Proteasome inhibitors induce Fas-mediated apoptosis by c-Myc accumulation and subsequent induction of FasL message in human glioma cells

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Abstract Proteasome inhibitors were shown previously to induce mitochondria-independent and caspase-3-dependent apoptosis in human glioma cell lines by unknown mechanisms. Here, we showed that treatment with proteasome inhibitors, lactacystin or acetyl-leucinyl-leucinyl-norleucinal, led to elevation of the steady-state c-Myc protein but not c-*myc* mRNA, suggesting the accumulation of c-Myc protein by proteasome inhibitors. In addition, the marked association of c-Myc protein with ubiquitin by treatment with proteasome inhibitors indicated the involvement of proteasome in c-Myc proteolysis and the stabilization of c-Myc protein by proteasome inhibitors in vivo. The expression of Fas (also termed CD95 or APO-1) mRNA, if analyzed by reverse transcriptase polymerase chain reaction assay, was found to occur constitutively, and increased slightly by the treatment with proteasome inhibitors. In contrast, the expression of Fas ligand (FasL) mRNA was markedly induced temporarily before the activation of caspase-3 by the treatment. Agonistic anti-Fas antibody (CH11) induced apoptotic cell death, suggesting the presence of a functional Fas receptor. In addition, proteasome inhibitor-induced apoptosis was prevented by the addition of antagonistic anti-FasL antibody (4A5) or z-IETD.fmk, a potent inhibitor of caspase-8, indicating the involvement of the Fas receptor–ligand apoptotic signaling system in proteasome inhibitor-mediated apoptosis. Thus, it is suggested that proteasome inhibitors cause the accumulation of c-Myc protein which induces transiently FasL message to stimulate the Fas receptor–ligand apoptotic signaling pathway. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Lactacystin; Acetyl-leucinyl-leucinyl-norleucinal; c-Myc; Fas ligand; Caspase-8; Apoptosis; Glioma

1. Introduction

In higher eukaryotic cells, the proteasome is involved in the ATP/ubiquitin-dependent proteolysis of most of the nuclear and cytosolic proteins and in particular of short-lived proteins critical for cell proliferation and cell cycle regulation. The natural product lactacystin, a specific inhibitor of proteasome [1], and acetyl-leucinyl-leucinyl-norleucinal (AcLLNal), one of the peptide aldehyde inhibitors of proteasome, have been shown to induce apoptotic cell death by the activation of caspase-3 in tumor cells including human glioma cells [2–6]. In mammals, programmed cell death can be initiated by three distinct pathways [7,8]: (i) the extrinsic pathway, which can be triggered by 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, which cleaves off an N-terminal fragment of Bid, a proapoptotic member of the Bcl-2 family, in low caspase-8 activation, allowed by the tBid to translocate to mitochondria and induce cytochrome *c* release [9,10]; (ii) the intrinsic pathway, which is initiated by diverse apoptotic stimuli and converges at mitochondria to release cytochrome *c* from mitochondria into cytosol followed by activation of caspase-9; or (iii) 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.

Calphostin C (a specific inhibitor of protein kinase C)-induced apoptosis in human glioma cell lines (U-87MG and T98G) is associated with cytochrome *c* release from mitochondria into cytosol and dissipation of mitochondrial potential [11,12], indicating the presence of the intrinsic apoptotic pathway. Nevertheless, lactacystin- or AcLLNal-induced apoptosis in U-87MG and T98G glioma cell lines is not associated with cytochrome *c* release and loss of mitochondrial potential [6], indicating the presence of the extrinsic apoptotic pathway which induces mitochondria-independent apoptosis in U-87MG and T98G cell lines. In contrast, lactacystin- and MG132 (a proteasome inhibitor)-induced apoptosis in other human glioma cell lines (LN-18 and LN-18-R) is associated with cytochrome *c* release from mitochondria and loss of mitochondrial potential without any involvement of both Fas receptor–ligand interactions and CrmA-sensitive caspase [13]. The difference in findings regarding cytochrome *c* release and disruption of mitochondrial potential between two experiments on human glioma cell lines might possibly be caused by the different phenotypes and genotypes of these cell lines reported recently [14]. Furthermore, proteasome inhibitor-induced apoptosis in human leukemia cells [15] and cultured rat cortical neurons [16] involves a cytochrome *c*-dependent pathway, raising the possibility that the execution of apopto-

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Abbreviations: AcLLNal, acetyl-leucinyl-leucinyl-norleucinal; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; z-IETD.fmk, benzyloxycarbonyl-Ile-Glu-Thr-Asp fluoromethylketone

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sis induced by proteasome inhibitor is likely to be mediated, depending upon the cell types and cellular factors, through several mechanisms including both cytochrome *c*-dependent and -independent pathways. Recently, proteasome inhibitors have been reported to sensitize human vascular smooth muscle cells to Fas receptor–ligand-mediated apoptotic cell death [17]. Our data show here that proteasome inhibitors induce the accumulation of c-Myc protein and subsequent induction of FasL message in human glioma cell lines U-87MG and T98G to mediate mitochondria-independent apoptosis via the Fas receptor–ligand apoptotic pathway.

2. Materials and methods

2.1. Glioma cell culture

Two human glioma cell lines, wild-type p53-positive U-87MG and mutant p53-positive T98G cells, were obtained from American Type Culture Collection, Rockville, MD, USA, and were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum and antibiotics in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were exposed to 50 μM lactacystin or 50 μM AcLLNal (Cosmo Bio Inc., Tokyo, Japan) for indicated times, and were stained with 2.5 μg/ml Hoechst 33258 after fixation with 4% paraformaldehyde. The number of apoptotic cells was assessed based on the expression of morphological features characteristic of apoptosis, and viability of the cells was confirmed by the trypan blue dye exclusion method.

2.2. Cell lysis, immunoprecipitation, and immunoblotting

Cells were lysed on ice in lysis mixture containing 2 mM Na_3VO_4, 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF) in phosphate-buffered saline (PBS). In ubiquitin blotting experiments the cells were lysed in boiling lysis buffer containing 1% sodium dodecylsulfate (SDS) in PBS in order to avoid isopeptidase activity. The lysate was heated at 100°C for 5 min, sheared to break DNA and centrifuged for 5 min at $10\,000 \times g$. Immunoprecipitations were carried out in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% bovine serum albumin, 1 mM EDTA, 2 mM Na_3VO_4, 0.1 M NaF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF in PBS. The reactions were incubated for 2 h at 0°C with anti-c-Myc antibody and protein A-agarose (Repligen, Cambridge, MA, USA) was used to isolate the immune complexes. The immunoprecipitates were washed twice with the same buffer and once with 10-fold diluted PBS, and immune complexes were analyzed by 7.5% polyacrylamide gel electrophoresis in the presence of SDS. For immunoblotting following immunoprecipitation, the immunoprecipitates obtained with anti-c-Myc antibody were transferred to polyvinylidene difluoride membrane, and immunostained using anti-ubiquitin conjugate antibody (FK1) [18] or anti-c-Myc antibody. In addition, levels of c-Myc protein were probed immunologically by immunoblot analysis. The antigens were visualized using ECL system (Amersham, UK).

2.3. RT-PCR for c-myc, Fas and FasL expression

The expression of c-*myc*, Fas and FasL mRNA was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Total RNA samples were prepared with Isogen (Nippon Gene Co., Toyama, Japan) according to the manufacturer's recommended protocol. For the first strand cDNA synthesis, 0.5 μg of total RNA was primed with oligo[dT] in reverse transcription with the AMV RNA PCR Kit Ver. 2.1. (Takara Shuzo, Kyoto, Japan). Subsequently, c-*myc* cDNA was amplified by 30 cycles of PCR with primers of 5′-CTGGTGCTCCATGAGGAG-3′ and 5′-AGGTGATCCAGACTCTGAC-3′, Fas cDNA was amplified by 30 cycles of PCR with primers of 5′-TCTAACTTGGGGTGGCTTTGTCTTC-3′ and 5′-GTGTCATACGCTTTCTTTCCAT-3′, and FasL cDNA was amplified by 30 cycles of PCR with primers of 5′-GGATTGGGCCTGGGGATGTTTCA-3′ and 5′-AGCCCAGTTTCATTGATCACAAGG-3′. As a control, glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA was amplified from the same first strand cDNA reactions, with primers 5′-GGCGTCTTCACCACCATGGAG-3′ and 5′-AAGTTGTCATGGAATGACCTTGG-3′ (Central Laboratory, Nippon Flour Mills, Kanagawa, Japan). The PCR products, 135-bp c-*myc* fragment, 365-bp Fas fragment, 278-bp FasL fragment, and 206-bp GAPDH fragment, were separated by electrophoresis on a 1.5 or 2.0% agarose gel and visualized by ethidium bromide staining. PCR amplifications were performed on a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA).

3. Results

3.1. Inhibition of proteasomal activity is accompanied by accumulation of c-Myc

Immunoreactive steady-state levels of c-Myc protein were increased as early as 6 h after treatment with lactacystin and 8 h after treatment with AcLLNal in U-87MG cells as well as 1–3 h after treatment with lactacystin and 3 h after treatment with AcLLNal in T98G cells (Fig. 1A,B). The c-Myc protein up-regulation was transient in U-87MG cells: the c-Myc protein levels were returned to normal at 24 h after lactacystin treatment and at 32 h after AcLLNal treatment. The increase in c-Myc protein levels in T98G cells was fixed uniformly for at least 15 h after treatment with lactacystin or AcLLNal. Recent evidence has indicated that the ubiquitin proteolytic system plays a major role in targeting short-lived key regulatory proteins for degradation [19–21], and degradation of a protein by means of the ubiquitin system involves two discrete steps, conjugation of multiple molecules of ubiquitin to the target protein and degradation of the tagged substrate by the 26S proteasome. Since the degradation of c-Myc protein in vitro and in vivo is reported to be mediated by the ubiquitin system [22,23], we examined the possible involvement of the proteasome in c-Myc proteolysis of U-87MG and T98G cells in vivo. Addition of the proteasome inhibitor led to accumulation of high-molecular-mass derivatives in c-Myc immunoprecipitates, and immunoblot analysis with anti-ubiquitin antibody showed that these compounds were composed of c-Myc protein–ubiquitin complexes (Fig. 1B), suggesting that c-Myc protein is proteolysed by proteasomes and stabilized by proteasome inhibition. In contrast, there was no evidence of any changes of c-*myc* mRNA expression in U-87MG and T98G cells assessed by RT-PCR analysis during the c-Myc protein up-regulation induced by the proteasome inhibitor (Fig. 1C). Thus, it is suggested that c-Myc protein in vivo is degraded by proteasomes and that proteasome inhibitors are shown to induce stabilization and accumulation of c-Myc protein, which has an extremely short half-life of about 30 min [24].

3.2. Fas receptor is constitutively expressed

To define the cell surface expression of Fas receptor, we analyzed the expression of Fas mRNA in U-87MG and T98G cells by RT-PCR assay. As shown in Fig. 2A, the expression of Fas mRNA was detected before and during lactacystin or AcLLNal treatment in U-87MG and T98G cells, suggesting that both glioma cells constitutively express Fas receptor. There was a slight enhancement of Fas mRNA levels, without any significant differences, concurrently with the increase in c-Myc protein levels by treatment with either proteasome inhibitor in both cells. To functionally assess the Fas receptor expression on cells we performed a cytotoxic assay using an agonistic anti-Fas monoclonal antibody (CH11). Agonistic stimulation with anti-Fas antibody induced apoptotic cell death as shown in Fig. 2B, suggestive of the presence of a functional Fas receptor in human glioma cells.

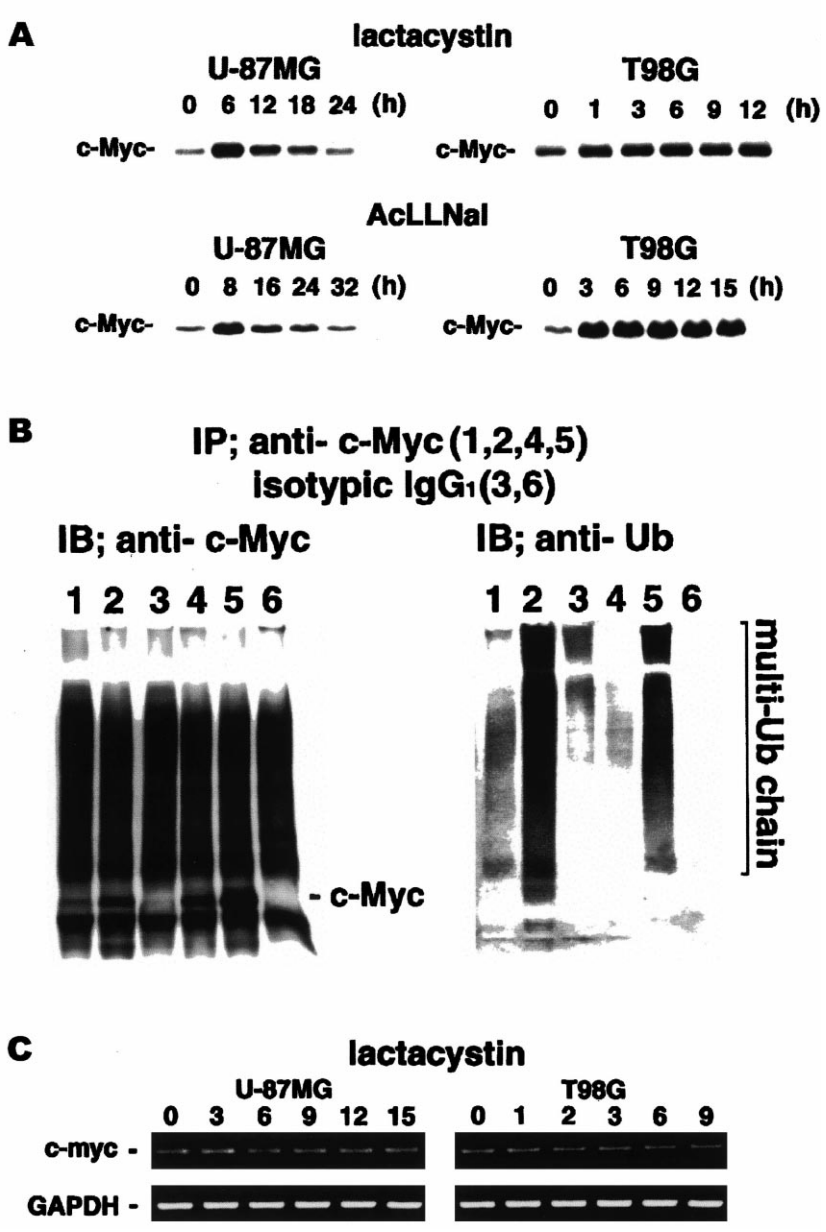

Fig. 1. Effects of lactacystin and AcLLNal on the steady-state level of c-Myc, on the formation of c-Myc–ubiquitin conjugates, and on c-*myc* mRNA expression. A: Human glioma cell lines (U-87MG and T98G) were exposed to 50 μM lactacystin or 50 μM AcLLNal for the indicated times. Steady-state level of c-Myc protein in U-87MG and T98G cells was monitored by immunoblot analysis. B: After treatment with 50 μM lactacystin for 6 h in U-87MG cells and for 3 h in T98G cells, lysates of both cells were immunoprecipitated with anti-c-Myc antibody (lanes 1, 2, 4, and 5) or isotypic IgG_1 (lanes 3 and 6). Immunoprecipitates were resolved by means of SDS polyacrylamide gel electrophoresis, and following transfer of the proteins to polyvinylidene difluoride membrane the conjugates were detected by anti-c-Myc antibody (left) or anti-ubiquitin antibody (right). c-Myc protein in U-87MG and T98G cells was increased by treatment with lactacystin (left). Adducts of c-Myc protein with ubiquitin were accumulated by treatment with lactacystin (right). IP, immunoprecipitation; IB, immunoblot; Ub, ubiquitin; 1–3, U-87MG; 4–6, T98G; 2 and 5, treatment with lactacystin; 1 and 4, no treatment. C: c-*myc* mRNA expression was analyzed by RT-PCR performed on total RNA isolated from U-87MG and T98G cells after treatment with lactacystin for the indicated times. A 206-bp GAPDH fragment was used to control for equal amounts of cDNA synthesized in the RT reactions. The results are from a representative study performed three times with comparable outcomes.

3.3. Inhibition of proteasomal activity enhances FasL mRNA

Effects of lactacystin or AcLLNal on the expression of FasL mRNA were analyzed by RT-PCR assay. As shown in Fig. 2A, the expression of FasL mRNA in U-87MG and T98G cells was not detected before lactacystin or AcLLNal treatment but markedly induced transiently after the treatment. The induction of FasL mRNA was detected 6–9 h in U-87MG cells and 2 h in T98G cells after lactacystin treatment and 12–16 h in U-87MG cells and 3–6 h in T98G cells after AcLLNal treatment. Therefore, the induction of FasL message by treatment with lactacystin or AcLLNal was detected concurrently with or after the increase of c-Myc protein levels in U-87MG and T98G cells.

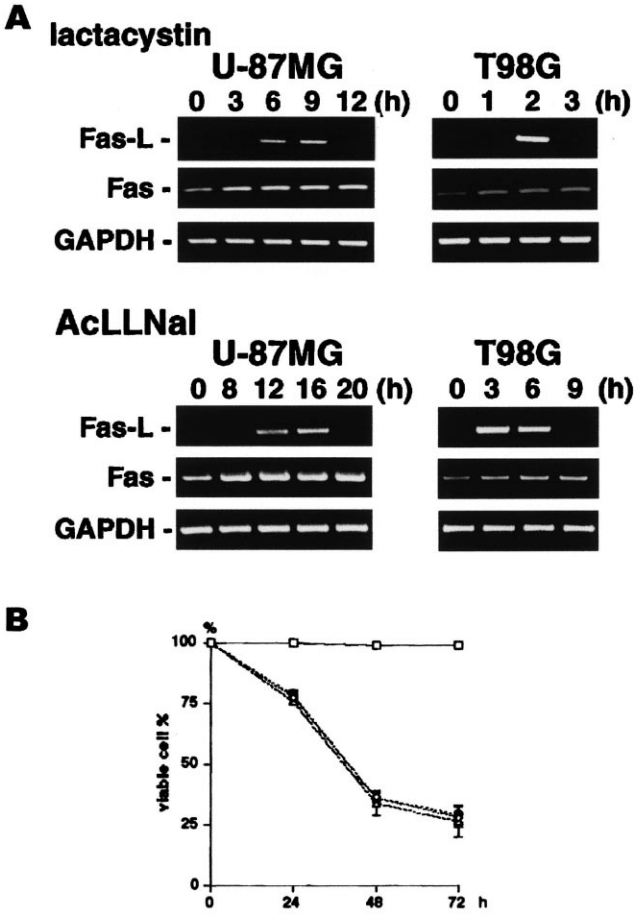

Fig. 2. Sensitivity of human glioma cells to Fas-mediated apoptosis. A: Analysis of Fas and FasL mRNA expression was performed by RT-PCR on total RNA isolated from U-87MG and T98G cells treated with 50 μM lactacystin or 50 μM AcLLNal for the indicated times. A 206-bp GAPDH fragment was used to control for equal amounts of cDNA synthesized in the RT reactions. B: Fas killing was triggered by the addition of agonistic anti-Fas antibody (CH11) at various concentrations to T98G cells and cumulative cell death is shown plotted against time. Open squares, treatment with mouse isotypic IgG as control antibody; open diamonds, treatment with 0.5 μg/ml CH11; closed circles, treatment with 1.0 μg/ml CH11; open triangles, treatment with 2.0 μg/ml CH11. Similar results were achieved in three separate experiments.

3.4. *Fas receptor–ligand system is involved in proteasome inhibitor-induced apoptosis*

To directly analyze whether the Fas receptor–ligand apoptotic pathway is involved in proteasome inhibitor-mediated apoptosis, we employed 10 μg/ml antagonistic anti-FasL antibody (4A5), which interferes with the Fas receptor–ligand interaction. If added to the culture medium 60 min prior to the treatment with lactacystin or AcLLNal, 4A5 antibody prevented the proteasome inhibitor-induced apoptotic cell death of U-87MG and T98G cells as shown in Fig. 3A. In addition, to analyze whether the Fas receptor–ligand apoptotic pathway has a direct role in proteasome inhibitor-induced cell death of U-87MG and T98G cells, we investigated the effects of 20 μM z-IETD.fmk, a potent inhibitor of caspase-8, on proteasome inhibitor-mediated cellular responses by adding it to the culture medium 60 min prior to the treatment with lactacystin or AcLLNal. Z-IETD.fmk prevented the proteasome inhibitor-mediated cell death (Fig. 3B) and morphological features characteristic of apoptosis (Fig. 3C), such as cell shrinkage and surface blebbing by phase contrast images as well as nuclear condensation and fragmentation by Hoechst 33258 stain.

4. Discussion

The degradation of c-Myc protein in vitro [22] and in vivo [23] has been reported to be mediated by the ubiquitin system. The present study clearly demonstrates that lactacystin or AcLLNal induces the stabilization and accumulation of c-Myc protein through the inhibition of the ubiquitin proteolytic system. The inhibition of proteasome activity is reported to cause accumulation of the c-Myc protein and induces apoptosis of preferentially c-*myc*-transformed fibroblasts and lymphoblasts [25]. In addition, the reduction of proteasome inhibitor-induced apoptosis by protein synthesis inhibitor was reported previously [6], suggesting that the accumulation of short-lived proteins such as c-Myc protein is associated with proteasome inhibitor-mediated apoptosis. Recently, *Bax* has been reported to be a direct transcriptional target of c-Myc and contributes to c-Myc-induced apoptosis [26], but Bax protein levels in U-87MG and T98G cells were not changed by the treatment with lactacystin or AcLLNal [6].

Fas mRNA was constitutively expressed, and the Fas message, when analyzed by RT-PCR assay, was slightly increased concurrently with the enhancement of c-Myc protein by the treatment with lactacystin or AcLLNal. Recently, proteasome inhibitors have been reported to up-regulate Fas of human vascular smooth muscle cells [17], and the interaction of Fas with the ubiquitin-conjugating enzyme [27,28] may be directly involved in ubiquitin-dependent degradation of Fas by proteasome. However, nothing is known on possible ubiquitination or stability of Fas. In contrast, the expression of FasL mRNA was markedly induced transiently by the treatment with lactacystin or AcLLNal, and the induction of FasL message was detected concurrently with or after the increase of c-Myc protein levels, suggesting that c-Myc protein plays a fundamental role in the regulation of the expression of FasL but not Fas as reported in T cells [29,30]. In addition, Brunner et al. [30] have reported recently that FasL promoter activity in T cells is driven by overexpression of c-Myc protein and inhibited by expression of dominant negative mutants of c-Myc. Since the activation of caspase-3 was detected as early as 18 h after lactacystin treatment and 24 h after AcLLNal treatment in U-87MG cells and as early as 9 h after lactacystin or AcLLNal treatment in T98G cells [6], both the increase of c-Myc protein levels and the induction of FasL message occur before the activation of caspase-3.

Fas and FasL expression is reported in human glioma cells [31–36] and a significant number of gliomas co-express both Fas and FasL [31,32,36]. Agonistic anti-Fas antibody (CH11) induced the apoptotic cell death in the present and other human glioma cells [37], suggesting the presence of a functional Fas receptor and an intact Fas apoptotic signaling pathway in the present glioma cell lines. In addition, proteasome inhibitor-induced apoptosis was prevented by addition of antagonistic anti-FasL antibody (4A5), which neutralizes FasL. Thus, both the apoptotic effects of the agonistic anti-Fas antibody and the apoptosis-inhibiting effects of the antagonistic anti-FasL antibody indicate the involvement of Fas receptor–ligand interactions in the proteasome inhibitor-medi-

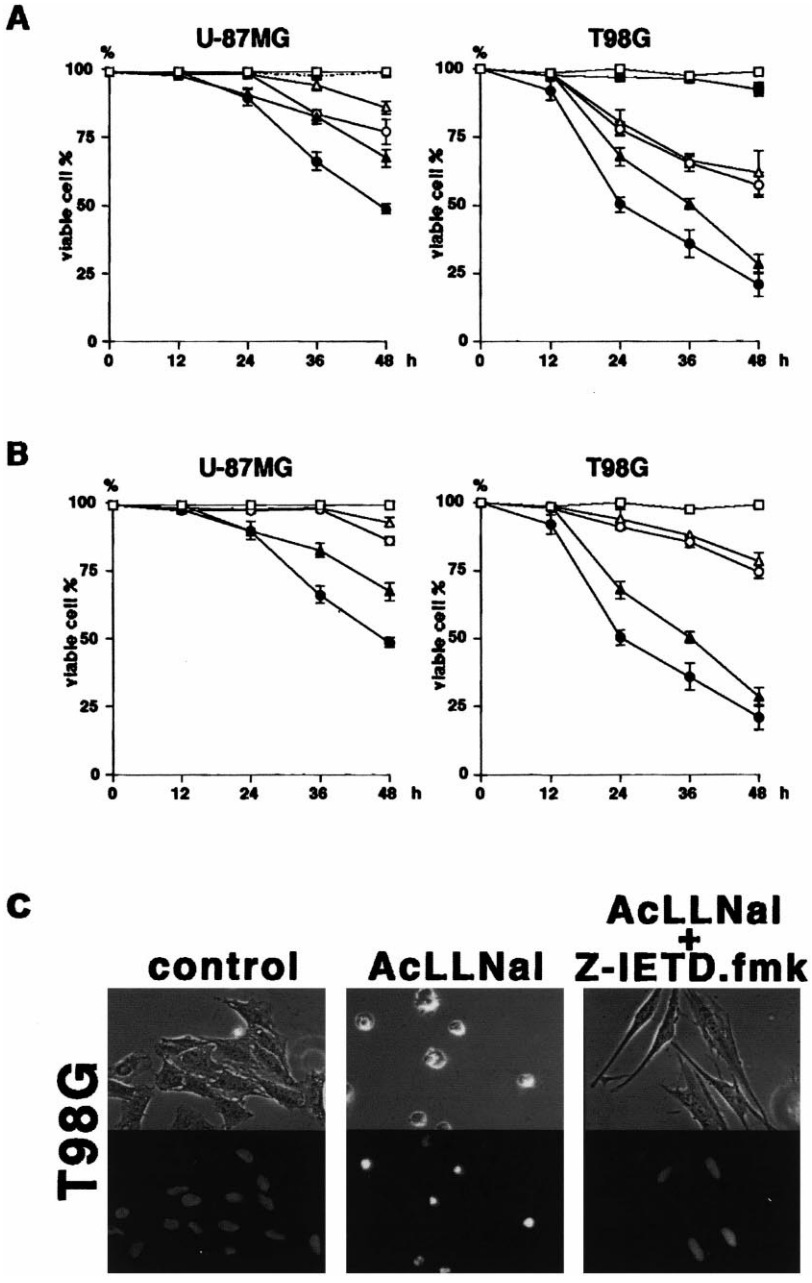

Fig. 3. Effects of antagonistic anti-FasL antibody (4A5: A) and caspase-8 inhibitor (z-IETD.fmk: B) on proteasome inhibitor-induced cell death. 4A5 (10 μg/ml) or z-IETD.fmk (20 μM) was added to the culture medium 1 h prior to the addition of 50 μM lactacystin or 50 μM AcLLNal, and thereafter 4A5 or z-IETD.fmk was given every 12 h. After treatment with lactacystin or AcLLNal for the indicated times, U-87MG and T98G cells were subjected to examination of cell viability. A: Open squares, no treatment; closed squares, treatment with mouse isotypic IgG as control antibody; closed circles, treatment with 50 μM lactacystin; open circles, treatment with 50 μM lactacystin and 10 μg/ml 4A5; closed triangles, treatment with 50 μM AcLLNal; open triangles, treatment with 50 μM AcLLNal and 10 μg/ml 4A5. B: Open squares, treatment with 0.1% DMSO as control; closed circles, treatment with 50 μM lactacystin; open circles, treatment with 50 μM lactacystin and 20 μM z-IETD.fmk; closed triangles, treatment with 50 μM AcLLNal; open triangles, treatment with 50 μM AcLLNal and 20 μM z-IETD.fmk. C: Representative photomicrographs of phase contrast images and Hoechst 33258 stain of T98G cells treated with 50 μM AcLLNal for 24 h in the absence or the presence of 20 μM z-IETD.fmk. Phase contrast was ×100; Hoechst 33258 stain was ×100. The data shown were obtained from three separate experiments.

ated apoptotic pathway. A functional relation between c-*myc* and Fas apoptotic signaling was already reported in activation-induced T cell death [22,38,39]. Recently, c-Myc-induced apoptosis in fibroblasts and T cells has been shown to require the Fas receptor–ligand apoptotic pathway [29,30,40].

The assembly of the Fas receptor death-inducing signaling complex occurs in a hierarchical manner: the death domain of Fas binds to the corresponding domain in the adapter molecule Fas-associated death domain FADD, which in turn recruits the zymogen form of caspase-8 by a homophilic interaction involving the death effector domains [41–44]. Immediately after recruitment, the single polypeptide caspase-8 zymogen is proteolytically processed to the active dimeric species composed of large and small catalytic subunits

that amplify the apoptotic signal by activating other downstream caspases such as caspase-3 [45–48]. Studies with cells derived from caspase-8 knock-out mice indicate that caspase-8 is absolutely required for Fas receptor-induced apoptosis [49]. Thus, caspase-8 is the most apical caspase in Fas-induced apoptosis [41,42]. Proteasome inhibitor-induced apoptosis was prevented by the addition of z-IETD.fmk, a potent inhibitor of caspase-8, indicating that efficient proteasome inhibitor-induced apoptosis requires cell surface interaction between Fas receptor and its ligand FasL and subsequent processing of caspase-8 through FADD. Recently, proteasome inhibitors have been shown to sensitize human vascular smooth muscle cells to Fas receptor–ligand-mediated apoptotic cell death [17]. In contrast, lactacystin has been reported recently to overcome Fas resistance in T cell leukemia cell lines by bypassing the proximal part of the Fas signal to activate caspase-8 [50]. However, the present study shows that antagonistic FasL antibody prevents proteasome inhibitor-induced apoptosis and agonistic Fas antibody induces apoptotic cell death, suggesting that Fas receptor–ligand interaction is necessary for proteasome inhibitor-induced apoptosis in the present human glioma cell lines. The absence of evidence of cytochrome *c* release from mitochondria into cytosol in the present proteasome inhibitor-induced apoptosis [6] indicates that cleavage of Bid by active caspase-8 [9,10] does not occur. In conclusion, the proteasome inhibitor-induced apoptosis in the present human glioma cell lines is mediated via the Fas receptor–ligand apoptotic pathway by the accumulation of c-Myc protein and subsequent induction of FasL message.

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