Inhibition of ubiquitin-proteasome pathway activates a caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells

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The ubiquitin-proteasome pathway is the principal mechanism for the degradation of short-lived proteins in eukaryotic cells. Here we examine the possibility that ubiquitin-proteasome is involved in regulating the levels of Bcl-2, which is abundantly expressed in M-07e cells, a granulocyte/macrophage colony-stimulating factor (GM-CSF)-dependent human leukaemic cell line. Apoptosis in M-07e cells, induced by GM-CSF withdrawal, was associated with a gradual cleavage of Bcl-2 into a 22 kDa fragment. Treatment of M-07e cells with benzyloxycarbonyl-Leu-Leu-L-leucinal (Z-LLL-CHO; MG-132), a reversible ubiquitin-proteasome inhibitor, markedly accelerated the cleavage of Bcl-2 and promoted cell death through the apoptotic pathway. The cleavage of Bcl-2 was inhibited by a caspase-3 (CPP32)-specific inhibitor [acetyl-Asp-Glu-Val-Asp-CHO (DEVD-CHO)] but not caspase 1 inhibitor (acetyl-Tyr-Val-Ala-Asp-CHO), suggesting that Bcl-2 is a proteolytic substrate of a caspase-3-like protease activated during apoptosis. The simultaneous addition of recombinant human GM-CSF (rhGM-CSF) to M-07e cultures delayed the activation of caspase 3 and Bcl-2 cleavage triggered by Z-LLL-CHO, suggesting that the activation of the GM-CSF signalling pathway can partly overcome the apoptotic effect induced by Z-LLL-CHO. Apoptosis induced by inhibition of the proteasome pathway was verified in studies with lactacystin, a highly specific and irreversible proteasome inhibitor. Lactacystin-induced apoptosis in M-07e cells was remarkably similar to that induced by Z-LLL-CHO, which included caspase 3 activation, cleavage of Bcl-2 into a 22 kDa fragment and, ultimately, cell death. These results showed that inhibition of the ubiquitin-proteasome pathways can lead to the activation of a DEVD-CHO-sensitive caspase and induces Bcl-2 cleavage, which might have a role in mediating apoptosis in M-07e cells.

Key words: apoptosis, benzyloxycarbonyl-Leu-Leu-L-leucinal, lactacystin.

INTRODUCTION

Apoptosis is an important process of cell death, characterized initially by a series of stereotypic biochemical and morphological changes [1,2]. Morphological features of apoptosis include chromatin condensation, nuclear fragmentation, membrane blebbing and the formation of apoptotic bodies. In the past, the cleavage of cellular DNA into oligonucleosomal fragments has been regarded as a hallmark of apoptosis and was the only biochemical marker associated with this event. Recently, the focus of interest in apoptosis has shifted towards the cleavage of several vital intracellular proteins by a family of cysteine proteases collectively termed caspases [3,4]. The substrates for the activated caspases include protein kinases, DNase, retinoblastoma protein, cytoskeletal proteins, auto-antigens and possibly other caspases. Cleavage of these proteins during apoptosis by caspases might either activate or inactivate essential functions of the proteins or produce cleaved products with altered activities.

Bcl-2 family proteins have an important role in regulating the programmed cell death induced by various stimuli [1,2]. Bcl-2 and Bax are the two prototype members of this large family of proteins: Bcl-2 is the apoptosis inhibitor and Bax the apoptosis promoter. Although the detailed mechanism is unknown, it is thought that they form protein dimers with each other and that the relative ratio of these two proteins can determine the balance between life and death. Current results favour the concept that Bcl-2 functions upstream of caspase activation and prevents apoptosis by suppressing caspase activity [5,6]. However, the biochemical connection between the Bcl-2 and caspases has been elusive. Other investigators reported that Bcl-2 prevents apoptosis by inhibiting the mitochondrial permeability transition pore and blocking the release of cytochrome *c* from mitochondria [7,8].

Although the role of caspases in apoptosis has been investigated extensively [3,4,9,10], much less is known about the ubiquitin-proteasome system and its potential role in mediating programmed cell death. Ubiquitin-proteasome is the cell's major non-lysosomal tool for rapidly degrading and processing proteins for disposal by ATP/ubiquitin-dependent or ubiquitin-independent proteolysis mechanisms [11–13]. The substrates of the proteasome system include regulatory proteins of cell growth and differentiation and several transcription factors such as p53. Ubiquitin-proteasome is expressed abundantly in leukaemic cells [14]. Its expression increases greatly during the malignant transformation of normal blood mononuclear cells. These findings suggest that ubiquitin-proteasome is essential for cell growth and cell cycle progress in actively dividing cells [12,14,15]. The importance of proteasomes in the processing of these events has been established largely by the use of specific proteasome inhibitors. More recently, proteasome inhibitors have been shown to induce apoptosis in several human cell lines [14,16]. In the present study we sought to examine the effect of proteasome inhibitors on the expression of Bcl-2 during apoptosis. We now report that the inhibition of ubiquitin-proteasome pathway with

Abbreviations used: Ac-DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide; CPP32, caspase-3; DEVD-CHO, acetyl-Asp-Glu-Val-Asp-CHO; rhGM-CSF, recombinant human granulocyte/macrophage colony-stimulating factor; YVAD-CHO, acetyl-Tyr-Val-Ala-Asp-CHO; Z-LLL-CHO, benzyloxycarbonyl-Leu-Leu-L-leucinal.

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benzyloxycarbonyl-Leu-Leu-L-leucinal (Z-LLL-CHO; MG-132) and lactacystin led to an extended cleavage of endogenous Bcl-2 and apoptotic death in M-07e leukaemia cells. Further, we showed that the cleavage of Bcl-2 was mediated through a caspase-3 (CPP32)-like protease during apoptosis induced by Z-LLL-CHO and lactacystin.

MATERIALS AND METHODS

Reagents

Recombinant human granulocyte/macrophage colony-stimulating factor (rhGM-CSF) was purchased from Washington Wholesale Drug Exchange (Savage, MD, U.S.A.). Mouse anti-(Bcl-2) monoclonal antibody (SC-509) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal rabbit anti-(caspase 3) antibodies were purchased from PharMingen (San Diego, CA, U.S.A.). Fetal calf serum was a product of GIBCO Co. (Grand Island, NY, U.S.A.). Protease inhibitors and other reagents were purchased from CalBiochem (San Diego, CA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.).

Cells

The M-07e megakaryoblastic leukaemia cell line was a gift from Dr. S. Clark (Genetic Institute, Boston, MA, U.S.A.). This cell line was maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10 % fetal calf serum and 1–10 ng/ml rhGM-CSF [17].

Caspase 3 assay

Caspase 3 activity assay was performed with the use of a highly sensitive colorimetric substrate in accordance with the protocol supplied by the manufacturer (CalBiochem). In brief, cells (10^6 per sample) were lysed in lysis buffer [50 mM Hepes (pH 7.4)/100 mM NaCl/0.1 % (v/v) CHAPS/1 mM dithiothreitol/0.1 mM EDTA] on ice for 5–10 min, then centrifuged at 10000 ***g*** for 10 min for collection of the supernatant. Equal volumes of the lysates were added to equal volumes of assay buffer [50 mM Hepes (pH 7.4)/100 mM NaCl/0.1 % (v/v) CHAPS/10 mM dithiothreitol/0.1 mM EDTA/10 % (v/v) glycerol] and incubated at 37 °C for 10 min. Thereafter, freshly prepared colorimetric substrate [*N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA), final concentration 200 μM] was added to the mixtures. The samples were mixed and A_{405} was recorded every 30 min for a total of 2–4 h at room temperature. Control cultures without cell lysates were used as controls. Enzyme activity was calculated, by using the formula provided by the manufacturer, as pmol/min.

Apoptosis assay

Apoptotic cell staining was performed as described previously [18,19]. In brief, cells were stained with Acridine Orange (10 μl of a 100 μg/ml solution into 100 μl of cell suspension) in the dark. At least 100 cells were counted under a fluorescence microscope. Cells with condensed chromatin were counted as positive, whereas those with a normal chromatin pattern were counted as negative. Apoptosis also was confirmed by using a TdT FragEL™ DNA fragmentation detection kit (Oncogene Research, Cambridge, MA, U.S.A.) performed in accordance with the manufacturer's instructions.

Immunoblotting

Total cell lysates were boiled in an equal volume of 2 × SDS sample buffer with 2-mercaptoethanol (1 mM) for 5 min. The samples were subjected to one-dimensional SDS/PAGE [8 cm × 10 cm; 8–10 % (w/v) gel]. After electrophoresis, proteins were transferred from the gel to a nitrocellulose filter (0.2 μm pore size; Schleicher and Schuell, Keene, NH, U.S.A.) overnight at 14 V and 4 °C, or for 4 h at 40 V and room temperature. Non-specific binding sites on the filter were blocked by incubating the nitrocellulose filter in blocking buffer [3 % (v/v) milk] for 1 h at room temperature [20]. The blots were washed in Tris-buffered saline and incubated for 1–2 h at room temperature with the primary antibodies [anti-(Bcl-2) monoclonal antibody or rabbit anti-(caspase 3) antibodies]. After the removal of primary antibodies with extensive washes, the blots were incubated with a secondary antibody (goat anti-mouse or goat anti-rabbit antibodies conjugated with horseradish peroxidase). The blots were developed with the enhanced chemiluminescence (ECL) substrate in accordance with the manufacturer's protocol (Amersham, Arlington Heights, IL, U.S.A.).

RESULTS

Z-LLL-CHO triggers extended cleavage of Bcl-2 in M-07e cells

Apoptosis in M-07e cells, induced by depletion of rhGM-CSF, was accompanied by a gradual cleavage of Bcl-2 into a 22 kDa fragment (Figure 1A). The cleaved 22 kDa product was quite stable and accumulated steadily with time during apoptosis. At 72 h after the removal of rhGM-CSF, approximately two-thirds of the natural Bcl-2 had been cleaved into 22 kDa fragments. Unexpectedly, treatment of M-07e cells with Z-LLL-CHO, a reversible proteasome inhibitor, markedly accelerated the process of Bcl-2 cleavage (Figure 1B). In the absence of exogenous rhGM-CSF, Z-LLL-CHO (20 μM) induced a complete cleavage of Bcl-2 within 24 h. The degree of Bcl-2 cleavage induced by Z-LLL-CHO (at 0.1 and 1.0 μM) was slightly inhibited in the presence of exogenous rhGM-CSF (Figures 1B and 1C). At high concentrations of Z-LLL-CHO (10 μM or more), rhGM-CSF had essentially no effect in preventing the cleavage of Bcl-2.

Effects of Z-LLL-CHO on the activation of caspase 3

To analyse further the underlying mechanism responsible for the cleavage of Bcl-2 by Z-LLL-CHO, we studied the possibility that Z-LLL-CHO-induced Bcl-2 cleavage was mediated through caspase 3 or related proteases. Caspase 3 is activated by multiple proteolytic cleavages of its 32 kDa precursor form to generate an enzymically active p12/p17 complex, which has been used to monitor the activation of caspase 3 [3–6,9]. Control cultures of M-07e cells express high levels of the 32 kDa caspase 3 precursors. Treatment of the cells with Z-LLL-CHO induced a rapid activation of caspase 3, which was accompanied by the appearance of the p17 fragment and a gradual decrease in the levels of caspase 3 precursors (Figure 2A). Prolonged treatment with Z-LLL-CHO (24 h or more) resulted in complete degradation and the loss of the 32 kDa precursor form. Similarly, in the presence of rhGM-CSF (50 ng/ml), the extent of caspase 3 cleavage induced by low concentrations of Z-LLL-CHO (0.1 and 1 μM) was partly inhibited. At high concentrations of Z-LLL-CHO, rhGM-CSF showed only a limited effect, if any, in preventing the cleavage and activation of caspase 3 (Figure 2B).

Effects of acetyl-Asp-Glu-Val-Asp-CHO (DEVD-CHO) and acetyl-Tyr-Val-Ala-Asp-CHO (YVAD-CHO) on Bcl-2 and caspase 3

The preceding experiments show that Z-LLL-CHO can activate caspase 3 and induce Bcl-2 cleavage in M-07e cells. To determine whether the cleavage of Bcl-2 induced by Z-LLL-CHO was

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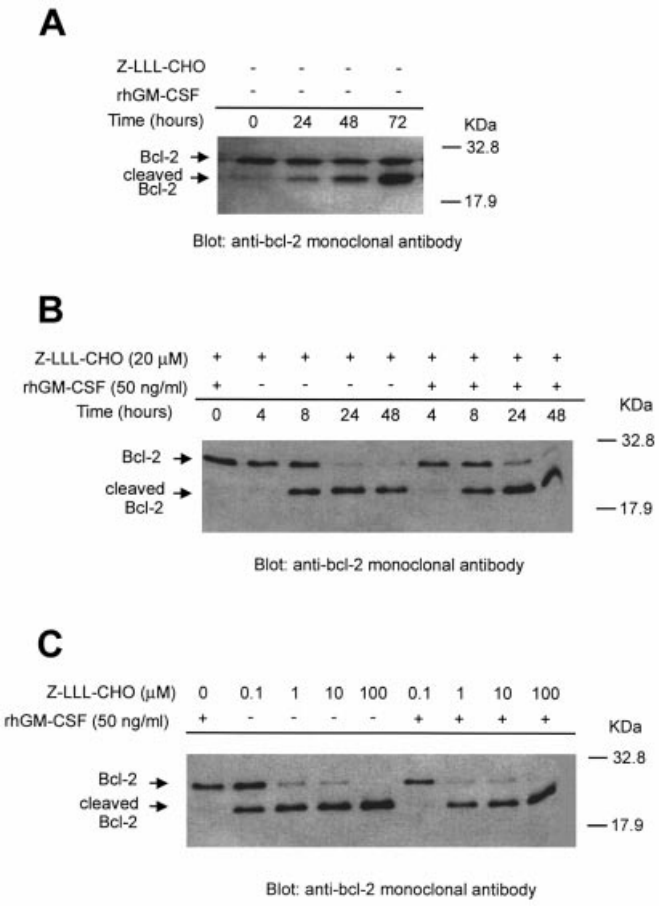

Figure 1 Induction of Bcl-2 cleavage by Z-LLL-CHO in M-07e cells

(**A**) M-07e cells were starved in cytokine-free medium for 24, 48 and 72 h. Cells were lysed and immunoblotted with anti-(Bcl-2) antibodies. Note the appearance of a cleaved 22 kDa Bcl-2 fragment during apoptosis. (**B**) M-07e cells were treated with Z-LLL-CHO (20 μM) in the presence or absence of rhGM-CSF (50 ng/ml) for 4, 8, 24 and 48 h. At the end of the treatment, cells were lysed and immunoblotted with anti-(Bcl-2) antibodies. (**C**) M-07e cells were exposed to various concentrations of Z-LLL-CHO as indicated, in the presence or absence of rhGM-CSF (50 ng/ml) for 24 h. Total cell lysates were subjected to an immunoblot analysis with anti-(Bcl-2) antibodies. In all panels the positions of molecular mass markers are indicated (in kDa) at the right.

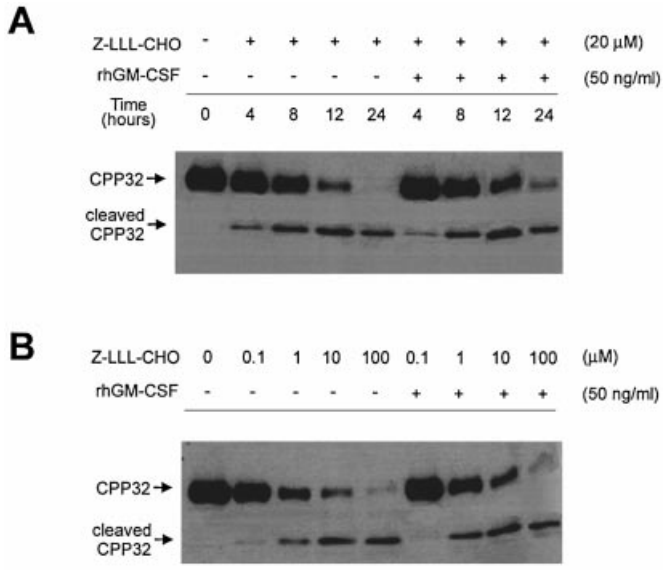

Figure 2 Induction of CPP32 activation by Z-LLL-CHO

(**A**) Induction of caspase 3 (CPP32) activation by Z-LLL-CHO. M-07e cells were treated with Z-LLL-CHO in the presence or the absence of rhGM-CSF for 4, 8, 12 and 24 h. At the end of the treatment, total cell lysates were prepared and subjected to an immunoblot analysis with anti-(caspase 3) antibodies. Note the appearance of the p17 subunit from cleaved caspase 3. (**B**) M-07e cells were treated with various concentrations of Z-LLL-CHO as indicated in the presence or absence of rhGM-CSF (50 ng/ml) for 24 h. At the end of the treatment, cells were lysed and the lysates were subjected to an immunoblot analysis with anti-(caspase 3) antibodies.

processed through activated caspase 3 or related proteases, we studied the effects of DEVD-CHO (an inhibitor of caspase 3/caspase 7) and YVAD-CHO (an inhibitor of caspase 1/interleukin-1β-converting enzyme) on the cleavage of Bcl-2 in M-07e cells induced to undergo apoptosis. As shown in Figure 3(A), treatment of M-07e cells with DEVD-CHO effectively inhibited the cleavage of Bcl-2, whereas YVAD-CHO had no obvious effect in preventing the cleavage of Bcl-2 during apoptosis induced by cytokine depletion. In contrast, treatment with Z-LLL-CHO caused an accelerated cleavage of Bcl-2 in these cells, similar to that shown in Figure 1. In addition, prolonged (40 h) treatment of M-07e cells with Z-LLL-CHO resulted in the almost complete degradation of the 32 kDa caspase 3 precursors (Figure 3B). The levels of p17 fragment also decreased markedly after prolonged treatment with Z-LLL-CHO. Exposure of M-07e cells to DEVD-CHO 2 h before the addition of Z-LLL-CHO also markedly prevented Z-LLL-CHO-induced Bcl-2 cleavage in M-07e cells (Figure 3C).

Effect of rhGM-CSF on the activation of caspase 3 induced by Z-LLL-CHO

To study further the role of the GM-CSF signalling pathway on the activation of caspase 3 and cleavage of Bcl-2, we employed a highly sensitive colorimetric assay to quantify the levels of caspase 3 activity. A control culture of M-07e cells containing rhGM-CSF exhibited a low but detectable level of caspase 3 activity (Figure 4A). Elevation of caspase 3 activity was detected within 2 h of the removal of exogenous rhGM-CSF. The levels of caspase 3 activity increased gradually, reaching a plateau level 24 h later (Figure 4A). Treatment of M-07e cells with Z-LLL-CHO after rhGM-CSF depletion induced a rapid elevation of caspase 3 activity, reaching a maximum level within 8 h. By comparison, in the presence of rhGM-CSF the activation of caspase 3 induced by Z-LLL-CHO was markedly delayed, reaching a maximum level in 24 h instead of 8 h. Similarly, the effect of rhGM-CSF in preventing the activation of caspase 3 was most prominent when low concentrations of Z-LLL-CHO (0.1 and 1.0 μM) were used to induce apoptosis in M-07e cells. At high concentrations of Z-LLL-CHO (more than 10 μM), rhGM-CSF had only limited or no inhibitory effect on the activation of caspase 3 (Figure 4B).

Z-LLL-CHO promotes cell death through apoptotic mechanisms

The survival of M-07e cells *in vitro* is strictly dependent on exogenous rhGM-CSF. A control culture of M-07e cells containing rhGM-CSF exhibited approx. 1–3% 'spontaneous' cell death. Treatment of M-07e cells with Z-LLL-CHO markedly

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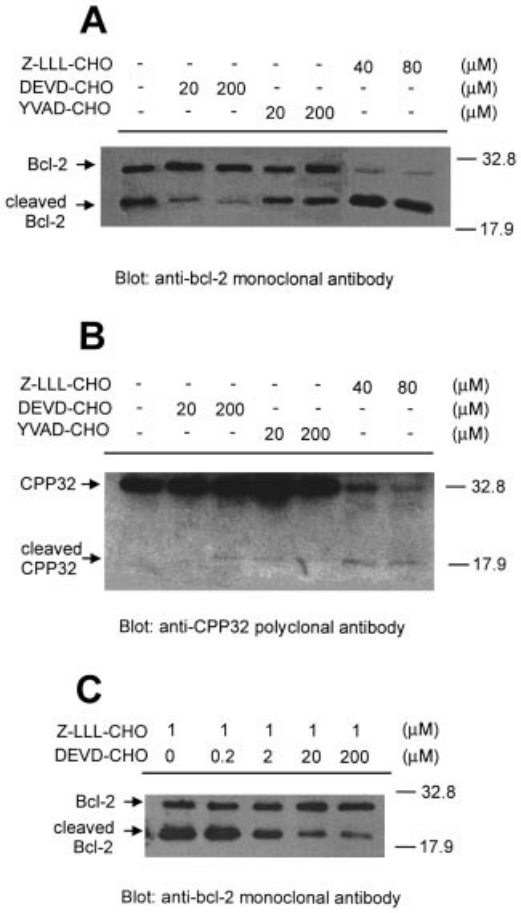

Figure 3 DEVD-CHO inhibits Bcl-2 cleavage

(**A**) Inhibition of Bcl-2 cleavage by DEVD-CHO during apoptosis. After the removal of rhGM-CSF, M-07e cells were incubated in the presence of DEVD-CHO, YVAD-CHO or Z-LLL-CHO as indicated for 40 h. Total cell lysates were prepared and subjected to immunoblot analysis with anti-(Bcl-2) antibodies. Note the absence of the 22 kDa Bcl-2 fragment from DEVD-CHO-treated cells. (**B**) Degradation of caspase 3 (CPP32) by prolonged treatment with Z-LLL-CHO but not with caspase inhibitors. M-07e cells were treated as described in (**A**) for 40 h. Total cell lysates were prepared and subjected to immunoblot analysis with anti-(caspase 3) antibodies. Note the disappearance of caspase 3 from M-07e lysates after prolonged treatment with Z-LLL-CHO. (**C**) The caspase 3 inhibitor DEVD-CHO inhibits Z-LLL-CHO-induced Bcl-2 cleavage in M-07e cells. Cells were exposed to DEVD-CHO as indicated for 2 h followed by treatment with Z-LLL-CHO for an additional 8 h. Thereafter, cells were lysed and total cell lysates were subjected to an immunoblot with Bcl-2 antibodies. In all panels the positions of molecular mass markers are indicated (in kDa) at the right.

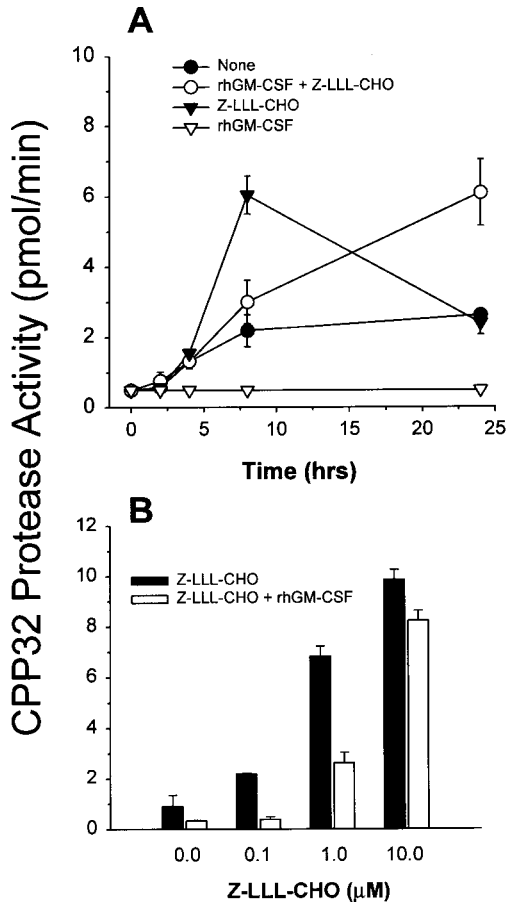

Figure 4 Activation of caspase 3 (CPP32) by Z-LLL-CHO

(**A**) M-07e cells (10^6/ml) were treated with Z-LLL-CHO (1 μM) or rhGM-CSF (10 ng/ml) or both as indicated for 0, 2, 4, 8 and 24 h. After treatment, cells were removed, washed and lysed for caspase 3 assay with the colorimetric substrate Ac-DEVD-pNA. (**B**) Inhibition of caspase 3 activation by rhGM-CSF in M-07e cells. M-07e cells (10^6/ml) were incubated in the presence of various concentrations of Z-LLL-CHO as indicated with or without rhGM-CSF (10 ng/ml) for 8 h. At the end of the treatment, cells (10^6 per sample) were lysed and the lysates were assayed for caspase 3 activity. Results are means $\pm$ S.D. for duplicate experiments.

accelerated the process of cell death in a dose-dependent manner. As shown in Figure 5(A), at the highest dose used in this study (100 μM), Z-LLL-CHO caused 95–100 % cell death in M-07e cell cultures within 24 h (Figure 5A). That Z-LLL-CHO induced apoptosis in M-07e cells was demonstrated by the appearance of marked nuclear fragmentation and chromatin condensation, a morphology indicative of apoptosis, in cells after staining with Acridine Orange (Figure 5B). In addition, Z-LLL-CHO-induced apoptotic cell death was confirmed by using a DNA fragmentation assay (X.-m. Zhang and B. Chen, unpublished work). In the absence of rhGM-CSF, Z-LLL-CHO further accelerated the process of apoptotic death in M-07e cells. In the presence of exogenous rhGM-CSF, cell death induced by a low dose of Z-LLL-CHO (1 μM or less) was partly inhibited (Figure 5C).

Induction of Bcl-2 cleavage and caspase 3 activation by lactacystin

To establish further the role of the proteasome pathway in apoptosis, we performed additional experiments using a different type of ubiquitin proteasome inhibitor, lactacystin. Lactacystin blocks proteasome activity by targeting the catalytic β-subunit of the 20 S proteasome in a highly specific and irreversible manner. As shown in Figure 6, treatment of M-07e cells with lactacystin induced the cleavage of Bcl-2 into a 22 kDa fragment similar to that caused by Z-LLL-CHO (Figure 1). Bcl-2 cleavage was detected as early as 4 h after the addition of lactacystin. The cleavage of Bcl-2 was slightly inhibited in the presence of rhGM-CSF. Similarly, the cleavage of Bcl-2 induced by lactacystin was associated with the activation of a caspase-3-like protease. As shown in Figure 7(A), lactacystin treatment induced a transient elevation of caspase 3 activity in M-07e cells, reaching a peak 8 h after the treatment. The activation of caspase 3 activity was slightly but not significantly inhibited in the presence of rhGM-CSF (Figure 7B). In comparison with Z-LLL-CHO, lactacystin

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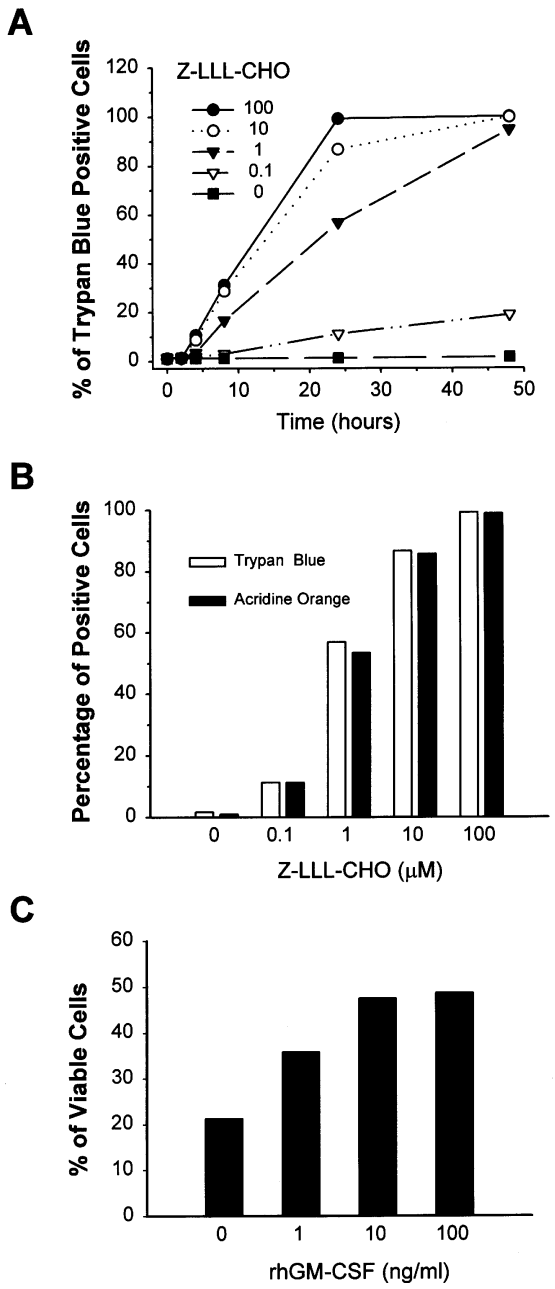

Figure 5 Induction of apoptotic cell death in M-07e cells by Z-LLL-CHO

(**A**) M-07e cells were incubated with various concentrations of Z-LLL-CHO as indicated plus 10 ng/ml rhGM-CSF for various periods. At the end of the incubation, cell viability was determined with the Trypan Blue uptake method. (**B**) M-07e cells were incubated with various concentrations of Z-LLL-CHO as indicated plus 10 ng/ml rhGM-CSF for 24 h. Apoptotic cells were determined by morphological examination after staining with Acridine Orange. (**C**) Inhibition of Z-LLL-CHO-induced cell death by rhGM-CSF. M-07e cells were treated with Z-LLL-CHO (1 μM) plus various concentrations of rhGM-CSF as indicated for 24 h; 200 cells were scored per sample. Results are the means for duplicate determinations from one representative experiment.

exerted a more potent effect on the induction of apoptotic cell death in M-07e cells. Treatment of the cells with lactacystin (0.1 μM) for 24 h was sufficient to induce nearly 50 % cell death in M-07e cultures (Figures 7C and 7D). At 10 μM, lactacystin caused more than 90 % cell death 24 h after treatment. There was a slight but non-significant effect of exogenous rhGM-CSF in preventing apoptotic cell death induced by lactacystin.

DISCUSSION

In the present study we have shown that apoptosis in M-07e cells proceeds with a gradual cleavage of Bcl-2 into a 22 kDa fragment. The cleaved Bcl-2 fragment seems to be quite stable, as evidenced by the steady accumulation of the cleaved fragments inside the cells. In an attempt to analyse further the mechanism of Bcl-2 cleavage, we found unexpectedly that blocking the ubiquitin-proteasome pathway by Z-LLL-CHO markedly promoted the cleavage of Bcl-2 in M-07e cells. Z-LLL-CHO is a leupeptin analogue that can decrease the degradation of ubiquitin-conjugated proteins by the 26 S protease complex without affecting its ATPase or isopeptidase activities. We showed that the inhibition of the proteasome pathway by Z-LLL-CHO can induce an almost complete cleavage of endogenous Bcl-2 within 24 h (Figure 1), an event that has not been reported previously in apoptotic cells induced by various stimuli. The mechanism whereby Z-LLL-CHO induces Bcl-2 cleavage is not known at present. However, with the use of specific caspase inhibitors, our results showed that Bcl-2 was cleaved by a caspase-3-like protease. The cleavage of Bcl-2 was inhibited by DEVD-CHO, an inhibitor of caspase 3/caspase 7, but not other inhibitors (Figure 3). Further, we showed that, before the cleavage of Bcl-2, caspase 3 was activated in M-07e cells by Z-LLL-CHO as determined by two different approaches. Induction of apoptosis by the inhibition of proteasome pathway also was verified in studies using lactacystin, a highly specific proteasome inhibitor. Lactacystin blocks proteasome activity irreversibly by targeting the catalytic β-subunit of the 20 S proteasome. We showed that lactacystin-induced apoptosis in M-07e cells was remarkably similar to that induced by Z-LLL-CHO, which included the activation of caspase 3, the cleavage of Bcl-2 into a 22 kDa fragment and, ultimately, cell death. Taken together, our findings show that the inhibition of proteasomes induces Bcl-2 cleavage by a caspase-3-like protease and promotes apoptosis in M-07e cells.

The importance of Bcl-2 in regulating apoptosis has been addressed previously in various cell types and tissues [2,5–8,21]. However, the occurrence of Bcl-2 cleavage as described in this study has not been widely reported. Hsu et al. [19] recently showed that retinoid-induced apoptosis in human leukaemic cells was associated with the cleavage of Bcl-2 into a shortened fragment. Using transfected cells overexpressing caspase 3, Cheng et al. [22] showed that Bcl-2 can be transformed into a Bax-like death-effector by cleavage of caspase 3 during apoptosis induced by Fas ligation and interleukin 3 depletion. In contrast, no Bcl-2 cleavage was detected in GM-CSF-dependent TF-1 leukaemic cells induced to undergo apoptosis by ionizing radiation [23]. Therefore the occurrence of Bcl-2 cleavage might depend primarily on the degree of caspase 3 activation and the levels of Bcl-2 in the cells, both of which are expressed abundantly in M-07e cells. The lack of reports of Bcl-2 cleavage also could be attributable to some trivial causes such as the anti-(Bcl-2) antibodies used in the immunoblot. It is conceivable that many of the commercially available antibodies do not recognize the cleaved Bcl-2 fragment.

The detailed mechanisms responsible for the activation of caspase 3 by Z-LLL-CHO and lactacystin are not known. However, Drexler [24] reported that proteasomal inhibitors can induce caspase 3 activation and apoptosis in HL60 human leukaemic cells primarily in the G_1 phase of the cell cycle. Furthermore, proteasome inhibitors even can protect quiescent

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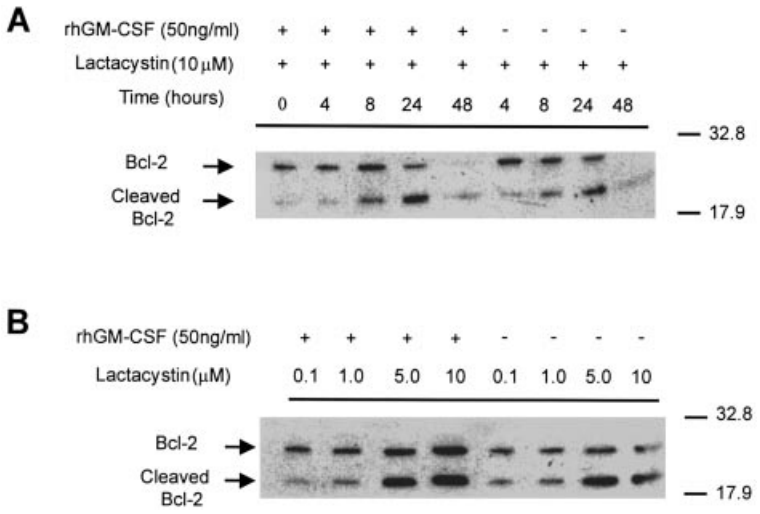

Figure 6 Induction of Bcl-2 cleavage by lactacystin in M-07e cells

(**A**) M-07e cells were treated with lactacystin (10 μM) in the presence or absence of rhGM-CSF (50 ng/ml) for 4, 8, 24 and 48 h. At the end of the treatment, cells were lysed and immunoblotted with anti-(Bcl-2) antibodies. (**B**) M-07e cells were exposed to various concentrations of lactacystin as indicated in the presence or absence of rhGM-CSF (50 ng/ml) for 24 h. Total cell lysates were subjected to an immunoblot analysis with anti-(Bcl-2) antibodies. In both panels the positions of molecular mass markers are indicated (in kDa) at the right.

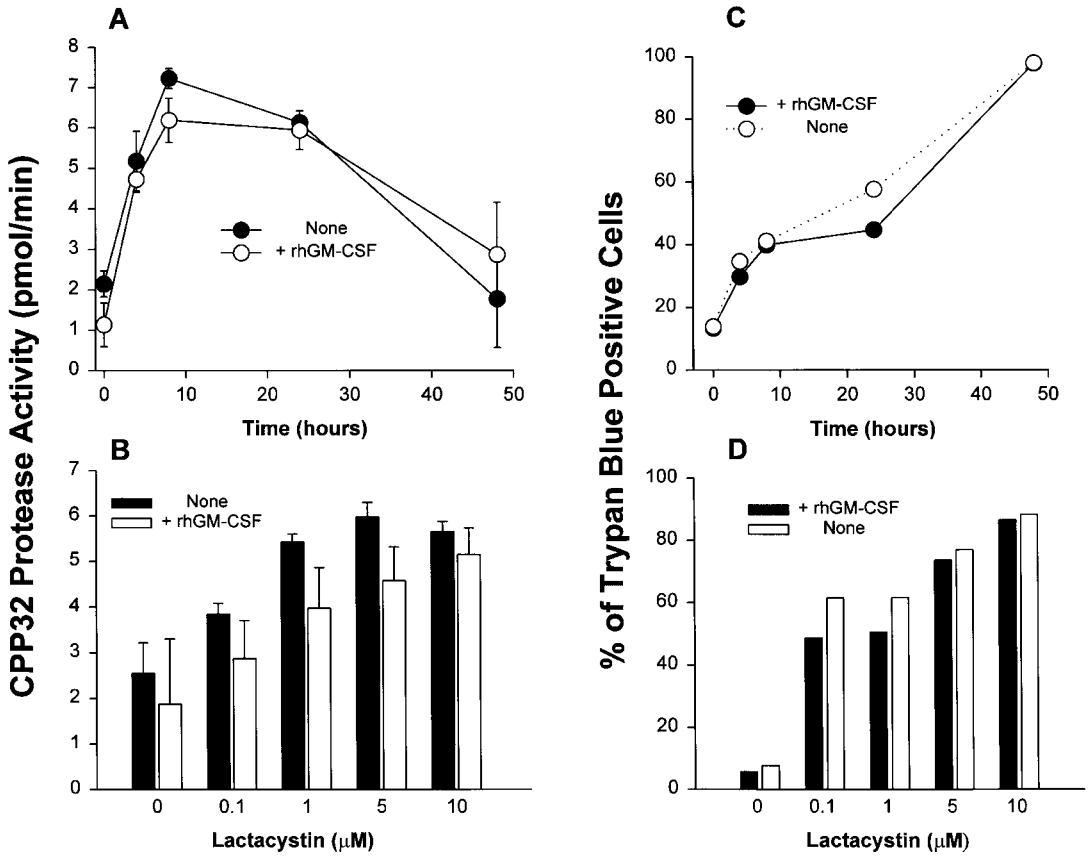

Figure 7 Induction of CPP32 activation by lactacystin

(**A**) Activation of caspase 3 in M-07e cells (10^6/ml) by lactacystin (10 μM) in the presence or absence of rhGM-CSF (50 ng/ml). Caspase 3 activity (10^6 cells per sample) was determined at the time indicated with the use of the colorimetric substrate Ac-DEVD-pNA. (**B**) M-07e cells (10^6/ml) were treated with various concentrations of lactacystin as indicated in the presence or absence of rhGM-CSF (50 ng/ml) for 24 h. Results are means $\pm$ S.D. for duplicate experiments. (**C**) Induction of apoptotic cell death in M-07e cells by lactacystin. M-07e cells (10^6/ml) were incubated with 10 μM of lactacystin in the presence or absence 50 ng/ml rhGM-CSF. At the end of the incubation period, cell viability was determined with the Trypan Blue uptake method. (**D**) M-07e cells were incubated with various concentrations of lactacystin as indicated in the absence or presence of rhGM-CSF (50 ng/ml) for 24 h. Cell death was determined with the Trypan Blue uptake method; 200 cells were scored per sample. Results are the means for duplicate determinations from one representative experiment.

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cell cultures such as differentiated and normal tissue cells from apoptosis. This finding suggests that proteasomal activity is required in proliferating, but not quiescent, cells for survival and normal progression through the cell cycle, and inhibition of proteasomal activity in G_1 can inevitably trigger programmed cell death. This finding raises the possibility of developing novel anti-cancer drugs that can specifically trigger apoptosis in proliferating cancer cells but not in normal quiescent and differentiated cells. These inhibitors might be useful as a purging agent to eliminate metastatic cancer cells before autologous bone marrow transplantation.

The effect of proteasome inhibitors is not limited to the activation of caspase 3 as shown in the present study. Meriin et al. [25] recently reported that inhibition of proteasome pathway can lead to a steady increase in activity of c-Jun N-terminal kinase, JNK, a stress kinase that has been implicated in mediating apoptosis and inducing heat shock proteins (Hsp72). Furthermore, inhibition of the JNK signalling pathway by accumulated Hsp72 can suppress proteasome inhibitor-induced apoptosis. Thus the execution of apoptotic process might require the activation of multiple pathways including caspase 3 by the protease inhibitors. More recently, Beyette et al. [26] reported that apoptosis of thymocytes induced by dexamethasone was associated with a down-regulation of proteolytic activities in both 20 S and 26 S proteasomes. The decreases in trypsin-like and peptidylglutamylpeptide hydrolase activities of the proteasome complexes were reversed by the apoptosis inhibitors. However, the decrease in the chymotrypsin-like activity of the proteasomes was further decreased in the presence of the apoptosis inhibitors. Their results suggest that although proteasomes are required for apoptosis, they might be protective against apoptosis in some circumstances, illustrating the complex nature of the proteasome systems in regulating apoptosis.

The biological relevance of the 22 kDa Bcl-2 fragment in apoptosis is a matter for speculation. Cleavage of Bcl-2 might be a means of effectively removing the anti-apoptotic effect of Bcl-2. Current results favour the notion that Bcl-2 functions upstream of caspase activation and prevents apoptosis by suppressing caspase 3 [27–29]. Our results have demonstrated that Bcl-2 can act as a downstream substrate of caspase 3 or a caspase-3-like protease, raising the possibility that the cleaved 22 kDa fragment might have a direct role in mediating the amplification of the death effects during apoptosis [22]. For example, the C-terminal products from the cleaved Bcl-2 protein might participate actively in the apoptotic pathway and trigger cell death by forming ion channels in the membranes [2,7,8]. Bcl-2 contains a hydrophobic stretch of amino acids at their C-terminus that cause them to insert into membranes. Conceivably, cleavage at the N-terminus of Bcl-2 might further increase its pore-forming capacity and therefore the death effect in the apoptotic process. In support of this view, a recent study by Kim et al. [30] showed that suppression of apoptosis in MCF-7 human breast cancer cells by nitric oxide is associated with an inhibition of Bcl-2 cleavage and cytochrome *c* release. Interestingly, the cleavage of Bcl-2 in MCF-7 cells during apoptosis was also mediated through a caspase-3-like protease whose activation was inhibited by nitric oxide during the process.

Although our results do not support the notion that the overexpression of Bcl-2 inhibits caspase 3 activation in M-07e cells, we found that rhGM-CSF can partly inhibit the activation of caspase 3 and cleavage of Bcl-2 in M-07e cells treated with Z-LLL-CHO and lactacystin. Treatment of M-07e cells with rhGM-CSF can partly inhibit apoptosis induced by both inhibitors. Because Lyn kinase is a key signal transducing element of GM-CSF receptor [17,31], our findings suggest that the Lyn kinase pathway might have a role in the suppression of caspase 3 and Bcl-2 cleavage, both of which might be crucial in regulating apoptosis induced by proteasome inhibitors. This view is supported by a study by Wei et al. [32] showing that the activation of Lyn kinase by GM-CSF inhibited neutrophil apoptosis.

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