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ARTICLES

Activation of Multiple Caspases and Modification of Cell Surface Fas (CD95) in Proteasome Inhibitor-Induced Apoptosis of Rat Natural Killer Cells

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Abstract The proteasome is a multi-subunit protease complex that is involved in intracellular protein degradation in eukaryotes. Previously, we have reported that selective, synthetic chymotryptic proteasome inhibitors inhibit A-NK cell-mediated cytotoxicity by approximately 50%; however, the exact role of the proteasome in NK cell-mediated cytotoxicity remains unknown. Herein, we report that proteasome inhibitors, MG115 and MG132, decreased the proteasome chymotrypsin-like activity in the rat natural killer cell line RNK16 by 85% at a concentration of 5 μM. The viability of RNK16 cells was also reduced in the presence of these inhibitors. Both inhibitors induced the apoptosis of RNK16 cells, as shown by DNA fragmentation, caspase-3 activation and the appearance of sub-G cell populations. An increase in the fraction of apoptotic cells was observed in a dose- and time-dependent manner in our studies. In addition, the activity of caspase-1, -2, -6, -7, -8, and -9, was increased following the treatment of RNK16 cells with these inhibitors. Further investigation revealed that the expression of Fas (CD95) protein on the RNK16 cell surface was increased after the treatment by MG115 or MG132, indicating that apoptosis induced by proteasome inhibitors in RNK16 cells might be mediated through the Fas (CD95)-mediated death pathway as well. Our studies indicate, for the first time, that proteasomal chymotryptic inhibitors can reduce natural killer cell viability and therefore indirectly inhibit cell-mediated cytotoxicity via the apoptosis-inducing properties of these agents. J. Cell. Biochem. 88: 482–492, 2003.

Key words: proteasome; inhibitor; apoptosis; natural killer cell

The proteasome is a multicatalytic proteinase complex that is involved in the major extra-lysosomal pathway responsible for intracellular protein degradation in mammalian cells. It is characterized by the presence of multiple proteolytic activities, defined as the chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing (PGPH) activity [Coux et al., 1996]. Cell cycle-regulating proteins degraded through ubiquitin-proteasome pathway include cyclins and cyclin-dependent kinase (CDK) inhibitors p21 and $p27^{kip-1}$ [Pagano et al., 1995]. Some transcription factors, such as p53, c-*Fos*, and c-*Jun*, are also degraded by the proteasome [Drexler, 1997]. Dysregulation of the degradation of such proteins has profound effects on cell cycle control and can cause cells to undergo apoptosis [An et al., 1998]. In recent years, a number of proteasome inhibitors were reported to induce apoptosis in actively proliferating cell lines such as HL-60 cells [Drexler, 1997] and Ewing's sarcoma cells [Soldatenkov and Dritschilo, 1997]. These compounds, by preventing degradation of cyclins, CDK inhibitors, tumor suppressor proteins and transcription factors, ultimately lead to deregulation of cell cycle progression and apoptotic cell death.

Abbreviations used: AMC, 7-amido-4-methylcoumarin hydrochloride; AFC, 7-amido-4-trifluoromethyl-coumarin; Ac, *N*-Acetyl; Z, carbobenzyloxy; Suc, succinyl; Dnp, 2,4-dinitrophenyl; MG132, Z-Leu-Leu-Leu-al; MG115, Z-Leu-Leu-Norvalinal; GGR, Gly-Gly-Arg; LLVY, Leu-Leu-Val-Tyr; LLE, Leu-Leu-Glu; WEHD, Trp-Glu-His-Asp; VDVAD, Val-Asp-Val-Ala-Asp; DEVD, Asp-Glu-Val-Asp; LEVDGWK, Leu-Glu-Val-Asp-Gly-Trp-Lys; VEID, Val-Glu-Ile-Asp; IETD, Ile-Glu-Thr-Asp; LEHD, Leu-Glu-His-Asp; FMK, fluoromethyl ketone; TPCK, N-p-tosyl-L-phenylalanyl chloromethyl ketone; ZPCK, *N*-carbobenzyloxy-L-phenylalanyl chloromethyl ketone.

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Natural killer (NK) cells are large granular lymphocytes that comprise 5–10% of peripheral blood mononuclear cells. By producing cytokines and exerting cytotoxicity, NK cells participate in resistance against microbial infections and malignant disease. In adoptive immunotherapy for cancer patients, NK cells are activated by IL-2 in vitro and then transfused back to the patients' body to locate within tumors and kill tumor cells [Basse et al., 1994]. Numerous studies have implicated proteolytic enzymes as crucial to the cytolytic mechanism of these cells [Goldfarb, 1986; Berke, 1994; Darmon and Bleackley, 1998]. The most studied proteolytic enzymes in cytotoxic lymphocytes are the granzymes, a series of serine proteases of varying specificities which are located within the cytolytic granules of these cells [Jenne and Tschopp, 1988; Heusel et al., 1994]. Our studies have focused on proteolytic enzymes other than those found only in cytolytic granules and have indicated that the chymotryptic activity of proteasomes in NK cells might play a role in their cell-mediated cytotoxicity [Goldfarb et al., 1992; Wasserman et al., 1994; Kitson et al., 1995].

Due to their ability to inhibit cell proliferation and induce apoptosis, together with their ability to inhibit angiogenesis, proteasome inhibitors have recently become attractive candidates as anti-cancer drugs. A boronate inhibitor MLN-341, previously called PS-341 (developed by Millennium Pharmaceuticals, Cambridge, MA), after showing impressive anti-proliferative effects in several animal model systems and cell culture, is currently in NCI-sponsored human clinical trials for the treatment of several types of cancer [Kisselev and Goldberg, 2001]. As indicated in our previous studies, proteasomal chymotryptic inhibitors substantially inhibit A-NK cell-mediated cytotoxicity against both NK-sensitive and -resistant targets [Kitson et al., 2000]. Therefore, with the spreading usage of the proteasome inhibitors in the clinical field, we considered it of importance to determine their potential toxicities on NK cells since unwanted inhibition of NK cells by such inhibitors could adversely impact on the overall potential benefit of such agents. In this study, we found that two proteasome inhibitors, MG115 and MG132, could induce the apoptosis of the rat NK cell line RNK16. In this proteasome inhibitor-induced apoptosis, activation of multiple caspases and modification of cell surface Fas (CD95) protein was observed. These results suggest a mechanism by which proteasomal chymotryptic inhibitors could reduce NK cell-mediated cytotoxicity due to their apoptosis-inducing properties.

MATERIALS AND METHODS

Reagents and Chemicals

RPMI-1640 tissue culture medium, nonessential amino acids, 2-mercaptoethanol, L-glutamine, and antibiotics were purchased from Gibco (Grand Island, NY). Sucrose (Ultrapure) was purchased from Beckman (Fullerton, CA). Ethylenedinitrilotetraacetic acid disodium salt (EDTA) was purchased from Fisher Scientific (Fair Lawn, NJ). Dithiothreitol (DTT), 2-mercaptoethanol, fluorogenic proteasome substrates Z-GGR-AMC, Suc-LLVY-AMC, Z-LLE-AMC, proteasome inhibitors MG115 and MG132, protease K, propidium iodide, and buffers HEPES and PIPES were purchased from Sigma (St. Louis, MO). Fluorogenic caspase substrates Ac-WEHD-AMC, Ac-VDVAD-AFC, Ac-DEVD-AMC, Ac-LEVDGWK(Dnp)-NH_2, Ac-VEID-AMC, Ac-IETD-AMC, Ac-LEHD-AFC were purchased from Bachem Bioscience (King of Prussia, PA). Amicon® nitrogen pressure-based concentration apparatus was obtained from Amicon (Beverly, MA). Sephacryl S-400 and heparin-Sepharose CL-6B chromatography media were purchased from Pharmacia (Piscataway, NJ). PE-conjugated Jo2 Fas antibody was purchased from Pharmingen (San Diego, CA).

Cell Culture and Treatment With Proteasome Inhibitors

Rat RNK16 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 1% (v/v) MEM nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (growth medium). Cells were treated with either a proteasome inhibitor, or DMSO (vehicle). During this process, morphological changes and cellular detachment were monitored. At each time point, cells were harvested, and used for measurement of apoptosis and other biochemical events.

Purification of Proteasome

20S proteasome was purified from RNK16 cells following methods described previously [Wasserman et al., 1994]. Briefly, subsequent to

harvesting RNK16 cells, postnuclear supernatants were collected after nitrogen cavitation at 325–350 psi for 30–40 min. Successively, isopycnic sucrose gradient fractionation, Sephacryl S-400 gel filtration chromatography, and heparin-Sepharose CL-6B chromatography were applied. In each step, the protein concentration and the specific activities for proteasomal chymotrypsin-like and trypsin-like activities were measured.

Cell Viability Assay

To quantitate cell death, cell viability was determined by exclusion of Trypan blue. At the indicated times after treatment with proteasome inhibitors, cells were detached, pelleted, and resuspended in RPMI 1640. After staining with Trypan blue, viable cells in five random fields of view were counted. The percentage of treated viable cells was determined as a percentage of control viability.

DNA Fragmentation Assays

At each indicated time point, RNK16 cells were washed in PBS and resuspended in 0.7 ml of a buffer containing 10 mM Tris-HCI, 10 mM EDTA, 0.5% SDS, and 200 μg/ml protease K. The cell mixtures were incubated at 55°C for 2 h and then treated with 25 μg/ml RNase at 37°C for 1 h. After incubation, DNA was precipitated with 1.5 volume of ethanol and resuspended in TE buffer (10 mM Tris-HCI, pH 8.0, 1 mM EDTA). The prepared DNA samples were analyzed in a 1% agarose gel containing ethidium bromide.

Detection of Apoptotic Cells by Flow Cytometry

Apoptosis can be detected by measuring the sub-G_1 population with flow cytometry. Cells were fixed in 70% ethanol and treated with 10 μg/ml of RNase. They were stained with 25 μg/ml of propidium iodide and then subjected to analysis on an EPICS XL-MCL flow cytometer with SYSTEM^{TM} II software (Beckman-Coulter Corporation, Miami, FL).

Whole Cell Extracts and Protein Determination

RNK16 cells were lysed in ice-cold homogenization buffer [20 mM Tris/HCl (pH 7.4)/0.1 mM EDTA/1 mM 2-mercaptoethanol/5 mM ATP/ 20% (v/v) glycerol, 0.04% (v/v) Nonidet P-40]. The buffer was supplemented with pepstatin (0.5 μg/ml), leupeptin (1.25 μg/ml), and phenylmethylsulfonyl fluoride (0.5 mM) to minimize activity of proteases other than the desired ones. The resulting lysates were placed in microfuge tubes and centrifuged at 13,000*g* at 4°C for 15 min to remove insoluble material. The resulting high-speed supernatant fractions were placed on ice and assayed for protein concentration by measuring the absorbance at 280 nm with bovine serum albumin as standard.

Assay of Proteasome Proteolytic Activities

Three major proteasome proteolytic activities, i.e., trypsin-like, chymotrypsin-like, and peptidylglutamyl-hydrolyzing activity, were assayed by using substrates CBZ-Gly-Gly-Arg-AMC, Suc-Leu-Leu-Val-Tyr-AMC, and Cbz-Leu-Leu-Glu-AMC, respectively. For trypsin-like activity, reaction buffer 1 mM dithiothreitol (DTT) and EDTA, 10 μg/ml BSA, 0.02% Triton X-100 (v/v) in 50 mM Tris/HCl buffer, pH 8.7, was used; for chymotrypsin-like activity, 1 mM DTT and EDTA, 10 μg/ml BSA, 0.02% SDS in 10 mM Tris/HCl buffer, pH 7.5 was used and for peptidylglutamyl-hydrolyzing activity, 1 mM DTT and EDTA, 10 μg/ml BSA, 0.02% SDS in 10 mM Tris/HCl buffer, pH 8.0, was used. All reaction mixtures contained peptide substrates at 20 μM concentrations unless otherwise noted. The rates of cleavage were determined by taking fluorescent intensity measurements using a Dynatech® fluorescence plate reader. Proteolytic activity was expressed as relative fluorescence units per second and per gram of protein.

Measurement of Caspase Activities

The following synthetic substrates were used to measure caspase activity: Caspase-1, Ac-WEHD-AMC; Caspase-2, Ac-VDVAD-AFC; Caspase-3, Ac-DEVD-AMC; Caspase-4, Ac-LEVDGWK(Dnp)-NH_2; Caspase-6, Ac-VEID-AMC; Caspase-8, Ac-IETD-AMC; and Caspase-9, Ac-LEHD-AFC. All caspase substrates were prepared as stock solutions of 10 mM in 100% DMSO. Before the assay, all caspase substrates were diluted, yielding final concentrations of 20 μM. To each well, 20 μl RNK16 cell lysates, 80 μl reaction buffer, and 100 μl of substrate solution were added and fluorescence of the cleavage product measured over time at room temperature in a microplate spectrofluorometer. For AMC substrates, excitation wavelength was 360 nm, emission 460 nm; for AFC substrates, excitation was 400 nm and emission 505 nm. Activity was

normally measured after incubating 2 h and was expressed as percentage of activity compared with the activity at $t = 0$ h. Mean $\pm$ SDs from triplicate wells are shown from experiments performed on three separate occasions.

Analysis of Cell Surface Fas

After treatment with proteasome inhibitors, cells were harvested and washed once by PBS. Then cells were resuspended in staining medium (1% FBS, 0.1% sodium azide in PBS) and incubated with PE-conjugated Jo2 antibody (PharMingen, San Diego, CA) for 30 min at room temperature. After the second wash in staining medium, cells were fixed by 1% paraformaldehyde in PBS and analyzed on an EPICS XL-MCL flow cytometer with SYSTEM II software (Beckman-Coulter Corporation, Miami, FL).

RESULTS

Effects of Protease Inhibitors on Proteasomal Proteolytic Activities In Vitro

After purification of 20S proteasome from RNK16 cells, we determined the specificity of some protease inhibitors on the RNK16 cell proteasome. Totally ten protease inhibitors were tested and four of them were found to achieve more than 80% inhibition on the proteasomal chymotryptic activity at the concentration of 5 μM (Fig. 1). The proteasomal inhibitors, MG115 and MG132, were among these four inhibitors.

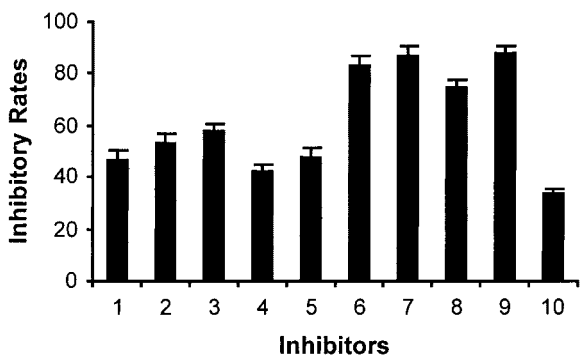

Fig. 1. Inhibitory effects of various protease inhibitors on the chymotrypsin-like activity of proteasome from RNK16 cells in vitro. Purified RNK16 cell proteasome (0.5 μg) was preincubated with various protease inhibitors for 10 min: (1) TPCK; (2) ZPCK; (3) H-Ala-Ala-Phe chloromethyl ketone; (4) L-Leucine chloromethyl ketone; (5) lactacystin; (6) MG-115; (7) Z-Ile-Glu-Ala-Leu-aldehyde; (8) Z-Leu-Leu-Phe-aldehyde; (9) Z-Leu-Leu-Leu-aldehyde; (10) TLCK. Then reaction buffer was added to the final concentration of inhibitors at 5 μM.

Next we investigated the inhibitory effects of these two inhibitors on the major three proteolytic activities of RNK16 cell proteasome, i.e., trypsin-like, chymotrypsin-like, and peptidylglutamyl-hydrolyzing activities. It is known that inhibition of all of multiple active sites in proteasome is not required to significantly reduce protein breakdown, and inhibition of the chymotrypsin-like site or its inactivation by mutation alone causes a large reduction in the rates of protein breakdown [Rock et al., 1994; Chen and Hochstrasser, 1996; Heinemeyer et al., 1997]. In our studies, we incubated MG115 or MG132 with the proteasome purified from RNK16 cells to test their inhibitory effects on the three major proteasomal proteolytic activities. Three specific substrates, GGR-AMC, LLVY-AMC, and LLE-AMC were employed to determine proteasomal trypsin-like, chymotrypsin-like, and peptidylglutamyl-hydrolyzing activities, respectively. At a concentration of 10 μM, MG132 inhibited the trypsin-like, chymotrypsin-like, and peptidylglutamyl-hydrolyzing activity of proteasome by 95.6, 94.2, and 90.1%, respectively, while the inhibition rates of MG115 were 90.8, 91.4, and 88.2% (Table I).

Effects of Proteasome Inhibitors on Proteasomal Proteolytic Activities in Cultured Cells

The proteasome inhibitors, MG115 and MG132, used in the present study were reported to efficiently block proteasomal activity in eukaryotic cells [Palombella et al., 1994]. To confirm the inhibitory effects of these proteasome inhibitors on proteasome in cultured NK cells, proteasomal proteolytic activities were assayed after RNK16 cells were incubated with inhibitors MG115 or MG132 in culture media. After lysates of incubated cells at indicated time points were collected, substrates GGR-AMC,

TABLE I. Effects of Proteasome Inhibitors MG115 and MG132 on Proteasomal Proteolytic Activities In Vitro

Inhibition rates	MG115 (%)	MG132 (%)
Trypsin-like activity	90.80	95.60
Chymotryptic activity	91.40	94.20
PGPH activity	88.20	90.10

Purified RNK16 cell proteasome (0.5 μg) was incubated with 10 μM MG115 or MG132 alone in the reaction buffer for 1 h at 37°C. The proteasomal trypsin-like, chymotrypsin-like, and PGPH activities were assessed by using 20 μM GGR-AMC, LLVY-AMC, and LLE-AMC in reaction buffer as substrates, respectively.

LLVY-AMC, and LLE-AMC were employed to determine any changes of the major three proteasomal proteolytic activities occurring during the incubation of RNK16 cells with these proteasome inhibitors. For the chymotrypsin-like activity, incubation of RNK16 cells with either 20 μM MG115 or MG132 did not cause as great a reduction in enzymatic activity as incubation of purified proteasome with either 10 μM MG115 or MG132 in the in vitro assay. After incubation for 12 h, MG115 caused 59% reduction in the chymotryptic activity while MG132 achieved 71% reduction of this activity (Fig. 2). Both MG115 and MG132 also inhibited the proteasomal trypsin-like and PGPH activity (data not shown). Indeed, we have also previously reported that certain selective inhibitors of proteasomal chymotryptic activities (e.g., CEP-1508) also substantially inhibit NK cell-mediated cytotoxicity [Kitson et al., 2000]. These results indicate that these two proteasome inhibitors are able to penetrate RNK16 cells and inhibit the proteasome.

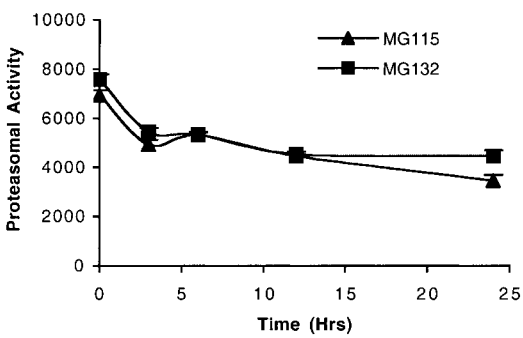

Fig. 2. Effects of proteasome inhibitors on proteasomal proteolytic activities in cultured cells. The chymotryptic activity of the proteasome was measured by Suc-LLVY-AMC hydrolysis. RNK16 cells were incubated with 20 μM MG115 or MG132 for 3, 6, 12, and 24 h. Activity was expressed as the relative fluorescence measured after 1 h of incubation with the substrates and reflected the mean of two independent experiments, each performed in triplicate.

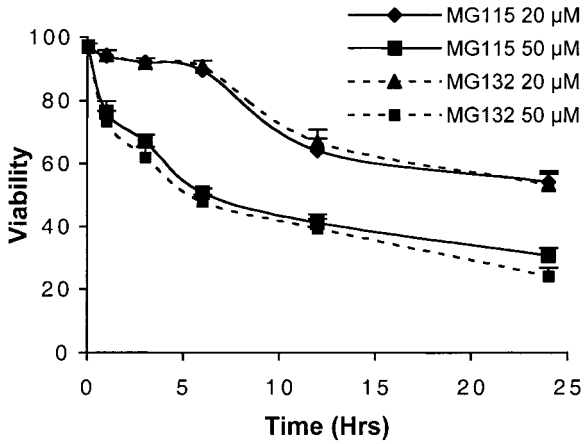

Fig. 3. Reduced viability of RNK16 cell in the presence of proteasome inhibitors. RNK16 cells were incubated for 1, 3, 6, 12, and 24 h with MG115 or MG132 alone at 20 or 50 μM, respectively. Viable cells were counted after staining with Trypan blue. The viability of untreated control cells was counted as 100%. The viability of treated cells was expressed as a percentage of control cells. Data represent average of three experiments.

Reduced Viability of RNK16 Cell in the Presence of Proteasome Inhibitors

To investigate any effects of these proteasomal inhibitors on RNK16 cells, the cells were incubated with proteasome inhibitors and morphological appearance of RNK16 cells were examined next. Although RNK16 cells in culture exist in an equilibrium between attached cells and cells in suspension, with the increasing treatment time more cells detached and were observed floating in the medium, indicating that these compounds upset this balance. After 24 h treatment, cell shrinkage could be found easily under microscope. To assess the extent of cell death at indicated time points, cell viability was measured by Trypan blue exclusion test. The viability of RNK16 cells was reduced to around 60% after 12 h with either 20 μM MG115 or MG132 alone, while 50 μM MG115 or MG132 caused a similar decrease in viability at 3–6 h (Fig. 3). These data demonstrate that proteasome inhibitors reduce the viability of RNK16 cells in a time- and dose-dependent manner.

Apoptosis of RNK16 Cells Induced by Proteasome Inhibitors

To determine the mechanism of reduced viability of RNK16 cells in presence of proteasome inhibitors, we investigated whether cell death was due to apoptosis. Initially, we used a DNA fragmentation assay to see any RNK16 cells undergoing apoptosis. Fragmentation of the genomic DNA is an irreversible event that commits the cell to die. These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180-bp subunit. An agarose gel of RNK16 cell DNA indicated that

DNA fragmentation occurred in these cells after they were treated with proteasome inhibitors, MG115 and MG132, at 20 μM for 10 h (Fig. 4).

We also performed flow cytometry analysis of propidium iodide stained cells to measure the DNA content and integrity of proteasome inhibitor treated RNK16 cells. The lower DNA content of apoptotic cells stained by a propidium iodide could be measured by flow cytometry. Our results showed that the percentage of apoptotic cells, characterized by the sub-G_1 peak, increased with longer incubation times with the proteasome inhibitors MG115 or MG132 (Fig. 5). When the concentration of MG132 was increased from 20 to 50 μM, the sub-G_1 cell population increased significantly at the indicated various time points. These results indicate that RNK16 cells undergo apoptosis in a time- and dose-dependent manner during their incubation with proteasome inhibitors.

Caspase-3 or DEVD-cleaving activity is an early marker of cells undergoing apoptosis. Ac-DEVD-AMC is a synthetic tetrapeptide fluorogenic substrate reported to be specific for caspase-3. By using this substrate, we identified that treatment of RNK16 cells with MG115 or MG132 led to a 7.8- or 7.2-fold respective increases in caspase-3 activity at 12 h (Fig. 6). This increase was not due to a direct effect of MG115 or MG132 on caspase-3, because in vitro incubation with MG115 or MG132 had no effect on Ac-DEVD-AMC hydrolysis (data not shown).

Fig. 4. DNA fragmentation of RNK16 cells during incubation with proteasome inhibitors, MG115 and MG132. RNK16 cells treated with 20 μM MG115 (**Lane 1**) or MG132 (**Lane 2**) alone for 10 h display DNA laddering analyzed by agarose gel electrophoresis. M: DNA size markers.

Proteasome Inhibitors Induce the Processing of Multiple Caspases in RNK16 Cells

Despite the diversity of signals, which can induce apoptosis, these pathways can share several features in their execution. One mechanism, which has been consistently implicated in the apoptosis, is the activation of a series of cytosolic proteases, the caspases. To examine processing of caspases in cell apoptosis mediated by proteasome inhibitors, we sought to measure the proteolytic activity of caspases-1, -2, -6, -7, -8, and -9 by fluorogenic assay. The results indicate that all six of these caspases were induced to active status by proteasome inhibitors at different amplitudes. Most caspases achieved their activity peaks around 12 h after exposure of RNK16 cells to the proteasome inhibitors (Fig. 7). Some caspases showed earlier activation by MG132 than that by MG115. caspase-9 showed greater amplification of activity than that of caspase-8, achieving a 23.4-fold increase after 12 h of treatment by MG115 and 29.5-fold after treatment by MG132 compared to 0 h, respectively.

Modification of Cell Surface Fas by Proteasome Inhibitors

Since the activity of caspase-8 was also seen to increase significantly by the treatment of RNK16 cells with MG115 or MG132, we examined the possible upregulation of Fas (CD95) on cell surface induced by proteasome inhibitors. RNK16 cells were incubated with 20 μM MG115 (panel A) or MG132 (panel B) for 3 and 20 h and then the level of cell surface Fas protein was determined by flow cytometry after staining with PE-conjugated monoclonal Jo2 which recognizes mouse Fas (Fig. 8). Compared with untreated cells, cells treated for 20 h with MG115 or MG132 showed distinct shifts in the distribution of fluorescence for Fas, while the curves for cells treated for 3 h with same concentration of inhibitors almost overlaid with that of control cells (data not shown). The data

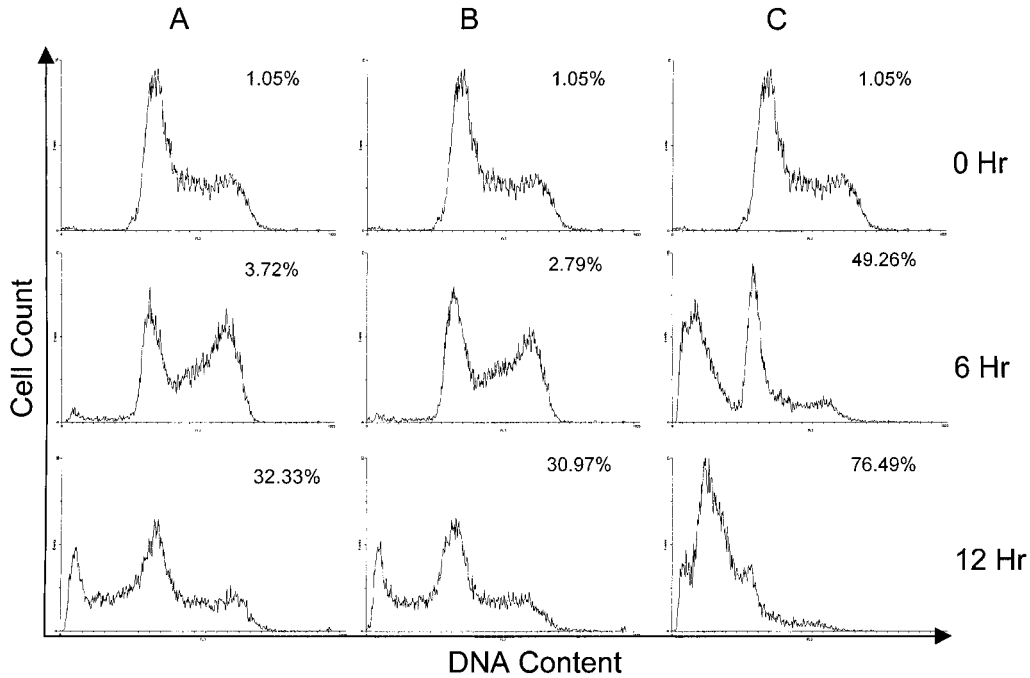

Fig. 5. Apoptosis of RNK16 cells induced by proteasome inhibitors evidenced by sub-G_1 cell population in flow cytometry analysis. Exponentially grown RNK16 cells (0 h) were treated with 20 μM MG115 (**Column A**) or MG132 (**Column B**), or 50 μM MG132 (**Column C**) for indicated time duration, followed by measurement of sub-G_1, G_1, S, and G_2-M cell populations (see Materials and Methods). The units of the Y-axis represent cell numbers (in total 10,000 events) and those of the X-axis represent cellular DNA content. The data indicate the percentage of sub-G_1 cells in the cell cycle. All experiments were performed twice independently and gave similar results.

indicate that proteasome inhibitors increased cell surface Fas of rat NK cells and imply that proteasome inhibitor-induced apoptosis of RNK16 cells might be mediated by the Fas-mediated pathway as well.

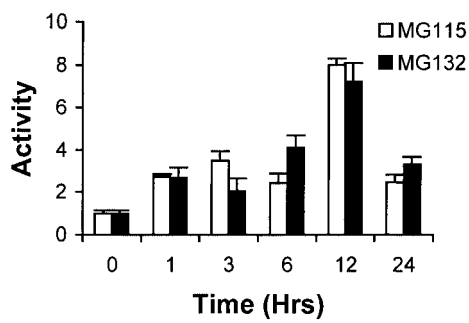

Fig. 6. Activation of caspase-3 of RNK16 cells during incubation with proteasome inhibitors, MG115 and MG132. RNK16 cells were incubated with the inhibitors for the times indicated, lysed, and caspase-3 activity was determined using Ac-DEVD-AMC, as described in Materials and Methods. The caspase-3 activity of cells at 0 h was counted as 100%. Data represent average of three experiments.

DISCUSSION

The proteasome is a 700-kDa protease multicomponent complex that is thought to be responsible for turnover of defective proteins in all cells including eukaryotic cells [Coux et al., 1996; Rivett et al., 1997]. It consists of 28 subunits which are arranged in a set of four stacked rings. To date the proteasome has been found to cleave peptides following basic amino acids (trypsin-like), hydrophobic amino acids (chymotrypsin-like), glutamic acid, branched chain amino acids and small neutral amino acids [Coux et al., 1996]. Unlike any other protease, all the proteolytic sites in the proteasome utilize N-terminal threonines of the beta subunits as active site nucleophiles. Most proteasome inhibitors have been designed to attack this N-terminal threonine by reacting with the catalytic hydroxyl or thiol groups in the active sites of the proteasome to form a reversible hemi(thio)acetal, which resembles a transition state analogue of the enzymatic reaction [Lowe et al., 1995; Groll et al., 1997; Kisselev

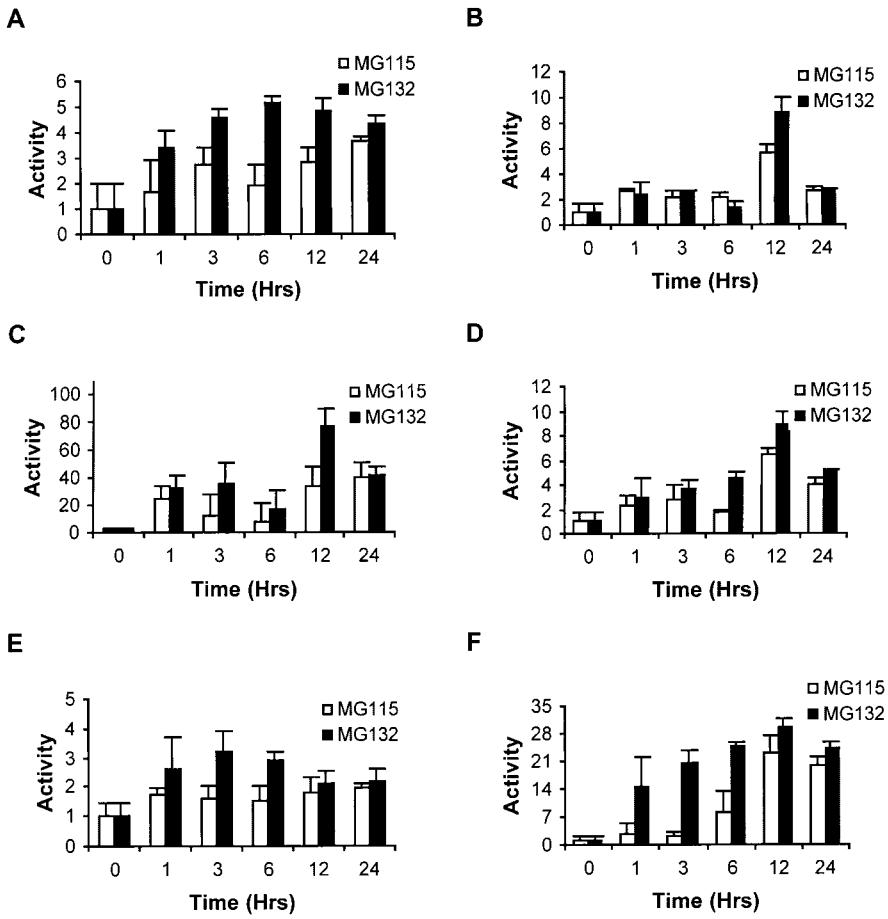

Fig. 7. Activation of multiple caspases in RNK16 cells during their incubation with 20 μM MG115 or MG132. Fluorogenic substrates used for these caspases were Ac-WEHD-AMC (**A**: Caspase-1), Ac-VDVAD-AFC (**B**: Caspase-2), Ac-LEVDGWK(Dnp)-NH_2 (**C**: Caspase-4), Ac-VEID-AMC (**D**: Caspase-6), Ac-IETD-AMC (**E**: Caspase-8), and Ac-LEHD-AFC (**F**: Caspase-9). Activity is expressed as a fold increase in activity at t = 0 h (control activity). Means ± SD of three separate experiments are shown.

and Goldberg, 2001]. Among these activities, the cleavage by the chymotrypsin-like sites appears to be rate-limiting in protein breakdown. Studies have indicated that the inhibition of the chymotrypsin-like site or its inactivation by mutation alone can cause a large reduction in the rates of protein breakdown [Rock et al., 1994; Chen and Hochstrasser, 1996; Heinemeyer et al., 1997], while inactivation of trypsin-like or PGPH activities has little effect on overall proteolysis [Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997; Kisselev et al., 1999]. In this study, the potency of MG115 and MG132 was also measured against the trypsin-like and PGPH activities with purified proteasomes, in addition to chymotrypsin-like activity. Our results indicate that these two inhibitors act on these three major active sites in the proteasome from RNK16 cells. In addition, with the highly hydrophobic properties of most chymotrypsin-like inhibitors, these two inhibitors were consequently confirmed to be cell-permeable.

By preventing degradation of cyclins, CDK inhibitors, tumor suppressor proteins and transcription factors, proteasome inhibitors lead to deregulation of cell cycle progression and apoptotic cell death. However, some studies have indicated that proteasome inhibition may be protective against some apoptotic stimuli in differentiated cells such as thymocytes [Grimm et al., 1996; Drexler, 1997]. Even though there have been an increasing numbers of reports demonstrating pro- or anti-apoptotic effects of proteasome inhibitors, their effects on NK cells have not been fully investigated [Wasserman et al., 1994; Kitson et al., 2000]. The present study sought to examine the potential effects of

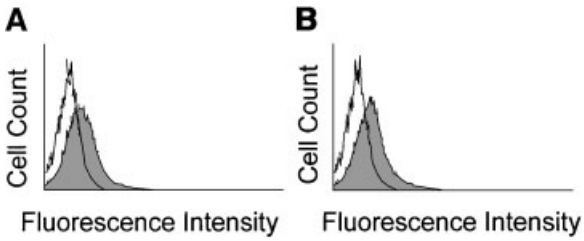

Fig. 8. Modification of cell surface Fas (CD95) of RNK16 cells by proteasome inhibitors, MG115 and MG132. RNK16 cells were incubated for 0 (line) and 20 h (solid line) with 20 μM MG115 (**Panel A**) or MG132 (**Panel B**). Cells were harvested and stained live for Fas on cell surface with PE-conjugated Jo2 antibody. The fluorescence was determined by flow cytometry.

proteasome inhibitors on NK cells. By investigating effects of proteasome inhibitors MG115 and MG132 on proteasomal activities in vitro and in vivo, their inhibition of the proteasome of NK cells was confirmed. In addition, this inhibition by the proteasome inhibitors MG115 and MG132 was shown to cause apoptosis in cultured RNK16 cells, as evidenced by appearance of the apoptotic population with sub-G_1 DNA content, internucleosomal DNA fragmentation, and activation of caspase-3.

In mammals, programmed cell death can be initiated by three distinct pathways [Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998]: (1) the extrinsic pathway, which can be triggered by the ligation of death receptors, such as Fas (also termed CD95 or APO-1), tumor necrosis factor receptor, or TRAIL receptor, and subsequent mitochondria-independent caspase-8 activation [Li et al., 1998; Luo et al., 1998]; (2) the intrinsic pathway, which is initiated by diverse apoptotic stimuli and followed by activation of caspase-9; or (3) the granzyme B pathway, where the cytotoxic cell protease granzyme B is delivered to sensitive target cells. Each of these pathways converges to a common execution phase of apoptosis that requires the activation of caspase-3 from its inactive zymogen form to its processed, active form. In this study, we have shown that the induction of cell death by proteasome inhibitors involves the activation of multiple caspases. In this report, we show the processing of caspases-1, -2, -3, -4, -6, -8, and -9 after the treatment of RNK16 cells with MG115 or MG132. Therefore, RNK16 cells seem to undergo apoptosis through a caspase-dependent pathway. However, some studies have observed that there is also caspase-independent apoptosis, led by the blocking of the ubiquitin-dependent pathway, in which accumulation of p53, p27, and cyclins D1 and B1 was seen [Monney et al., 1998]. So, it is therefore important to also examine the changes of signaling molecules in the apoptosis of NK cells induced by proteasome inhibitors. The use of caspase inhibitors allows us to clarify whether caspase-independent pathways are involved in the proteasome inhibitor-induced apoptosis of rat NK cells. For instance, Z-DEVD-FMK is a potent inhibitor of caspase-3, but it can inhibit other caspases as well when used in cell-culture experiments [Villa et al., 1997]. Such studies are currently under investigation in our laboratory.

Besides the controversy of the role of caspases in apoptosis, there is also contradictory evidence for the role of each individual caspase in proteasome inhibitor-induced apoptosis. In some studies, caspase-8 activation is not a critical step in the killing cascade and to date the role of endogenous death ligand/receptor interactions has not been reported [Wagenknecht et al., 1999]. However, in other studies, proteasome inhibitors did not influence expression of procaspase-8, procaspase-3, but did upregulate Fas and FADD [Kim, 2001], and the interaction of Fas with the ubiquitin-conjugating enzyme may be directly involved in ubiquitin-dependent degradation of Fas by proteasome [Wright et al., 1996; Becker et al., 1997]. Our results appear to be consistent with the latter studies. We suppose that the different findings regarding caspase-8 activation might possibly be caused by the different phenotypes and genotypes of these cell lines.

We have previously determined that some proteasomal inhibitors selective for chymotryptic domains of NK cell proteasome can inhibit NK cell viability (e.g., CEP-1612) [Kitson et al., 2000]. The current study extends and classifies this phenomenon. Indeed, this study reveals at least one mechanism by which apoptosis-inducing proteasomal chymotryptic inhibitors can contribute to reduced NK cell-mediated cytotoxicity.

A key prerequisite for optimal therapeutic application of proteasome inhibitors for treatment of malignant tumors should be the sparing of NK cells, i.e., immune effector cells which recognize and kill tumor cells. In this regard, this study also provides a potential approach towards clinical guidance for therapeutic application of proteasome inhibitors in malignant tumors, i.e., the negative effects on killer cells,

should also be considered to minimize unwanted adverse consequence that might arise through untoward inhibition of NK cell function.

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