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The CD99 signal enhances Fas-mediated apoptosis in the human leukemic cell line, Jurkat

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Abstract The CD99 antigen has been implicated in various cellular processes, including apoptosis in T cells. Previously, we reported two monoclonal antibodies that recognize different epitopes of the CD99 molecule, named DN16 and YG32. In this study, we investigated the role of each CD99 epitope in T cell apoptosis. Unlike the DN16 epitope, CD99 ligation via the YG32 epitope failed to induce T cell death. Surprisingly, however, the YG32 signal enhanced Fas-mediated apoptosis in Jurkat T cells. Augmentation of Fas-mediated apoptosis by YG32 ligation was inhibited by treatment with either of the caspase inhibitors z-VAD-fmk or z-IETD-fmk, and YG32 ligation appeared to induce Fas oligomerization. These results suggest that each CD99 epitope plays a distinct role in T cell biology, especially in T cell apoptosis.

Key words: Apoptosis; Caspase; CD99; Epitope; Fas aggregation

1. Introduction

Apoptosis is the process by which cells commit suicide, and is characterized by distinct morphological and biochemical changes [1–3]. The transmembrane protein CD95 (Fas, APO-1) is a death receptor expressed on a variety of cell types, including lymphocytes, hepatocytes, and cardiac muscle cells. Cross-linking of surface Fas molecules by the Fas ligand (FasL, CD95L) or agonistic anti-Fas antibodies activates apoptotic death programs [4,5] via homo-oligomerization and results in the cleavage of several intracellular substrates [6–8]. Although receptor-mediated death signaling plays a major role in classical apoptosis, an increasing number of antigens have been implicated in non-classical, programmed cell death [9–13]. These include CD45, CD47, CD99, and MHC class I molecules.

The CD99 (E2 or MIC2) molecule is a leukocyte surface protein with a molecular weight of 32 kDa encoded by the MIC2 gene [14], which is located in the pseudo-autosomal region of the human X and Y chromosomes [15]. Although the function of the CD99 molecule is still unclear, it has been implicated in various cellular processes, including homotypic aggregation, apoptosis [16–19], vesicular protein transport [20–22], cell adhesion [23], Th1 cell differentiation [24], and migration of monocytes [25]. Apoptosis induced via the CD99 molecule has been demonstrated in double-positive thymocytes [16] and immature T cell lines [13,19] and appears to be caspase-independent [13].

A number of monoclonal antibodies (mAbs) that react with the CD99 molecule have been produced and each mAb recognizes a different epitope on CD99 with distinct functions [13,26]. For example, Pettersen et al. [13] reported that certain epitopes on the CD99 molecule induced rapid death signaling in Jurkat E6 cells, suggesting that CD99 is a death receptor. We previously developed two anti-CD99 mAbs, DN16 and YG32, which appear to recognize different epitopes on CD99 molecules [26,27]. Although the affinity of YG32 to the CD99 molecule is stronger than that of DN16 [26], most of our studies were restricted to the DN16 epitope [17,20–22,28].

To investigate the biological roles of distinct CD99 epitopes in T cell signaling, we examined the impact of the YG32 epitope in T cell apoptosis. Here, we report that while CD99 ligation via the YG32 epitope did not induce apoptosis, YG32 signaling enhanced Fas-mediated apoptosis via acceleration of Fas aggregation. Therefore, our results suggest that each CD99 epitope is involved in T cell death through divergent pathways.

2. Materials and methods

2.1. Cells and reagents

Jurkat T cells were cultured at 37°C in 5% CO_2 in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 50 μM 2-mercaptoethanol (2-ME), 2 mM L-glutamine, 10 mM HEPES, and 100 U/ml each of penicillin and streptomycin. The following antibodies against Fas protein were purchased from the sources shown: clone CH11 (Medical and Biological Laboratory, Nagoya, Japan), and clone C20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-mouse Ig antise-

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Abbreviations: 7-AAD, 7-aminoactinomycin D; AMC, aminomethylcoumarin; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; FasL, Fas ligand; HRP, horseradish peroxidase; 2-ME, 2-mercaptoethanol; mAbs, monoclonal antibodies; PBS, phosphate-buffered saline; $\Delta\psi_m$, mitochondrial membrane potential

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rum coupled to horseradish peroxidase (HRP) was from Dako Cytomation (Glostrup, Denmark). The caspase inhibitors z-VAD-fmk and z-IETD-fmk were from Calbiochem (La Jolla, CA, USA). Anti-CD99 mAbs, clones YG32 and DN16, were purchased from DiNonA (Seoul, Korea). FITC-labeled annexin V was purchased from BD Biosciences (San Diego, CA, USA) and 7-aminoactinomycin D (7-AAD) and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were obtained from Sigma.

2.2. Antibody treatment and flow cytometric analysis

Jurkat cells were plated in 24-well plates at 2×10^5 cells/well in a final volume of 1 ml of RPMI 1640 medium. These cells were treated with 2 or 10 μg/ml anti-CD99 mAb (YG32 or DN16) with or without anti-Fas Ab (CH11). At the indicated times, cells were harvested and then analyzed for apoptosis or subjected to immunoblotting. To detect apoptosis by flow cytometry, annexin V-FITC and 7-AAD were used. To evaluate the mitochondrial potential ($\Delta\psi_m$) by flow cytometry, the cationic lipophilic fluorochrome $DiOC_6$ was used. Flow cytometric analysis was performed using a FACSCalibur™ flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and the data were analyzed using CellQuest™ (Becton-Dickinson) software.

2.3. Assay for caspase activity

To detect caspase activity, the Caspase 3 Activity Assay kit (Roche, Mannheim, Germany) was used. Cytosolic extracts from 2×10^6 cells were incubated in anti-caspase 3 antibody-coated plates, followed by washing and adding the fluorescent tetrapeptide substrate (Ac-DEVD-AMC). Free aminomethylcoumarin (AMC) accumulation, which resulted from cleavage of the aspartate–AMC bond, was monitored in each sample in 96-well microtiter plates using a fluorometer at 400 nm excitation and 505 nm emission wavelengths.

2.4. Treatment with caspase inhibitors

Jurkat cells were treated with YG32 (2 or 10 μg/ml) and CH11 (10 or 50 ng/ml) in the indicated combinations. Caspase inhibitors (20 μM z-VAD-fmk or 50 μM z-IETD-fmk) were added to the media to evaluate their effects. The cells were harvested and analyzed after 5 h.

2.5. Western blot analysis

Sodium dodecyl sulfate (SDS)-stable Fas microaggregates were analyzed as described previously [29]. Briefly, aliquots of 5×10^6 cells were stimulated with anti-CD99 mAb (2 or 10 μg/ml YG32) for 30 min or 2 h at 37°C. The cells were washed with phosphate-buffered saline (PBS) and lysed in buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM NaF, 10 mM iodoacetamide, 200 μM Na_3VO_4, and 1% NP-40. The post-nuclear supernatants were loaded onto SDS–polyacrylamide gel electrophoresis gels, separated by electrophoresis, and transferred onto polyvinylidene difluoride Immobilon

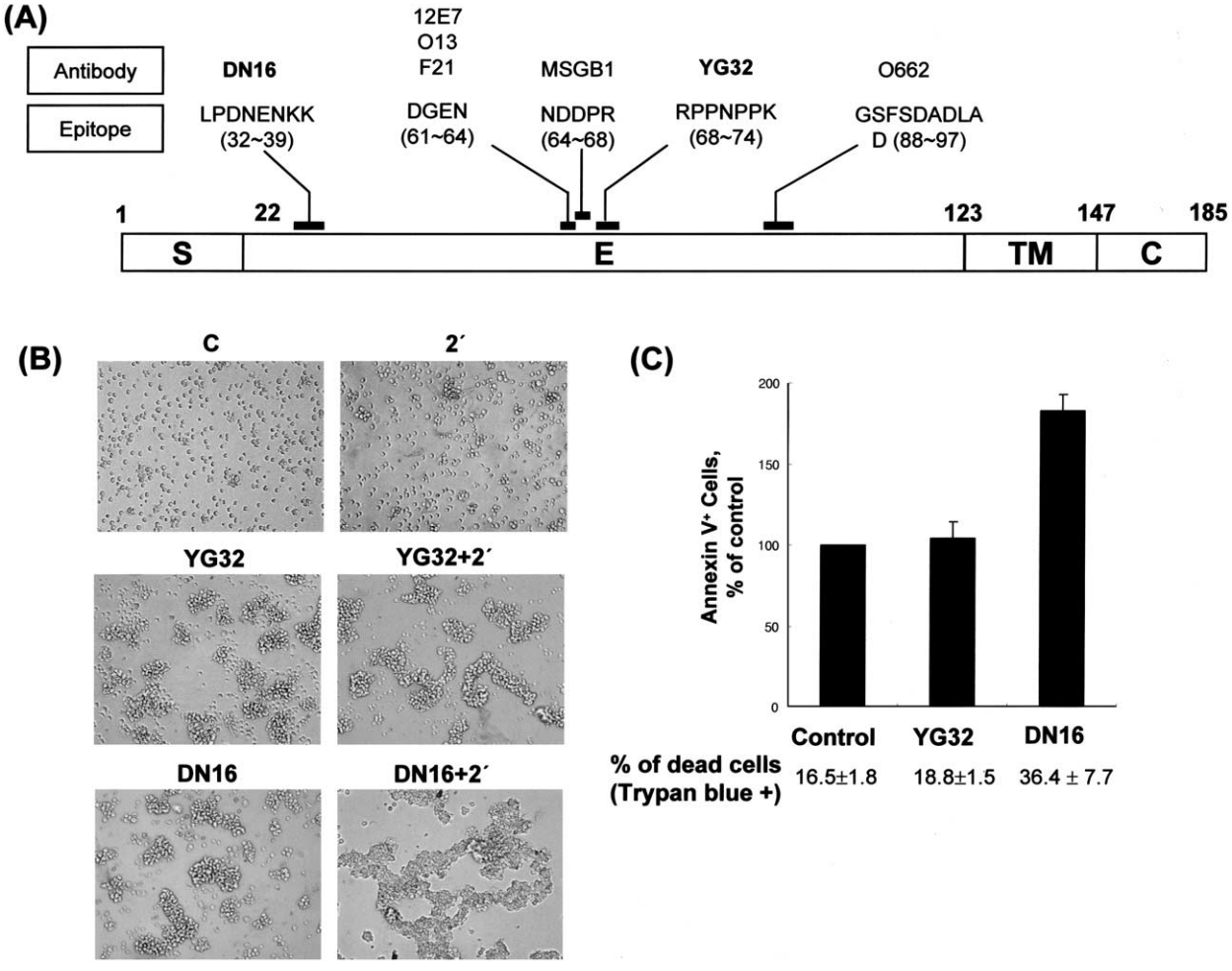

Fig. 1. Comparison of death responses mediated by mAbs YG32 and DN16, recognizing different epitopes and domains on the CD99 molecule. A: Schematic diagram of the CD99 molecule [16,26]. Locations of epitopes of anti-CD99 mAbs are shown. S, signal sequence; E, extracellular domain; TM, transmembrane domain; C, cytoplasmic domain. B: Jurkat T cells were stimulated with DN16 anti-CD99 mAb (10 μg/ml) and YG32 anti-CD99 mAb (10 μg/ml) for 30 min in the presence (right panels) or absence (left panels) of secondary Ab (20 μg/ml of anti-mouse Ig) (C; no stimulation, 2′; secondary Ab). C: Jurkat cells were cultured with mAb as indicated for 5 h. Cell death was assayed by annexin V staining. Specific cell death (% cell death) was calculated as a normalized value as follows: 100×[% of apoptotic cells (stimulated)−% of apoptotic cells (unstimulated)]/% of apoptotic cells (unstimulated). The real percentages of dead cells were assayed by trypan blue exclusion and the ranges are shown.

membranes (Millipore, Bedford, MA, USA). The blotted membranes were incubated with C20 Ab against Fas followed by HRP-conjugated anti-mouse Ab. The specific bands were visualized using an Enhanced Chemiluminescent kit from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.6. Immunofluorescence staining and confocal analysis

Jurkat cells were stimulated with anti-CD99 mAb. After washing in cold PBS, the cells were adhered to poly-L-lysine-coated slides. The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and incubated with biotinylated anti-Fas mAb (clone DX2, BD Biosciences) and streptavidin-Alexa 488 (Molecular Probes, Eugene, OR, USA). The stained preparations were mounted with coverslides and observed with a confocal microscope (Radiance 2000, Bio-Rad Laboratories, Hercules, CA, USA).

3. Results

3.1. Treatment with the anti-CD99 mAb YG32 induces homotypic aggregation, but does not induce apoptosis in Jurkat T cells

Two anti-CD99 mAbs, DN16 and YG32, were used to study the functional role of the CD99 molecule in T cell biology. Based on previous studies, a schematic diagram of the CD99 molecule with binding epitopes of mAbs is shown in Fig. 1A. We examined whether homotypic aggregation was induced by incubating Jurkat T cells with each anti-CD99 antibody in the presence or absence of a second cross-linking Ab. As reported previously [21], ligation via the DN16 epitope induced homotypic aggregation (Fig. 1B). Likewise, signaling via the YG32 epitope of the CD99 molecule also resulted in homotypic aggregation of Jurkat T cells (Fig. 1B) suggesting that both epitopes play roles in T cell functioning. In addition, with both mAbs, CD99-induced homotypic aggregation occurred, even in the absence of a second cross-linking Ab (Fig. 1B).

As we were interested in the role of CD99 epitopes in T cell death, we next examined whether both mAbs triggered the apoptotic signaling pathway. In fact, CD99 engagement has been reported to induce apoptosis in T cells using various mAbs and a second cross-linking Ab [13,16,19,20]. In this study, as no second cross-linking Ab was needed for homotypic aggregation (Fig. 1B), only the primary mAb, DN16 or YG32, was added to induce apoptosis in Jurkat cells and then flow cytometric analysis was used to detect apoptotic cells. Ligation of the CD99 molecule via the DN16 epitope successfully induced apoptosis, but no apoptosis was observed in YG32-treated Jurkat cells (Fig. 1C) implying that each epitope of the CD99 molecule has a distinct function in T cell death. Similar results were seen in the presence of a second cross-linking Ab (data not shown).

3.2. Signaling via the YG32 epitope enhances Fas-mediated cell death in Jurkat cells

Although YG32 signaling failed to induce apoptosis in Jurkat cells, we examined the involvement of the YG32 epitope in T cell death. Jurkat cells are widely used to study Fas-mediated cell death. Therefore, we evaluated the role of the YG32 epitope in Fas-mediated cell death. Cells treated with an agonistic anti-Fas Ab, CH11, were treated with or without YG32 mAb and the apoptosis was analyzed by flow cytometry (Fig. 2A,B). Ligation of the YG32 epitope in the CD99 molecule augmented the degree of Fas-mediated apoptosis (Fig. 2A,B). This was confirmed by trypan blue exclusion (Fig. 2C) and measuring the reduction in the mitochondrial membrane potential, $\Delta\psi_m$ (Fig. 3). In addition, YG32-en-

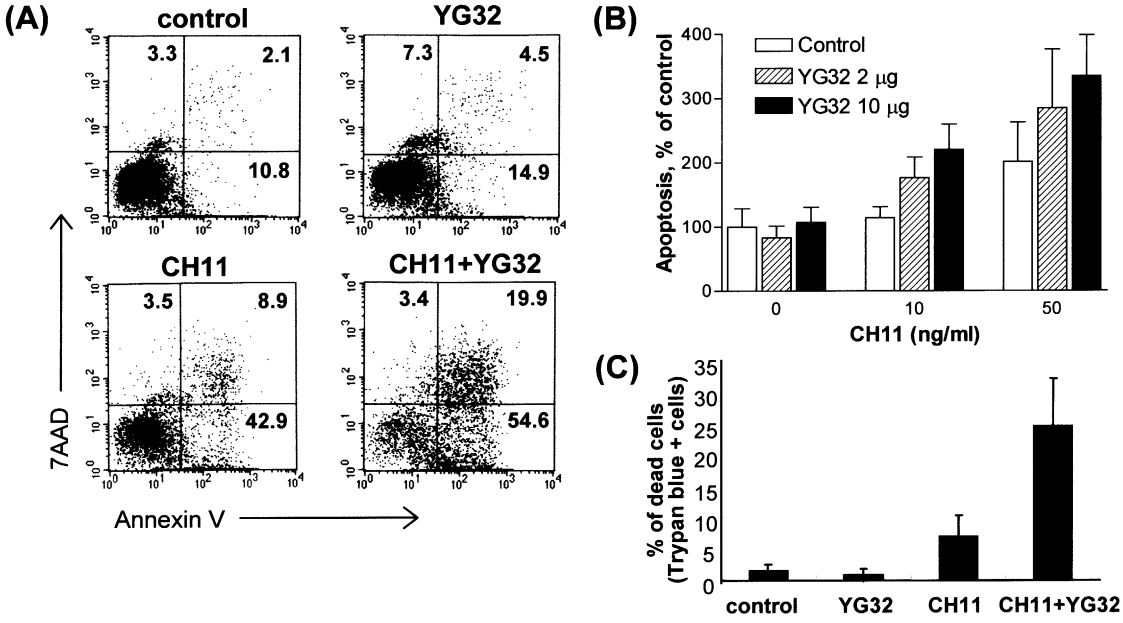

Fig. 2. Fas-mediated apoptosis was increased by CD99 ligation through the YG32 epitope. Addition of the anti-CD99 mAb YG32 enhanced the cell death induced by Fas ligation. A: Jurkat cells (2×10^5/ml) were incubated for 5 h with mAbs (YG32, 10 μg/ml; CH11, 50 ng/ml) as indicated combinations and stained with annexin V and 7-AAD. Representative result of three separate experiments. B: The degree of cell death was measured by annexin V staining after 5 h. After 5 h incubation with indicated combinations, apoptotic cells were measured by annexin V staining. Specific cell death (% of annexin V-positive cells) was calculated as a normalized value as described in Fig. 1C. The real percentages of annexin V-positive apoptotic cells are as follows. Control, 16.1 ± 4.7; YG32 2 μg/ml, 13.4 ± 2.9; YG32 10 μg/ml, 17.2 ± 3.9; CH11 10 ng/ml, 18.4 ± 2.8; CH11 10 ng/ml+YG32 2 μg/ml, 28.4 ± 5.2; CH11 10 ng/ml+YG32 10 μg/ml, 35.5 ± 6.4; CH11 50 ng/ml, 32.4 ± 9.98; CH11 50 ng/ml+YG32 2 μg/ml, 45.84 ± 14.8; CH11 50 ng/ml+YG32 10 μg/ml, 54.0 ± 10.3. C: The real percentages of dead cells were measured by trypan blue exclusion. Jurkat cells were incubated with mAbs (YG32, 10 μg/ml; CH11, 50 ng/ml) for 18 h and the dead cells were calculated.

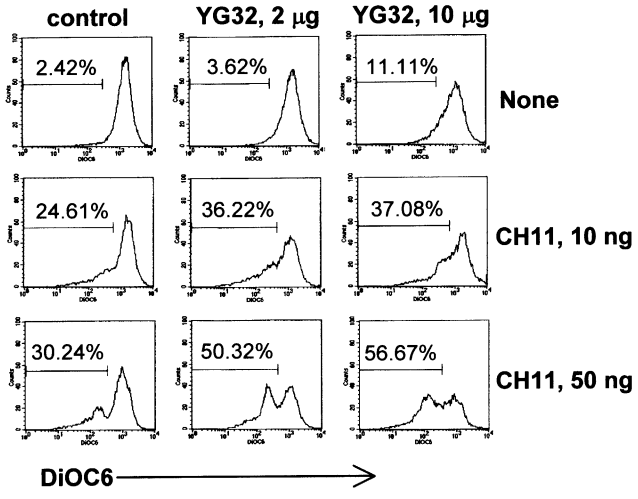

Fig. 3. Augmentation of reduction in $\Delta\psi_m$ in Fas-mediated apoptosis by CD99 ligation. The CH11-induced cell death was increased by YG32 mAb treatment, and the degree of augmentation was parallel to the amount of mAb added. Jurkat cells were incubated with mAbs for 5 h, and cell death was assayed by $DiOC_6$ staining.

hanced cell death even occurred with 10 ng of anti-Fas Ab, which did not cause apoptosis of Jurkat cells in 5 h incubation (Fig. 2B).

We next examined whether ligation via the YG32 epitope up-regulated the level of Fas expression in Jurkat cells. With YG32 treatment, FACS analysis showed no difference in the Fas expression on the cell surface as compared with control cells (data not shown). Reverse transcription polymerase chain reaction (RT-PCR) analysis also revealed that the messengers for the Fas molecule were identical in both untreated and YG32-treated Jurkat cells (data not shown). These observations suggest that the augmentation of Fas-mediated cell death was not due to the up-regulation of Fas expression.

3.3. *The augmentation of Fas-mediated apoptosis via the YG32 epitope is related to the caspase activation*

Caspases play a pivotal role in Fas-mediated apoptosis. On binding to cognate ligands or agonistic antibodies, Fas aggregates initiate the activation of caspase 8, which is followed by a cascade of caspase activation including caspase 3. We measured caspase 3 activities upon treatment of mAbs, CH11 and/or YG32, if there is an augmentation of caspase 3 functions by CD99 ligation via the YG32 epitope. As shown in Fig. 4A, though the YG32 signal itself did not induce caspase 3 activation, it was able to augment the activation of caspase 3 in

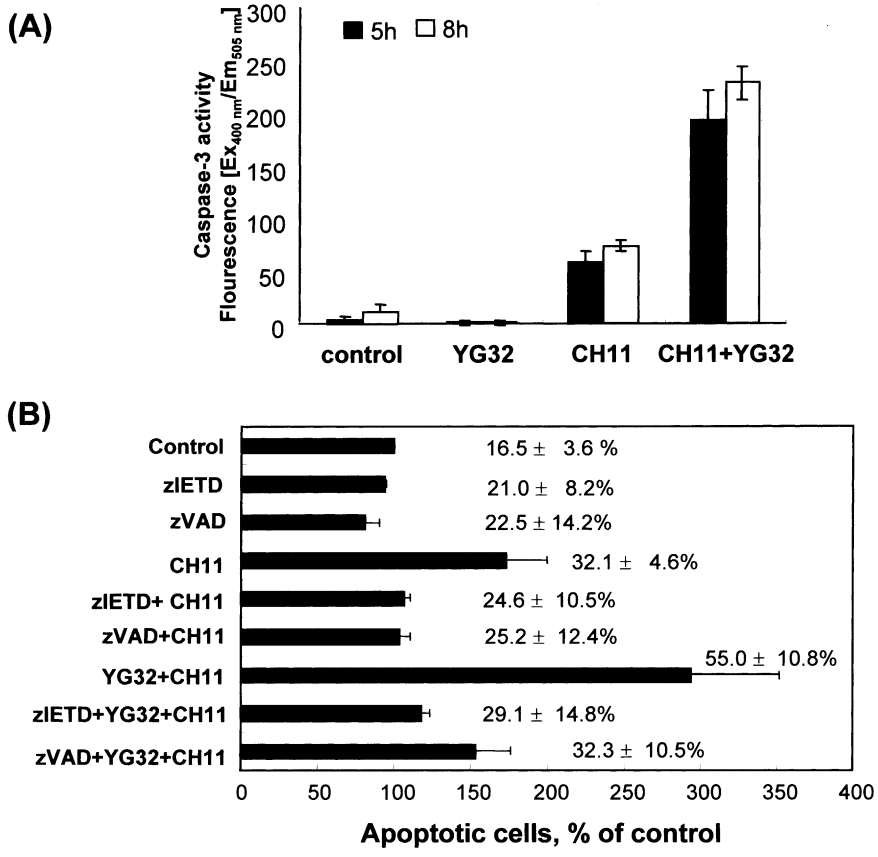

Fig. 4. Activation of caspase 3 and the effect of caspase inhibitors on YG32-amplified apoptosis in Jurkat cells. A: Activation of caspase 3 was measured upon ligation of CH11 and/or YG32 mAbs (CH11, 50 ng/ml; YG32, 10 μg/ml). Jurkat cells were incubated with mAbs as indicated and the cellular extracts were collected after 5 or 8 h. Caspase 3 activity was assayed as described in Section 2. B: Cells (2×10^5/ml) were incubated with YG32 (10 μg/ml) and/or CH11 (50 ng/ml) for 5 h. 20 μM of z-VAD-fmk or 50 μM of z-IETD-fmk was added to block the caspase activation. Specific cell death (% cell death) was calculated as a normalized value as described in Fig. 1C. After 5 h incubation, the degree of apoptosis was determined by using annexin V staining and analyzed by flow cytometry. The real percentages of annexin V-positive apoptotic cells are shown as numbers.

the Fas signaling pathway. We next used caspase inhibitors to elucidate whether the inhibition of caspase activation could prevent the Jurkat cells from apoptosis by engagement of both Fas and CD99. We first used a pan-caspase inhibitor, z-VAD-fmk, and found that the YG32-mediated augmentation of Fas-mediated cell death was completely inhibited (Fig. 4B). Next, a caspase 8 inhibitor, z-IETD-fmk, was used to block the early events in the Fas pathway. As shown in Fig. 4B, the caspase 8 inhibitor also blocked the YG32-mediated augmentation of Fas-mediated cell death, as well as Fas-mediated cell death itself. These findings imply that YG32 itself could not induce the apoptosis of Jurkat cells, when the Fas-mediated signal is blocked, confirming that YG32-mediated apoptosis of Jurkat cells is dependent on Fas signal.

3.4. The YG32/CD99 signal induces the aggregation of Fas molecules

When Fas interacts with FasL or agonistic anti-Fas Abs, Fas molecules are trimerized and caspase 8 is subsequently activated [30]. As the YG32 treatment did not affect the expression of Fas molecules on the surface of Jurkat cells, we assessed whether YG32 ligation enhanced Fas-mediated apoptosis via an effect on Fas aggregation. For this evaluation, Jurkat cells were treated with 2 or 10 μg of YG32 mAb for 30 min or 2 h, and Western blot and confocal microscope analyses were performed (Fig. 5A,B). As shown in Fig. 5A, stimulation of Jurkat cells with anti-CD99 mAb (YG32, 2 or 10 μg/ml) induced the formation of SDS- and 2-ME-stable, high M_r aggregates (>200 kDa) of Fas antigen, as reported previously [31–33]. The formation of the high M_r aggregates increased with time and the amount of mAb added, while no aggregation was observed in the control cells treated with irrelevant mAb (Fig. 5A). These results indicate that CD99 ligation via the YG32 epitope induced Fas aggregation in the absence of Fas engagement. This was confirmed by confocal analysis. After Jurkat cells were treated with the anti-CD99 mAb YG32 for the indicated times, Fas molecules were visualized and analyzed by confocal microscopy. Patch-shaped Fas aggregates were detected when cells were treated with YG32 mAb, while none were noted in the negative controls (Fig. 5B). In particular, almost all of the cells showed clumped patches of Fas molecules after 2 h of stimulation (Fig. 5B). Therefore, the CD99 signal via the YG32 epitope induced aggregation of Fas molecules in Jurkat T cells without FasL or agonistic anti-Fas mAb treatment.

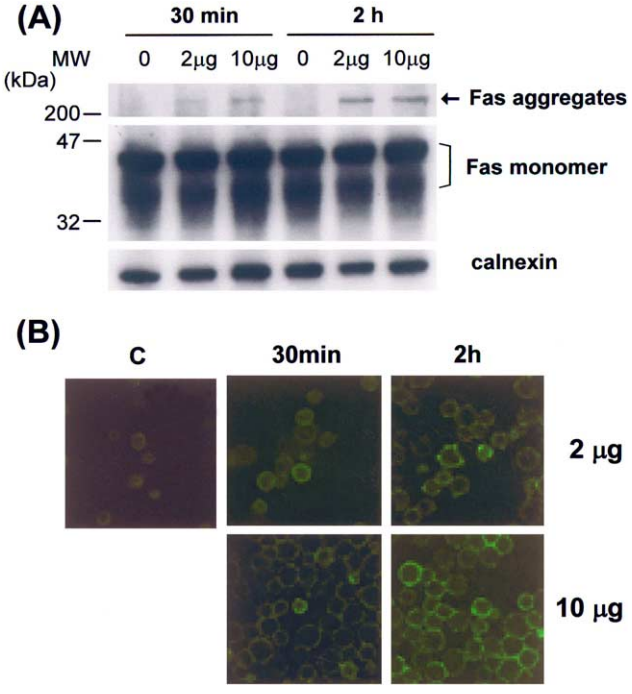

Fig. 5. YG32-induced aggregation of the Fas molecules. A: Jurkat cells (5×10^6/ml) were incubated with anti-CD99 (2 μg/ml or 10 μg/ml) for 30 min and 2 h. Total cell lysates were prepared and analyzed as described in Section 2. B: Jurkat cells were treated with anti-CD99 mAb, YG32, for 30 min and 2 h. Fas aggregates were detected by staining cells with biotinylated anti-Fas mAb and streptavidin-Alexa 488. Stained cells were analyzed by confocal microscopy. Prominent patch-like aggregations of Fas were observed in anti-CD99 mAb-treated Jurkat cells.

4. Discussion

In this study, we dissected the functional role of each CD99 epitope in T cell biology. Although the function of the CD99 molecule is still unclear, the CD99 antigen has been implicated in various cellular processes, including homotypic aggregation, apoptosis [16,17,21], vesicular protein transport [20,22,28], activation of mitogen-activated protein (MAP) kinase [21], up-regulation of VLA-4-mediated adhesion of T cells to endothelium [23], Th1 cell differentiation [24], and migration of monocytes across endothelial cells into an inflammatory site [25]. Moreover, apoptosis induced via the CD99 molecule has been demonstrated in thymocytes [16], Ewing's sarcoma cells [17], and Jurkat T cells [13]. In particular, Pettersen et al. [13] investigated the role of CD99 epitopes in T cell death using a number of mAbs to CD99 antigen and found that each epitope possessed a distinct function in apoptosis.

Previously, we described two mAbs recognizing the CD99 molecule, DN16 and YG32, which appear to recognize different epitopes of the CD99 antigen (Fig. 1A) [26]. In this study, we examined the role of the YG32 epitope in T cell death, as most of our work has been restricted to the DN16 epitope. In fact, the affinity of YG32 mAb to CD99-GST fusion protein seemed to be stronger that that of DN16 mAb, although it is not certain if the affinity differences are also applicable to the natural form of CD99 [26]. Consequently, we focused on the function of the YG32 epitope in the CD99 molecule. Unlike DN16, the YG32 epitope did not seem to act as a death receptor in Jurkat cells (Fig. 1C). This was interesting as the YG32 signal successfully induced homotypic aggregation even in the absence of a second cross-linking Ab (Fig. 1B) and led to the activation of MAP kinase, as did the DN16 signal [21].

Proper activation of death receptors plays a major role in the regulation of T cell development and function. Of these, Fas plays an important role in regulating the immune response and controlling peripheral B and T cell survival, which is critical for the maintenance of immune cell homeostasis [34]. The contribution of the Fas-mediated cell death pathway to controlling the peripheral T cell immune response has been well characterized [35–37]. In addition, Jurkat cells are widely used to investigate Fas-mediated cell death. On binding to cognate ligands or agonistic antibodies, the Fas receptors homo-oligomerize via the death domain [1], and the involvement of a lipid raft in Fas-mediated cell death was recently

demonstrated [38,39]. The YG32 signal appears to play a role in the vesicular transport of transmembrane proteins [22] and lipid rafts from the intracellular location to the cell surface [28]. In addition, CD99 ligation appeared to be involved in T cell death [13,16,19,20]. As the CD99 molecule plays a role in a number of biological functions, we tested if the YG32 epitope could also act as a death receptor. However, unlike the DN16 epitope, YG32 ligation failed to induce apoptosis in Jurkat cells. As the Jurkat cells are sensitive to Fas-mediated cell death, we next examined whether the YG32 epitope is involved in Fas-mediated cell death. CD99-mediated cell death has been reported to be Fas-independent in thymocytes [16] and leukemic cell lines [13]. As shown in Fig. 2, although the YG32 signal alone did not induce apoptosis, it enhanced Fas-mediated cell death in Jurkat cells, implying that the role of the YG32 epitope differs from that of DN16. Pettersen et al. [13] also reported that a distinct epitope of CD99 recognized by clone Ad20 possessed a specific role in T cell death, while another epitope of CD99 recognized by clone O662 failed to kill Jurkat T cells. By contrast, Bernard et al. [16] reported that engagement of CD99 by O662 antibody had been known to induce the apoptosis of immature thymocytes. These results suggest that each CD99 epitope has a distinct role in T cell function.

Fas ligation via FasL or agonistic anti-Fas Ab induces Fas oligomerization [4,5], recruits adapter and effector cytoplasmic proteins to the cell surface [1], and results in caspase activation. Next, we considered which steps in the Fas pathway are the targets of the YG32 signal in Jurkat cells. We first considered the possibility that the expression of Fas molecules on the surface of Jurkat cells is enhanced by the YG32 treatment. However, flow cytometry and RT-PCR analyses revealed that this was not the case (data not shown). Thus, we next assessed whether YG32 signal induced oligomerization of Fas molecules. As shown in Fig. 5A,B, oligomerized Fas molecules were readily observed on treatment with the anti-CD99 mAb YG32. We reported previously that the CD99 signal is involved in the vesicular transport of transmembrane proteins [22] and lipid rafts [28], and the involvement of lipid rafts in Fas-mediated cell death was demonstrated recently. Therefore, we investigated whether Fas oligomers resided in lipid rafts of the cell membrane. When stained simultaneously with cholera toxin, Fas oligomers did not appear to reside in the lipid raft (data not shown). This implies that the effect of the YG32 signal on Fas signaling differs from that on vesicular transport of the lipid raft [28].

In summary, the YG32 epitope of CD99 antigen conveys a different signal from that of DN16, as no apoptosis occurred on YG32 ligation. In addition, the YG32 signal activates Fas oligomerization, enhancing Fas-mediated cell death in T cells via an as yet undefined pathway. Therefore, the CD99 molecule can induce caspase-independent non-classical apoptosis and is involved in classical receptor-mediated apoptosis in T cells. It would be interesting to determine whether the YG32 epitope of the CD99 molecule enhances physiological activation-induced cell death.

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