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Peptidyl Aldehyde Inhibitors of Proteasome Induce Apoptosis Rapidly in Mouse Lymphoma RVC Cells[1]

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Proteases play an important role in regulation of apoptosis. To elucidate the role of proteasome in apoptosis, we examined the effects of the proteasome inhibitors, carbobenzoxy-L-isoleucyl-γ-t-butyl-L-glutamyl-L-alanyl-L-leucinal and carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal on RVC lymphoma cells. Cells exposed to the proteasome inhibitors arrested at G2/M phase followed by internucleosomal DNA cleavage, chromatin condensation, and formation of apoptotic bodies dose- and time-dependently. Ubiquitinated histone H2A levels decreased in the exposed cells, suggesting a relationship between deubiquitination of histone H2A and the chromatin disarray seen in apoptosis. Northern blots showed an increase in expression of polyubiquitin genes early in the incubation. These findings suggest that the ubiquitin-mediated proteasome-proteolytic system is involved in regulating the cell cycle and apoptosis in RVC cells.

Key words: apoptosis, cell cycle, lymphoma cells, proteasome inhibitor, ubiquitin.

Apoptosis, a mode of cell death, plays a significant role in normal embryonic development and physiological tissue homeostasis (*1, 2*). Apoptosis entails characteristic morphological and biochemical changes such as cell shrinkage, chromatin condensation, cell fragmentation called "apoptotic bodies" and chromosome DNA cleavage at internucleosomal sites (*1, 2*). However, the molecular mechanisms of apoptosis remain unknown. We and others have shown that thymocyte apoptosis, by which self-reactive cells are eliminated, is regulated by cross-talking of various signaling pathways (*3, 4*). Triggered by diverse stimuli, these pathways are reversibly regulated *via* protein phosphorylation and dephosphorylation by protein kinases and phosphatases (*5*). Intracellular proteases may play important roles in the commitment to and execution of apoptosis. Protease inhibitors such as *N*-tosyl-L-lysyl-chloromethylketone (TLCK) and *N*-tosyl-L-phenylalanyl-chloromethylketone (TPCK) prevent internucleosomal DNA cleavage in thymocytes and in a tumor cell line (*6-8*). Both TLCK and TPCK inhibit some cysteine and serine proteases. A more general role of proteases in apoptosis is indicated by the observation that the product of the cell death gene, *ced-3*, in the nematode *Caenorhabditis elegans* shares sequence homology with the well-characterized human protein interleukin-1β-converting enzyme (ICE) and that both proteins contain a conserved pentapeptide domain in the active site (*9*). A number of polypeptides with structural homology to ICE and CED-3 have been identified and evaluated in terms of a role in apoptosis (*10, 11*). However, in ICE-knockout mice, negative selection through apoptosis occurs in the thymus, suggesting that a protease(s) other than ICE or its homologs is involved in apoptosis (*12*).

The ubiquitin-mediated proteasome-proteolytic system is involved in many physiological functions including the cell cycle, cellular stress response, ribosome biogenesis, and signal transduction (*13, 14*). Ubiquitin is a highly conserved, 76-amino-acid-residue protein that conjugates to the ε-amino group of lysine residues of target proteins through an isopeptide linkage. Protein degradation by the ubiquitin-mediated proteasome-proteolytic system consists of two separable steps: ubiquitin conjugation of target proteins by an ATP-dependent enzymatic pathway composed of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3), and successive degradation of multiubiquitinated proteins by the 26S proteasome (*13, 14*). In addition to the degradation of denatured proteins, tumor suppressor protein p53 or oncogene products c-Myc and c-Fos are selectively degraded by the ubiquitin-proteasome pathway (*13, 14*). These proteins are related to the regulation of apoptosis, indicating that ubiquitin-mediated protein degradation is involved in the regulation of apoptosis in mammalian cells. Ubiquitin is also involved in monoubiquitination of many proteins such as actin, platelet-derived growth factor receptor, growth hormone receptor, and histone H2A and H2B. About 10-15% of histone H2A is ubiquitinated (*15*). Deubiquitination of ubiquitinated histone H2A (uH2A)

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Abbreviations: AcYVAD, acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartinal; AcYVKD, acetyl-L-tyrosyl-L-valyl-L-lysyl-L-aspartinal; AcLLM, acetyl-L-leucyl-L-leucyl-L-methioninal; AcLLnL, acetyl-L-leucyl-L-leucyl-norleucinal; AcDEVD, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartinal; AUT, acid-urea-triton; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICE, interleukin-1β-converting enzyme; TLCK, *N*-tosyl-L-lysyl-chloromethylketone; TPCK, *N*-tosyl-L-phenylalanyl-chloromethylketone; ZIE(OBu)AL, carbobenzoxy-L-isoleucyl-γ-t-butyl-L-glutamyl-L-alanyl-L-leucinal; ZLLnV, carbobenzoxy-L-leucyl-L-leucyl-norvalinal.

that is implicated in chromatin condensation in mitosis (*16, 17*) is also observed in TGFβ1-induced apoptosis associated with chromatin condensation (*18*), suggesting that the state of monoubiquitination is closely related to apoptosis. In the present paper, we show that proteasome inhibitors induce cell cycle arrest at G2/M phase and apoptosis in RVC cells in an early period of incubation. With the progression of apoptosis, the histone uH2A content was reduced and polyubiquitin gene transcription was highly activated. These results suggest that apoptosis in RVC cells is in part regulated by the ubiquitin-proteasome pathway.

MATERIALS AND METHODS

Materials—Proteasome inhibitors carbobenzoxy-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal [ZIE(OBu)AL] and carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal (ZLLnV), interleukin-1β converting enzyme inhibitors acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartinal (AcYVAD), acetyl-L-tyrosyl-L-valyl-L-lysyl-L-aspartinal (AcYVKD), and the inhibitor of CPP32 acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartinal (AcDEVD) were purchased from Pep-tide Institute (Osaka). The calpain inhibitor I acetyl-L-leucyl-L-leucyl-L-norleucinal (AcLLnL), the calpain inhibitor II acetyl-L-leucyl-L-leucyl-L-methioninal (AcLLM), and the serine protease inhibitor TLCK were purchased from Sigma (St. Louis, MO). Culture medium was obtained from Immuno Biological Laboratories (Gunma).

Cell Culture—A murine T-cell lymphoma line, RVC (*19*), was routinely cultured in RPMI1640 medium containing 10% heat-inactivated fatal bovine serum, 50 μM 2-mercaptoethanol, 0.1 mg/ml of streptomycin, and 100 U/ml of penicillin at 37°C in a humidified incubator under a 5% CO_2 atmosphere.

Cellular Morphological Examination—RVC cells were observed by phase contrast microscopy and by light microscopy after May-Giemsa staining.

DNA Fragmentation and Cell Viability—After incubation, RVC cells were harvested and lysed in 0.5% SDS containing 25 mM EDTA and 75 mM NaCl for over 3 h on ice (*20*). Fragmented and intact DNA were separated by centrifugation at 100,000 $\times g$ for 30 min at 0°C. The DNA content in the supernatant and in the pellet was measured by the described fluorometric method (*20*). The percentage of fragmented DNA was defined as the ratio of DNA in the supernatant (fragmented DNA) to that in the precipitate plus supernatant (total DNA). Cell viability was assessed by Trypan blue exclusion.

DNA Electrophoresis—RVC cells (2×10^5) were harvested and lysed in 50 mM Tris-HCl, pH 7.8, containing 0.5% sodium lauroyl sarcosilate and 10 mM EDTA. The samples were treated with RNase A (0.5 mg/ml) at 50°C for 15 min, then with proteinase K (0.5 mg/ml) at 50°C for 30 min. The samples were loaded onto a 1.8% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) containing 0.5 mg/ml of ethidium bromide, and resolved using a submerged gel electrophoresis system. Thereafter, bands were visualized under UV light.

Cell Cycle Analysis—RVC cells (2×10^6) were harvested and washed with phosphate-buffered saline. The cells were suspended in propidium iodide (50 μg/ml) containing 0.1% NP-40, 10 μg/ml of RNase A, 10 mM NaCl, and 3.4 mM sodium citrate, then incubated at room temperature for 10 min. The nuclei were washed with 10 mM NaCl containing 3.4 mM sodium citrate, resuspended in the same solution, then the cell cycle population was analyzed by flow cytometry (Cyto Ace 150, Jasco, Tokyo).

Analysis of Ubiquitinated Histones—RVC cells (5×10^6) were suspended in phosphate-buffered saline, and an equal volume of 0.8 N sulfuric acid was added. The samples were briefly disrupted using a Sonifier (Branson) and kept on ice for 30 min to extract core histones. Core histones in the supernatant obtained by centrifugation at 12,000 $\times g$ for 20 min were precipitated by adding an equal volume of 40% (w/v) trichloroacetic acid and recovered by centrifugation at 20,000 $\times g$ for 20 min. The precipitate was washed twice with cold acetone and dried under vacuum. Dried samples were dissolved in 10 M urea, and samples with 20 μg of protein were resolved by acid-urea-Triton polyacrylamide gel electrophoresis for the first dimension and SDS-polyacrylamide gel electrophoresis for the second dimension (*21*). The gels were then stained with silver.

Northern Blots—Total RNAs of RVC cells exposed to proteasome inhibitor were isolated by the method of Chomczynski and Sacchi (*22*). Poly(A)$^+$ RNA was prepared by the method of Kuribayashi *et al.* (*23*) from RVC cells that had been incubated with proteasome inhibitor. The mRNA samples were resolved on a 1.5% agarose/formaldehyde gel in MOPS buffer and transferred to Nytran (Schleicher & Schuell, Keene, NH). The blotted membrane was hybridized with biotinylated probes. Mouse polyubiquitin mRNA transcripts were detected using a 30 mer biotinylated ubiquitin probe (5′GGTCTTGCCGGTCAGGGTCTTCACGAAGAT3′) complementary to the translation region (bases 7 to 36) of mouse polyubiquitin gene (*24*). Human GAPDH cDNA was used as a control probe (Clontech, Palo Alto, CA). Hybridization was performed as described by Sambrook *et al.* (*25*). After washing, the membrane was incubated with streptavidin-HRP (Vector, Burlingame, CA) and with Renaissance solution (DuPont NEN, Boston, MA) and exposed to X-ray film.

RESULTS

Proteasome Inhibitors Induced Apoptosis Rapidly in RVC Cells—To investigate the role of proteasome in apoptosis, we examined the effects of proteasome inhibitors as well as other protease inhibitors on RVC cells. RVC cells were incubated with various protease inhibitors for 6 h and the DNAs extracted were analyzed by agarose gel electrophoresis (Fig. 1). Proteasome inhibitors ZIE(OBu)AL and ZLLnV and the calpain inhibitor I, AcLLnL, induced a ladder DNA fragmentation with multiples of 200 bp. DNA fragmentation induced by ZIE(OBu)AL and ZLLnV reached about 33 and 34% at 6 h, respectively. AcLLnL induced 25% DNA fragmentation, but other protease inhibitors including TLCK, calpain inhibitor II (AcLLM), and peptidyl aldehyde inhibitors of ICE, AcYVAD, and AcYVKD, did not induce DNA fragmentation above the level in the control cells, being less than 6%. DNA fragmentation was induced by ZIE(OBu)AL at 4 h of incubation and increased to about 51% at 8 h (Fig. 2A). Cells were almost all viable at 2 h of incubation, but viability decreased progressively after 6 h, being about 50 and 40% at 6 and 8 h of incubation, respectively. DNA fragmentation by ZIE(OBu)AL was induced dose-dependently, being faint at

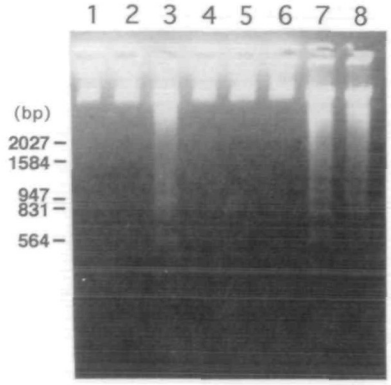

Fig. 1. **Effect of protease inhibitors on DNA fragmentation in RVC cells.** RVC cells were incubated for 6 h without (lane 1) or with protease inhibitors (lanes 2-8), then DNAs were isolated and resolved by electrophoresis on a 1.8% agarose gel. Lane 2, 100 μM TLCK; lane 3, 10 μM AcLLnL; lane 4, 10 μM AcLLM; lane 5, 50 μM AcYVAD; lane 6, 50 μM AcYVKD; lane 7, 10 μM ZIE(OBu)AL; lane 8, 10 μM ZLLnV.

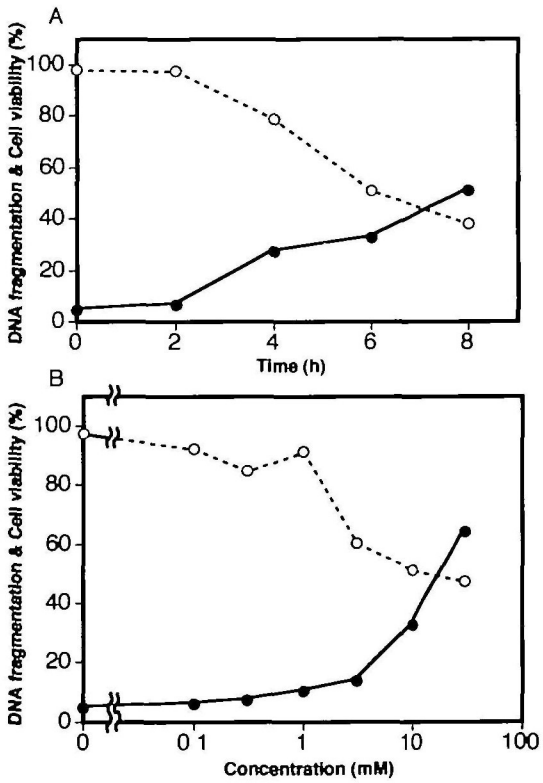

Fig. 2. **(A) Time course of proteasome inhibitor-induced DNA fragmentation in RVC cells.** RVC cells were incubated with 10 μM ZIE(OBu)AL. DNA fragmentation (closed circles) and cell viability (open circles) were determined at the indicated times. Data represent mean values of triplicate determinations. **(B) Dose dependence of proteasome inhibitor-induced DNA fragmentation in RVC cells.** RVC cells were incubated for 6 h with ZIE(OBu)AL at the concentrations indicated. DNA fragmentation (closed circles) and cell viability (open circles) were determined. Data show mean values of triplicate determinations.

3 μM and increasing to 33 and 65% at 10 and 30 μM, respectively (Fig. 2B). Fragmentation was almost at the control level at a concentration below 1 μM, being less than 6%. The activation of ICE family members during apoptosis is well documented, and inhibitors of ICE and its homologs have been shown to inhibit apoptosis in various cells (*10, 11*). We thus examined the effects of peptidyl aldehyde inhibitors of ICE and its homolog, CPP32, on the proteasome inhibitor-induced apoptosis in RVC cells (Table I). In the absence of the proteasome inhibitor, neither inhibitors of ICE nor of CPP32 affected the DNA fragmentation and cell viability even at 200 μM (data not shown). The proteasome inhibitor-induced DNA fragmentation was not inhibited by ICE inhibitors, AcYVAD and AcYVKD. However, an inhibitor of CPP32, AcDEVD, inhibited the DNA fragmentation dose-dependently (Table I), suggesting that CPP32, but not ICE, is involved in the apoptosis induced by proteasome inhibitors.

Table I. **Effect of ICE family protease inhibitors on proteasome inhibitor-induced DNA fragmentation in RVC cells.** RVC cells were preincubated with AcYVAD, AcYVKD, or AcDEVD for 30 min, then additionally incubated for 6 h in the presence of 10 μM ZIE(OBu)AL. DNA fragmentation was determined as described in the text. Data show mean values ± SE of triplicate determinations.

Treatment	DNA fragmentation (%)
Control	5.74±0.47
ZIE(OBu)AL	35.88±3.73
+AcYVAD 50 μM	32.71±2.77
100	25.83±2.24
200	33.91±2.29
+AcYVKD 50 μM	34.05±1.59
100	31.29±1.42
200	34.84±2.98
+AcDEVD 50 μM	21.48±1.15
100	16.66±2.78
200	13.96±1.18

Morphological Examination—Morphological changes during incubation with a proteasome inhibitor, ZIE(OBu)-AL, were analyzed by phase contrast microscopy. RVC cells grow as a single cell suspension, though the cells tended to aggregate during incubation as shown in Fig. 3A. However, aggregation did not affect cell viability in the absence of proteasome inhibitors. After 2 h of incubation with ZIE-(OBu)AL, cellular fragmentation characteristic of apoptosis appeared, becoming predominant at 6 h of incubation as shown in Fig. 3B. Light microscopic examination of cells stained with May-Giemsa showed chromatin condensation and fragmentation (Fig. 3D). These features reflected apoptotic bodies. Cellular shrinkage is another feature of typical apoptosis, but the cell size seemed to become larger (Fig. 3B). Light microscopic examination showed an increase in metaphase cells (Fig. 3D), suggesting that the increased cell size reflected arrest at the G2/M phase with a doubled DNA content. This was confirmed by flow cytometry as discussed below.

Cells Exposed to Proteasome Inhibitors Accumulated at G2/M Phase—Various proteins involved in the cell cycle machinery are degraded *via* the ubiquitin-proteasome pathway (*13, 14*). We examined the effect of proteasome inhibitors on perturbations in cell cycle and apoptosis in asynchronizing RVC cells. RVC cells proliferate with about an 8 h doubling time. The ratios of the cell cycle populations in non-treated cells at G0/G1, S, and G2/M phases were about 50, 30, and 20%, respectively, and the cell cycle distribution was not altered during incubation. After 2 h of

incubation with ZIE(OBu)AL, cells at G1 phase began to decrease followed by accumulation in G2/M phase (Fig. 4). At 6 h , the ratios of cells at G0/G1, S, and G2/M stages were 13.5, 17, and 41%, respectively. Cells containing less than a G1 DNA content accounted for less than 1% at 2 h, but they increased to about 8% at 4 h and about 28% at 6 h. Note that the decrease in cell viability and DNA fragmentation was observed after 4 h of incubation as seen in Fig. 2A. Cells exposed to ZLLnV, an another proteasome inhibitor, were also arrested at G2/M phase, similarly to those

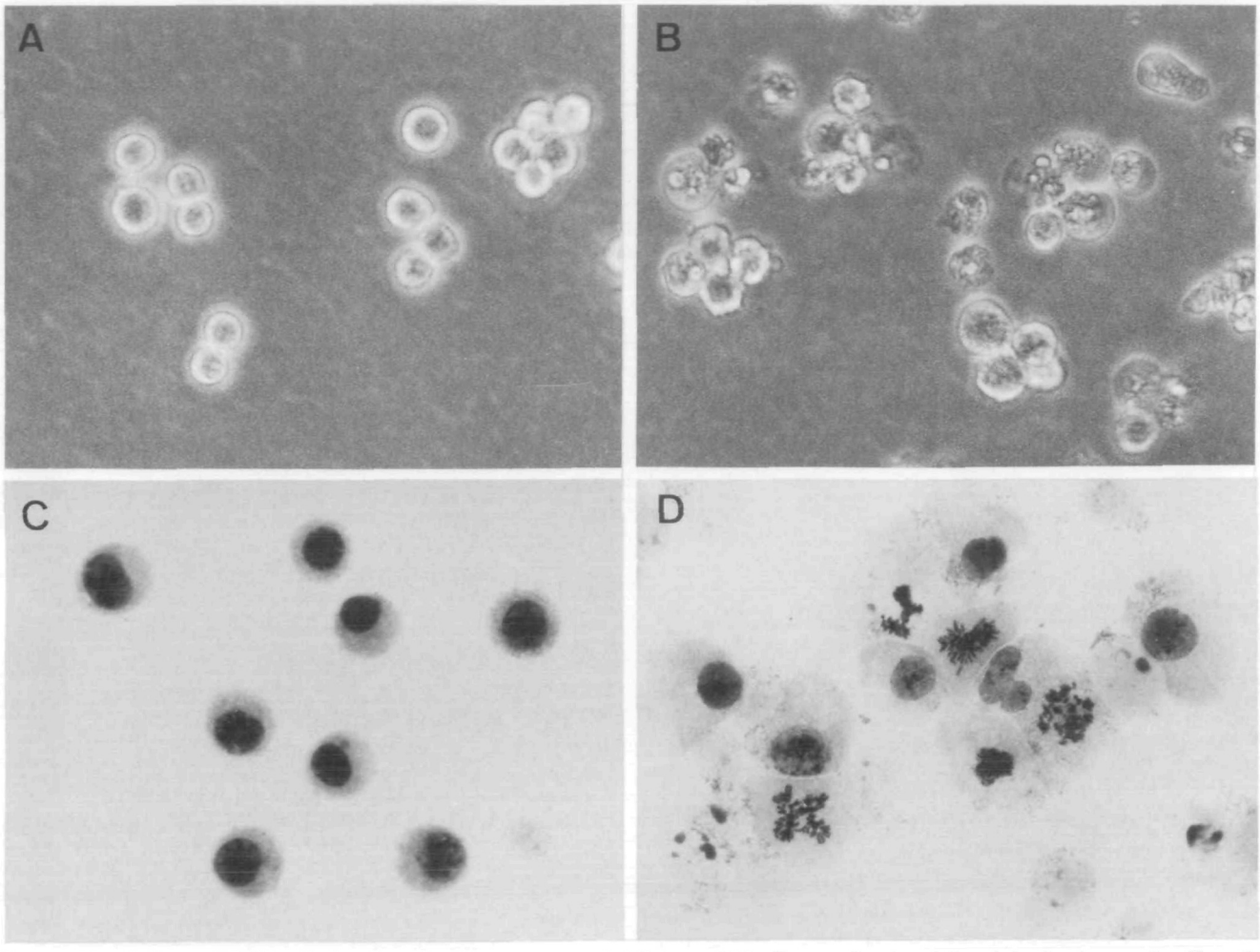

Fig. 3. **Morphology of RVC cells incubated with proteasome inhibitor.** RVC cells were incubated for 6 h in the absence (A, C) or the presence (B, D) of 10 μM ZIE(OBu)AL. The cells were directly observed by phase contrast microscopy (A, B), and by light microscopy after May-Giemsa staining (C, D).

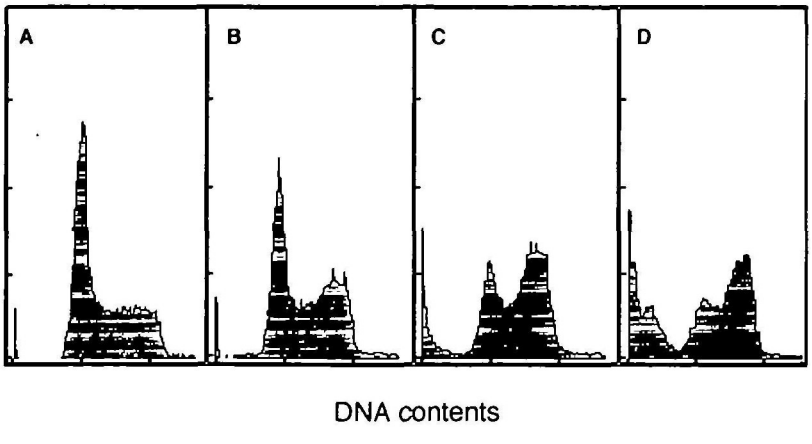

Fig. 4. **Cell cycle analysis of RVC cells incubated with proteasome inhibitor.** RVC cells were incubated with 10 μM ZIE(OBu)AL for 0 (A), 2 (B), 4 (C), and 6 h (D), and the cell cycle population was analyzed by flow cytometry.

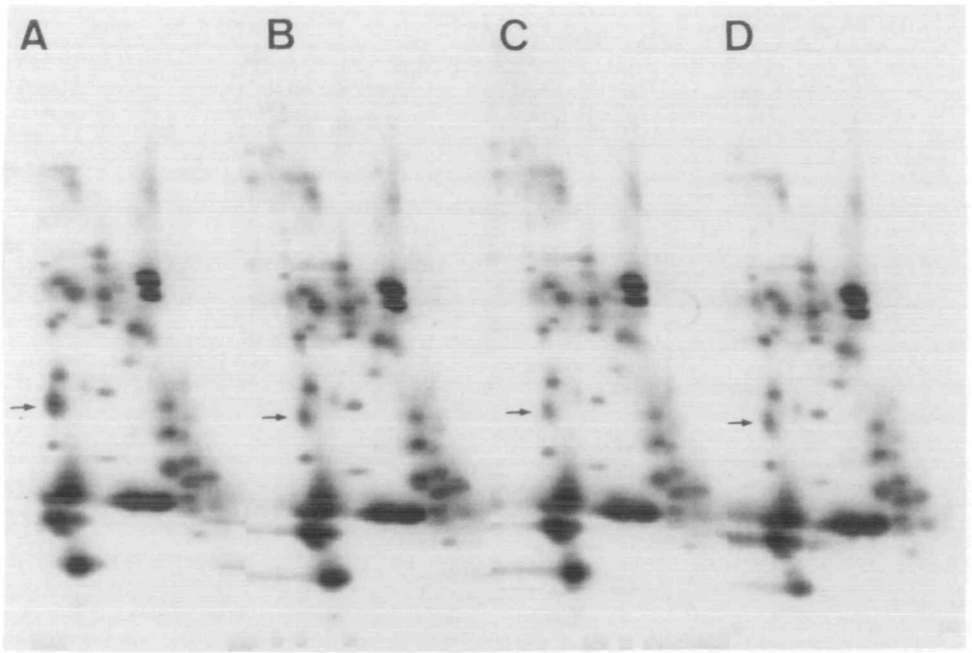

Fig. 5. **Time course of deubiquitination of histone uH2A in RVC cells incubated with proteasome inhibitor.** RVC cells were incubated with 10 μM ZIE(OBu)AL for 0 (A), 2 (B), 4 (C), and 6 h (D). Histones were extracted and resolved by two-dimensional AUT-SDS polyacrylamide gel electrophoresis. The gels were then stained with silver. The arrows denote ubiquitinated histone H2A spots.

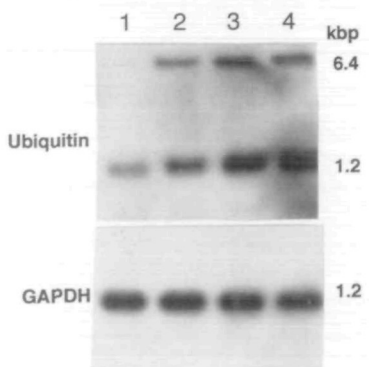

Fig. 6. **Expression of polyubiquitin genes in RVC cells incubated with proteasome inhibitors.** RVC cells were incubated with 10 μM ZIE(OBu)AL for 0, 2, 4, and 6 h (lanes 1-4, respectively). Messenger RNAs were isolated from the cells, then resolved by electrophoresis on a 1.5% agarose gel. The RNAs were transferred to a nylon membrane, then ubiquitin (A) and GAPDH (B) mRNA were analyzed by Northern blotting.

exposed to ZIE(OBu)AL. The flow cytometric findings were consistent with the microscopic observation of cellular enlargement.

Histone uH2A Decreased in Parallel with the Progression of Apoptosis—Chromatin condensation is a prominent feature of apoptosis (*1, 2*). However, the mechanism of chromatin condensation during apoptosis is uncertain. Chromatin condensation similar to that of apoptotic cells is seen in the cells entering metaphase, which is associated with deubiquitination of histone uH2A (*16, 17*). We analyzed histone uH2A in the cells exposed to ZIE(OBu)AL by two-dimensional AUT-SDS polyacrylamide gel electrophoresis. Histone uH2A was present in the control chromatin, and its level began to decrease after 2 h of incubation (Fig. 5). DNA fragmentation became evident after 4 h. At 6 h the level of histone uH2A in the exposed cells was about 25% of that in the controls. The levels of histone H2A and other histones in RVC cells did not change during incubation. Exposing the cells to inhibitors of ICE and its homolog did not alter the histone uH2A level (data not shown). These results suggest that apoptosis induced by proteasome inhibitors is associated with the deubiquitination of histone uH2A, similarly to that of metaphase cells.

Ubiquitin mRNA Expression Increased by Proteasome Inhibitors—We examined whether ubiquitin gene expression alters during apoptosis induced by proteasome inhibitors by Northern blotting of ubiquitin mRNA. In untreated cells, we detected polyubiquitin mRNAs of 6.4 and 1.2 kb, of which the latter was predominant. The expression of the mRNA began to increase after 2 h of incubation with ZIE(OBu)AL, when DNA fragmentation was undetectable, and it increased about 8-fold at 6 h (Fig. 6). Etoposide induced apoptosis in RVC cells (*26*) like the proteasome inhibitors. However, ubiquitin mRNA expression was not altered (data not shown), suggesting that the ubiquitin mRNA expression that increased early in the incubation was not a function of apoptosis, and seemed to be specific to treatment with the proteasome inhibitors.

DISCUSSION

RVC cells exposed to the peptidyl proteasome inhibitors, ZIE(OBu)AL and ZLLnV, underwent apoptosis early in the incubation. The process was time- and dose-dependent. The calpain inhibitor I AcLLnL also induced apoptosis, but TLCK and AcLLM, a calpain inhibitor II, did not. Calpain inhibitors AcLLnL and AcLLM also reversibly inhibit the chymotrypsin-like proteolytic activity of proteasomes (*27*). The K_i value of AcLLnL for proteasome is 5.7 μM and that of AcLLM is one order of magnitude higher than AcLLnL (*27*). The K_i value of AcLLnL for calpain is 0.5 μM (*28*). DNA fragmentation was induced by AcLLnL at concentrations above 10 μM, which is 20-fold higher than the K_i value for calpain. AcLLnL has a structure similar to the proteasome inhibitor ZLLnV, since both have a similar

C-terminus, norleucinal and norvalinal, respectively, whereas the C-terminus of the calpain inhibitor II AcLLM is methioninal, which has different properties from norleucinal or norvalinal. These findings suggest that, at the concentration used, AcLLnL inhibited proteasomes rather than calpain. The inhibitors of ICE and its homolog, AcYVAD, AcYVKD, and AcDEVD, did not induce apoptosis in RVC cells. Thus, among the protease inhibitors examined, only the proteasome inhibitors induced apoptosis in RVC cells early in the incubation.

ICE and its homologs are involved in apoptosis induced by various stimuli in a variety of cells, and inhibition of these proteases inhibits the progression of apoptosis (*10, 11*). Recently, evidence is growing that CPP32, a member of the ICE family, may play a more important role in the apoptotic pathway than ICE itself (*29, 30*). ICE itself neither induced nor inhibited the apoptosis in RVC cells (Fig. 1 and Table I), suggesting that ICE itself does not participate directly in the apoptosis induced by proteasome inhibitors. CPP32 may act downstream in the apoptotic pathway induced by proteasome inhibitors in RVC cells, since AcDEVD inhibited the apoptosis dose-dependently (Table I). The inhibitor of CPP32, but not of ICE, has been shown to inhibit the Fas-mediated apoptosis in human carcinoma-derived cell lines at similar concentrations to whose we used (*31*). However, it remains unclear how proteasomes and CPP32 are cooperatively regulated in the apoptosis of RVC cells.

Apoptosis is morphologically characterized by cellular shrinkage and cytoplasmic and nuclear condensation followed by the formation of apoptotic bodies (*1, 2*). RVC cells exposed to ZIE(OBu)AL formed apoptotic bodies with chromatin condensation early in the incubation as seen in Fig. 3, although the cellular volume seemed to increase. Cells exposed to proteasome inhibitors accumulated at G2/M phase (Fig. 4), suggesting that the increase in cellular size reflects cells with a doubled DNA content arrested at G2/M phase.

The ubiquitin-proteasome pathway seems to play a significant role in cell cycle regulation, since cell cycle regulatory factors such as cyclins or *c-mos* and *p53* oncogene products are degraded by the ubiquitin-proteasome pathway (*13, 14*). Lactacystin, a specific inhibitor of proteasome isolated from a microbe, inhibits cell growth and induces apoptosis in human monoblast U937 cells (*32*) and a peptidyl aldehyde inhibitor of proteasomes induces arrest at G2/M phase in HeLa cells (*33*). Mutation in a ubiquitin-activating enzyme leads Chinese hamster cells to arrest at G2 phase (*34*). Ubiquitin mutants in yeast that are unable to assemble ubiquitin chains on proteolytic substrates are inviable, with most cells arresting near the G2/M boundary of the cell cycle (*35*). Mutants in the 26S proteasome complex and a ubiquitin-conjugating enzyme also exhibit cell cycle defects (*36, 37*). These findings indicate that the ubiquitin-proteasome pathway has an important function in several steps of the RVC cell cycle and that proteasome inhibitors perturb the cell cycle resulting in cell cycle arrest and apoptosis. The mechanism of cell cycle arrest at G2/M phase induced by proteasome inhibitors is uncertain, but a proteasome-mediated step may be present in metaphase and essential for anaphase chromosome separation. The inhibitor of CPP32 inhibited apoptosis induced by proteasome inhibitors (Table I), but it did not affect the cell cycle of RVC cells either in the presence or absence of proteasome inhibitors (data not shown), indicating that CPP32 itself does not participate in the control of cell cycle.

The morphological changes are similar to those in cells undergoing mitosis and mitotic catastrophe which are characterized by chromatin condensation, spindle formation, rearrangement of the actin cytoskeleton and the nuclear membrane disassembly (*16, 17*). It is likely that some of the biochemical changes that occur during apoptosis and mitosis are similar. Ubiquitination of histone H2A occurs in active regions of chromatin, and histone uH2A disappears in metaphase chromosomes (*16, 17*), suggesting that the deubiquitination of histone uH2A is involved in chromatin condensation in apoptosis. In RVC cells, histone uH2A was deubiquitinated at 2 h of incubation with ZIE(OBu)AL (Fig. 5) and seemed to proceed to DNA fragmentation, since this was evident after 4 h of incubation (Fig. 2A). Histone uH2A also decreased in cells exposed to etoposide, an inhibitor of topoisomerase II (not shown), which induces rapid apoptosis in RVC cells (*26*). Marushige *et al.* reported that histone uH2A disappears in glioma and Schwannoma cells that undergo apoptosis in the presence of TGFβ1 (*18*). Considering that the ubiquitination of histone plays a role in preventing the formation of a higher order chromatin structure (*38*), deubiquitination of histone uH2A must play an active part in the process of apoptosis, particularly in altering chromatin structure. Ubiquitinated histones are not degraded in a ubiquitin-proteasome system but are relatively stable. Deubiquitination of histone uH2A is catalyzed by ubiquitin C-terminal hydrolase, which is integrated with the ubiquitin-proteasome system (*14*). The enzyme is induced in a differentiating lymphoblastic leukemia cell line that undergoes apoptosis as terminal differentiation (*39*). These results suggest that the enzyme plays an important role in apoptosis associated with deubiquitination of histones in RVC cells. However, the role and regulation of ubiquitin C-terminal hydrolase remain to be elucidated.

Ubiquitin is a stress protein and the ubiquitin-proteasome system may be activated under various stressed conditions when general intracellular protein degradation increases (*13, 14*). Under these conditions, some polyubiquitin genes may be induced. The mouse ubiquitin multigene family is composed of three classes: a monoubiquitin 52-amino acid carboxyl-extension protein fusion gene (UbA52), four-unit polyubiquitin genes (UbB), and multiple polyubiquitin genes (UbC) (*40*). Transcription of polyubiquitin gene in mouse, human, and yeast is activated by various types of stress such as heat shock, starvation, or γ-irradiation (*13, 14*), while the expression of UbA52 gene, which codes for an ubiquitin-ribosomal fusion protein, is not altered under stressed conditions; rather, the fusion proteins are constitutively expressed for maintaining survival (*13, 14*). In RVC cells, we detected two ubiquitin transcripts of 6.4 and 1.2 kb by Northern blotting (Fig. 6). The 6.4 and 1.2 kb transcripts correspond to the products of the polyubiquitin genes UbC and UbB, respectively (*40*). These polyubiquitin mRNAs were rapidly increased in the presence of the proteasome inhibitor, TIE(OBu)AL (Fig. 6). Ubiquitin expression increases in various tissues that undergo programmed cell death, such as the metamorphosis of insects, lens maturation and the maturation of

reticulocytes in mammals (*13, 14*). In the programmed cell death, increased ubiquitin levels are involved in the massive degradation of cellular proteins. Ubiquitin also appears to play an essential regulatory role in the γ-irradiation-induced apoptosis of human lymphocytes (*41*), However, levels of polyubiquitin mRNAs do not change in apoptotic RVC cells induced by etoposide, which induces DNA cleavable complex (*26*). Increases in ubiquitin do not appear to play a role in apoptosis induced by nerve growth factor in ganglial cells (*42*) or that induced by dexamethasone-treated thymocytes (*43*). These findings suggest that increased transcription is not a universal component of apoptosis, but is rather specific to the action of proteasome inhibitors, which induce the accumulation of intracellular ubiquitin-protein conjugates (*44*), resulting in a decrease in free ubiquitin in the cells. This decrease may activate the expression of polyubiquitin mRNA to maintain a constant free ubiquitin level. However, it is uncertain whether ubiquitin expression is directly involved in the induction of apoptosis or whether it is a secondary response to disturbed ubiquitin recycling induced by proteasome inhibitors.

In the present studies, we demonstrated that RVC lymphoma cells exposed to proteasome inhibitors were arrested at G2/M phase followed by apoptosis characterized by internucleosomal DNA fragmentation and formation of apoptotic bodies. The process was associated with deubiquitination of histone uH2A and increased expression of the polyubiquitin gene. Shinohara *et al.* (*45*) showed that peptidyl aldehyde inhibitors of proteasome induced apoptosis in human and murine leukemia cells accompanied by accumulation of p53 protein. These findings suggest that a certain short-lived regulatory protein-sensitive ubiquitin-proteasome pathway may be involved in cell cycle arrest and induction of apoptosis, although the accumulation of p53 does not explain the cell cycle arrest at G2/M phase observed in RVC cells. Contrary to the apoptosis-inducing action of proteasome inhibitors, they inhibited apoptosis in thymocytes (*46*) and neurons (*47*), which are both non-proliferating cells. We also observed that etoposide-induced thymocyte apoptosis is inhibited by the proteasome inhibitors (*48*). However, thymocyte apoptosis induced by phorbol ester plus calcium ionophore or by staurosporine was not inhibited by proteasome inhibitors (*46*). All these findings suggest that the involvement of proteasome in apoptosis depends on the types of cell and stimulation. Further studies on the mechanism of proteasome inhibitor-induced apoptosis, including identification of the proteins implicated in the regulation of both cell cycle and induction of apoptosis, are in progress at our laboratory.

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