molecular events involved in the activation of cell suicide triggered by different stimuli. Although the role of caspase protease activation in apoptosis is now becoming clear [9,10,21], little is known about the upstream events transducing the death message.

Etoposide, a topoisomerase II inhibitor, is a potent and widely used antineoplastic agent able to induce apoptosis in most cell

types; however, very little is known about its mechanism of action. This study demonstrates that proteasome inhibitors attenuate thymocyte apoptosis induced by etoposide, suggesting the involvement of proteasome in the signal transduction pathway used by this drug to kill cells.

MATERIALS AND METHODS

Materials and cells

Etoposide, other inducers of apoptosis, protease inhibitors, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) and other biochemicals were obtained from Sigma. Monoclonal anti-p53 antibody (Pab 240) was from Santa Cruz. Thymocytes were obtained from immature male Wistar rats. The isolated cells were suspended at the indicated concentration in RPMI 1640 medium containing 10 mM Hepes and 100 i.u./ml each penicillin and streptomycin. Etoposide (10 μ M final concentration) and proteasome inhibitors were prepared as 1000-fold concentrated solutions in DMSO. Each experimental point was derived from two separate wells, each assayed in duplicate. All the presented data were obtained from experiments performed at least twice with comparable results.

DNA fragmentation and cell death

A detailed description of the methodology routinely used for the determination of DNA fragmentation and cell death is given elsewhere [22]. In brief, to quantify fragmented DNA [23], the cells (2×10^7 cells/ml, 2 ml per well) were lysed and the lysate was centrifuged for 20 min at 13000 g. The DNA content of the detergent-soluble supernatant with respect to the total DNA in the lysate was assayed with the diphenylamine reagent.

Cell death was evaluated by flow cytometry, measuring the percentage of cells incorporating propidium iodide. Flow cyto-

Abbreviations used: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; AMC, 7-amino-4-methylcoumarin; LLL-al, *N*-carbobenzoxyleu-leu-leucinal; LLM-al, *N*-acetyl-Leu-Leu-methioninal; LLnL-al, *N*-acetyl-Leu-Leu-norleucinal; LLnV-al, *N*-carbobenzoxyleu-leu-norvalinal.

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metry was also used to detect apoptotic cells by the method of Dive et al. [24].

Fluorimetric determination of caspase activity

The enzymic activity cleaving the fluorogenic peptide substrate Ac-DEVD-AMC [25,26], which will be simply referred to here as caspase activity, was measured in 2×10^7 cells incubated in 1 ml of medium for the indicated period. At the end of the incubation the cells were collected, washed once in PBS, suspended in 150 μ l of lysis buffer [20 mM Hepes (pH 7)/5 mM dithiothreitol/2 mM EDTA/0.1% (v/v) CHAPS/0.1% (v/v) Triton X-100/1 mM PMSF/1 μ g/ml aprotinin/1 μ g/ml pepstatin/1 μ g/ml leupeptin], vortex-mixed and left for 10 min in ice. The lysates were centrifuged for 1 min at 28000 g and the supernatant was used as enzyme source. A sample (10 μ l) of this extract (approx. 10 μ g of protein) was combined with 20 μ l of assay buffer containing 100 mM Hepes, pH 7, 5 mM dithiothreitol, 0.1% (v/v) CHAPS, 10% (w/v) sucrose and 150 μ M Ac-DEVD-AMC and incubated for 15 min at 37 °C. The reaction was stopped in ice by adding 0.1 ml of 2% (w/v) sodium acetate in 0.2 M acetic acid. The samples were diluted with 2.5 ml of water and the specific cleavage of the fluorogenic peptide Ac-DEVD-AMC was monitored by 7-amino-4-methylcoumarin (AMC) liberation by using excitation at 370 nm and emission at 455 nm. Conversion to moles of substrate cleaved was obtained by using a standard curve of free AMC concentration against fluorescence. One unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per min under the conditions described.

Immunoblotting

At the end of the incubations, the cells (1.5×10^8) were collected, washed once in PBS, suspended in lysis buffer [150 mM NaCl/0.25% sodium deoxycholate/1% (v/v) Nonidet P40/1 mM NaF/1 mM EGTA/1 mM PMSF/1 μ g/ml aprotinin/1 μ g/ml leupeptin/1 μ g/ml pepstatin] and sonicated for 5 min in ice. The homogenates were centrifuged for 10 min at 28000 g and, after the determination of protein concentration, the supernatants were diluted in loading buffer and boiled for 3 min. Aliquots corresponding to 50 μ g of protein of each sample were analysed by SDS/PAGE on a 10% (w/v) polyacrylamide gel. Standard protein markers were used for molecular mass calibration. After blotting on nitrocellulose membrane, this was blocked with 3% (v/v) non-fat milk/2% (w/v) albumin, washed with 0.5% (v/v) Tween 20 in TBS [150 mM NaCl/10 mM Tris/HCl (pH 8)] and finally probed for 2 h with monoclonal anti-p53 antibody at a dilution of 1 μ g/ml. Immunoreactive bands were detected with an enhanced chemoluminescence kit (Amersham). Densitometric analysis was performed with SIGMA-SCAN image-measurement software after digitization of the images with an Epson GT-6500 scanner.

RESULTS

The peptide aldehyde *N*-carbobenzoxy-Leu-Leu-norvalinal (LLnV-al) is a powerful inhibitor of proteasome proteolytic activity that has been used successfully to elucidate the role of proteasome in antigen presentation [5] and in the regulation of NF κ B [6]. The effects of LLnV-al were tested on thymocyte apoptosis induced by the anticancer drug etoposide. The treatment of thymocytes with etoposide is known to rapidly induce internucleosomal DNA fragmentation [27]. In preliminary experiments we noted that LLnV-al attenuated DNA laddering; the extent of DNA fragmentation was then quantified by the

Table 1 LLnV-al inhibits etoposide-induced thymocyte apoptosis

The cells were incubated, in the presence or absence of 1 μ M LLnV-al, without any inducer of apoptosis (control) or with 10 μ M etoposide, 0.5 μ M calcium ionophore A23187 or 1 μ M staurosporine. DNA fragmentation was measured after 6 h of incubation; the number of apoptotic cells was determined by flow cytometry after 8 h of treatment. Results are means $\pm$ S.D. for four determinations.

Treatment	DNA fragmentation (%)		Apoptotic cells (%)	
	Without LLnV-al	With LLnV-al	Without LLnV-al	With LLnV-al
Control	12 $\pm$ 3	18 $\pm$ 4	9 $\pm$ 2	25 $\pm$ 3
Etoposide	44 $\pm$ 4	19 $\pm$ 3	50 $\pm$ 6	24 $\pm$ 4
A23187	27 $\pm$ 4	32 $\pm$ 4	24 $\pm$ 4	31 $\pm$ 5
Staurosporine	31 $\pm$ 3	36 $\pm$ 5	35 $\pm$ 2	46 $\pm$ 4

Figure 1 Inhibition of etoposide-induced DNA fragmentation by proteasome inhibitors

Thymocytes were treated with 10 μ M etoposide and incubated for 6 h with the indicated concentrations of peptide aldehyde inhibitors; DNA fragmentation was then measured. The lower horizontal line (control) represents the mean value of percentage DNA fragmentation in control untreated cells ($12 \pm 2\%$). Results are means of four determinations.

diphenylamine assay. Table 1 shows that the increase in DNA fragmentation caused by 10 μ M etoposide after 6 h of incubation was greatly diminished by 1 μ M LLnV-al. The same dose of the peptide inhibitor also decreased apoptosis in cells incubated for 8 h in the presence of etoposide. However, LLnV-al was not able to counteract apoptosis when the triggers were a calcium ionophore or staurosporine, in agreement with a previous paper [7]. It is noteworthy that, although inhibiting the cytotoxic effect of etoposide, LLnV-al itself caused a limited but significant increase in DNA fragmentation and apoptosis.

Because LLnV-al also inhibits calpains, whose activation might be related to some forms of apoptosis [28], the dose effects of three different peptide aldehyde inhibitors known to have different inhibitory potencies against proteasome, but a similar effect against other proteases including calpains [5,6], were compared (Figure 1). LLnV-al and *N*-carbobenzoxy-Leu-Leu-leucinal (LLL-al) are powerful inhibitors of proteasome, whereas *N*-acetyl-Leu-Leu-methioninal (LLM-al) is a much weaker inhibitor; their potency in inhibiting DNA fragmentation mirrored

Figure 2 Effect of proteasome inhibitors on caspase activity in untreated or etoposide-treated thymocytes

(A) Time course of caspase activity cleaving the fluorogenic substrate Ac-DEVD-AMC in thymocytes treated with 10 μ M etoposide or the corresponding volume (1 μ l) of DMSO (control). Results are means for three separate experiments. (B) Untreated thymocytes and etoposide-treated cells were incubated for 4 h with the indicated concentrations of proteasome inhibitors. Caspase activity was then measured. Results are expressed as percentages of the activity measured in control cells incubated without etoposide or proteasome inhibitors. This control activity (0.15 $\pm$ 0.02 units/mg of protein) was taken as 100%. Results are means $\pm$ S.D. for four determinations.

the effect against proteasome [5–7]. At concentrations as low as 0.5 μ M, LLL-al and LLnV-al inhibited the digestion of DNA induced by etoposide, the former being the most effective. In contrast, LLM-al exerted an inhibitory effect only at concentrations higher than 10 μ M. The differential dose effects of peptide aldehydes is considered a specific method for implicating proteasome in cell processes [5,6], and all the interpretation of the presented results is based on the assumption that proteasome is the target of peptide aldehydes.

The activation of caspase 3-like enzymes is a typical event of apoptosis [9,10]. Caspase activity was assessed by the cleavage of the specific fluorogenic substrate Ac-DEVD-AMC [25,26], which represents the cleavage site in poly(ADP-ribose) polymerase and other protein substrates of these zymogens [29]. Caspase activity began to increase significantly by 3 h after the treatment with etoposide; after 4 h of treatment the activity was 3–4-fold that in control thymocytes (Figure 2A). Again, caspase activity induced by etoposide was inhibited by LLL-al, LLnV-al and LLM-al in relation to their potency as proteasome inhibitors, the effect of LLM-al being detectable only at concentrations higher than 10 μ M (Figure 2B). The blocking of proteasome function, however, itself triggered caspase activity. LLL-al, LLnV-al, LLM-al and also *N*-acetyl-Leu-Leu-norleucinal (LLnL-al) (results not shown for the last inhibitor) activated caspase activity with a dose response that was correlated with their potency as proteasome inhibitors, but their effect was weaker than that of etoposide.

The apoptotic action of etoposide is thought to depend, at least partly, on p53 [30]. In contrast, p53 is a target of proteasome [31], and p53 accumulation is known to be caused largely by the stabilization of the protein [32]. Consequently proteasome inhibition should increase the p53 level, as observed in different cell models [18,31]. Etoposide caused a large increase in thymocyte p53 content (Figure 3A). This effect, as expected, was not inhibited by LLnV-al, which by itself increased the p53 level. LLnV-al also anticipated the time course of early p53 accumulation in etoposide-treated cells (Figure 3B). The increase in p53 protein caused by LLnV-al gave further evidence of proteasome-inhibiting activity of the peptide aldehyde.

It is acknowledged that etoposide requires protein synthesis *de novo* to induce apoptosis [27]. To define the point at which

Figure 3 Etoposide and LLnV-al induce p53 accumulation in thymocytes

(A) Thymocytes were incubated for 0 h (lane 1) or 3 h (lanes 2–5) with the following additions: none (control, lane 2), 10 μ M etoposide (lane 3), 1 μ M LLnV-al (lane 4) or 10 μ M etoposide plus 1 μ M LLnV-al (lane 5). After cell lysis, the extracts were subjected to SDS/PAGE followed by Western blotting. (B) Time course of early p53 accumulation in thymocytes treated with etoposide, etoposide plus LLnV-al or no addition (control). After Western blotting, the intensities of the bands corresponding to p53 were measured by densitometry and compared with the intensity of the p53 band in non-incubated cells, taken as unity.

proteasome function is essential in the response to etoposide, the effects obtained by blocking proteasome function and blocking protein synthesis were compared. Thymocytes were treated with etoposide, then 1 μ M LLnV-al or 2 μ M cycloheximide (this treatment decreased the incorporation of radioactive leucine in trichloroacetic acid-precipitable material by more than 90 %

Figure 4 LLnV-al and cycloheximide inhibit an early event in etoposide-induced apoptosis

Thymocyte suspensions were treated with 10 μ M etoposide; 1 μ M LLnV-al or 2 μ M cycloheximide (Chx) was then added after different times ranging from 0 h (inhibitors were added at the same time as etoposide) to 5 h. In each case the cells were collected after 6 h of treatment with etoposide for DNA fragmentation analysis. The percentage inhibition of DNA fragmentation was calculated as follows: $100 - [(DNA \text{ fragmentation in etoposide-inhibitor-treated cells} / DNA \text{ fragmentation in etoposide-treated cells}) \times 100]$. Results are means $\pm$ S.D. for four determinations.

without any significant cytotoxic effect) was added to cells at various times after the addition of the drug. After 6 h of treatment with etoposide the extent of DNA fragmentation was measured. These experiments (Figure 4) showed that DNA digestion was inhibited when LLnV-al or cycloheximide was added within 2 h of the induction of apoptosis. Partial inhibition was observed when LLnV-al or cycloheximide were added after 3 h, but when the inhibitors were given 4 h after the induction of apoptosis, DNA fragmentation was not blocked. In each case the time courses of the effect of LLnV-al and cycloheximide were identical, indicating that both proteasome and protein synthesis involvement must occur within the first 3 h after the induction of apoptosis.

DISCUSSION

Eukaryotic cells contain multiple proteolytic systems but most of the cell proteins are degraded in an extralysosomal process by the proteasomes [1–5]. The discovery of selective peptide inhibitors of proteasome have disclosed an important role of proteasomes in several cell processes. Inhibitor studies also demonstrated the involvement of proteasome in apoptotic cell death [7,8], according to independent results suggesting a role of ubiquitin-dependent proteolysis in different forms of programmed cell death [11–16,33,34]. However, the role of proteasome in apoptosis is controversial. On the one hand, proteasome inhibitors gave protection from apoptosis to sympathetic neurons deprived of nerve growth factor [8] and to thymocytes exposed to ionizing radiations, glucocorticoids or phorbol esters [7]. On the other hand, in several tumour cell lines the inhibition of proteasome function activated the death programme and induced apoptosis [17–20]. These apparently contradictory results can be explained on the basis of the different proliferative statuses of the cell [20], because proteasome is involved in the control of cell cycle

progression and its inhibition would not affect quiescent cells to the same extent as rapidly dividing cells. Proteasome inhibition also enhanced background apoptosis in untreated thymocytes, where proteasome inhibitors increased caspase activity and DNA fragmentation (Table 1 and Figure 2). The apoptotic effect of peptide aldehyde inhibitors in thymocytes, however, was not as marked as in tumour cell lines [17–20], in agreement with Grimm et al. [7], who reported only a delayed toxicity of proteasome inhibitors in thymus cells. Furthermore, although proteasome inhibitors increased spontaneous caspase activity, the extent of this activation was moderate and varied from one experiment to another. In contrast, the activation of caspase activity by etoposide was constantly high and reproducible. This fact could reflect a sensitivity to proteasome inhibitors limited to some cell subset(s) or to cells in active proliferation, whose numerical consistency would vary between different cell preparations obtained from different animals. Although inhibiting the apoptotic effect of etoposide in thymocytes, LLnV-al caused an accumulation of p53 in the same way as etoposide, suggesting that p53 alone is not sufficient to mediate etoposide-induced apoptosis. However, p53 could be responsible for the delayed cytotoxic effect of proteasome inhibitors, as recently suggested [35]. In each case, it is evident that the involvement of proteasome in the pathway leading to apoptosis must be studied in quiescent cells, where the effect of proteasome inhibitors is not masked by the marked toxic effect that they exert in cycling cells.

Despite their effect as weak inducers of apoptosis, proteasome inhibitors counteracted the apoptotic effect of etoposide. LLnV-al, LLL-al and LLnL-al (results not shown for the last peptide aldehyde) attenuated caspase activation and DNA fragmentation in etoposide-treated thymocytes, and their dose effect was correlated with their potency against proteasome [5–7], the effect of LLL-al and LLnV-al being detectable at submicromolar concentrations. In contrast, LLM-al, which exhibits a similar potency against calpains but a much weaker effect against proteasome with respect to LLL-al and LLnV-al [5], was effective only at concentrations higher than 10 μ M.

Etoposide, a topoisomerase II inhibitor, is a potent anti-neoplastic agent but very little is known about its mechanism of action. The results reported here demonstrate that degradation of at least one protein (that could be for example a transcription factor or an inhibitor of caspase activation) by proteasome is required for the transmission of the etoposide-triggered signal. Etoposide requires protein synthesis to trigger apoptosis [27,36] but the hypothetical death-protein induced by etoposide has never been identified. The strict temporal correlation in the apoptosis-inhibiting activity of LLnV-al and cycloheximide suggests that proteasome is involved in a step close to the synthesis of the putative death-protein. To exert an antiapoptotic effect, LLnV-al as well as cycloheximide have to be given to thymocytes within 3 h of etoposide (Figure 4), before the caspase activity has reached high levels (Figure 2), supporting the notion that caspase activation represents the ‘point of no return’ in the apoptotic process [10] and the central role of caspase 3 in etoposide-induced apoptosis [37].

It has been proposed that proteasomes are key regulators of apoptosis, placed upstream from the caspase cascade [7,8]. However, the finding that proteasome inhibitors can themselves induce caspase activity and, in the models in which they are inhibitors of apoptosis, affect apoptosis induced by some stimuli but not by others [7] (Table 1) does not support this hypothesis. Rather, these results suggest that proteasome is not a common regulator of caspase activation but is selectively involved in the signal transduction pathway(s) used by etoposide and some other triggers of apoptosis to induce cell suicide.

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