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Requirement of Cell–Cell Contact in the Induction of Jurkat T Cell Apoptosis: The Membrane-Anchored but Not Soluble Form of FasL Can Trigger Anti-CD3-Induced Apoptosis in Jurkat T Cells

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Fas ligand (FasL) has been shown to be processed by the action of certain metalloproteinase and released from the cell surface. However, it is unclear whether death of Fas-sensitive target cells is mediated by a membrane-bound form of FasL (mFasL) or by a soluble form of FasL (sFasL). In the present study, we demonstrated that JCaM, a p56*lck*-deficient mutant of Jurkat, underwent Fas-dependent apoptosis only upon physical contact with anti-CD3-stimulated Jurkat cells or with human FasL- expressing transfectant (hFas/L5178Y). Recombinant FasL or sFasL-containing supernatant failed to induce apoptosis in both Jurkat and JCaM. Moreover, addition of a metalloproteinase inhibitor, which led to the accumulation of mFasL in hFas/L5178Y, was found to augment apoptosis in both Jurkat and JCaM. These findings indicate that, in a physiologic setting represented by the activation-induced cell death in Jurkat T cells, cell–cell contact appears to be required for the induction of Fas-mediated killing. © 1997 Academic Press

T lymphocytes are known to undergo activation-induced cell death (AICD) via a mechanism of apoptosis when repeatedly stimulated through their T cell receptor/CD3 complex. Several studies have provided evidence that the interaction of Fas (APO-1/CD95) with its ligand (FasL) plays a critical role in the induction of apoptosis during the process of AICD (1-3). Fas ligand (FasL), a type II integral membrane protein homologous with tumor necrosis factor-alpha (TNFα), has recently been shown to undergo proteolytic cleavage by the action of some metalloproteinase and be released from the cell surface (4). In certain pathologic conditions, elevated levels of sFasL could be detected in the serum (5). However, it remains unclear whether death of Fas-sensitive target cells is mediated by membrane-bound form of FasL (mFasL) or by soluble form of FasL (sFasL) during the process of AICD.

To address this question, we investigated the apoptosis induction in AICD utilizing Jurkat cells and its p56*lck*-deficient mutant JCaM. We have previously reported that both cell lines expressed Fas antigen and were sensitive to undergo apoptosis by anti-Fas mAb (CH-11) treatment; however, in contrast to Jurkat, JCaM failed to undergo apoptosis by anti-CD3 stimulation which was associated with the absence of FasL mRNA induction (6). In the present study, we demonstrated that JCaM cells underwent Fas-dependent apoptosis only upon physical contact with anti-CD3-stimulated Jurkat cells or with human FasL-expressing transfectant (hFas/L5178Y). Addition of recombinant sFasL and sFasL-containing supernatant failed to induce apoptosis in both Jurkat and JCaM. These findings indicate that there are substantial differences in the biologic activity of mFasL and sFasL for the induction of Fas-mediated apoptosis during the process of AICD in T cells.

MATERIALS AND METHODS

Cells and culture conditions. Human T cell leukemia cell line Jurkat (clone E6-1, ATCC TIB-152) and its *lck*-deficient Jurkat mutant JCaM (JCaM1.6, ATCC CRL-2063) were obtained from American Type Culture Collection (Rockville, MD). RPMI 1640 supple-

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Abbreviations used: mFasL, membrane-bound Fas ligand; sFasL, soluble form FasL; AICD, activation-induced cell death.

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mented with 10% fetal calf serum, 2mM L-glutamine 100 U/ml penicillin G and 100 μg/ml streptomycin was used for all cultures. Generation of a stable human FasL transfectant (hFasL/L5178Y) was described previously (4). In some experiments, cells were separately cultured in a 24-well plate using the cell culture insert (pore size, 0.4μm, Falcon 3095, Becton Dickinson, Franklin Lakes, NJ).

Monoclonal antibodies (mAbs) and reagents. Reagents and mAbs used; mAb to CD3 (mAb 454, IgG2a, Ref. 7, a gift from N. Chiorazzi, North Shore University Hospital, Manhasset, NY), mAb to Fas (clone CH-11, IgM, Ref. 8, MBL, Nagoya Japan), mAb to FasL (NOK-1; IgG1, NOK-3; IgM) were prepared as described (4). Goat anti-human TNFα neutralizing antibody was from R&D System (Minneapolis, MN). The matrix metalloproteinase inhibitor KB8301 was kindly provided by Kanebo Ltd. (Osaka, Japan).

Immunofluorescence staining and flow cytometry. For estimation of the cell surface expression of Fas, cells were stained with FITC-conjugated anti-Fas mAb (clone UB2 IgG1, Cosmo Bio., Tokyo, Japan). FITC-conjugated mouse IgG1 antibody (Cosmo Bio.) was used as a control. For surface FasL expression, cells were incubated with 1 μg of biotinylated NOK-1 for 1 h at 4°C, followed by PE-labelled avidin (PharMingen, San Diego, CA). The PKH26-GL fluorescent cell linker compound (Sigma, St. Louis, MO) was used for cell labeling. Fluorescent cell labeling was performed according to the manufacturer s protocol. The cell-surface analysis and the cell sorting were performed on a flow cytometer (FACS Vantage, Becton Dickinson).

Measurement of apoptosis by flow cytometry. Unless otherwise noted, 14 h culture with 100 ng/ml of anti-Fas mAb (CH-11) or 48 h culture with anti-CD3 (mAb 454, 1:50 dilution of the supernatant of hybridoma) was used for the induction of apoptosis. The percentage of cells undergoing apoptosis was quantitated by a modification of the flow cytometric method as described (9). Briefly, following fixation in 75% ethanol, the cells were washed and resuspended in PBS containing RNase (0.25 mg/ml, type I, Sigma) and propidium iodide (PI, 50 μg/ml, Sigma). The PI fluorescence of individual cells was measured on a flow cytometer. A distinct cell cycle region of apoptosis (Ao) could be identified below the Go/G1 diploid peak. Percentage of cells in the Ao region were estimated by using the ModFit LT program. To analyze the cell surface phenotype in conjunction with apoptosis, we employed annexin V binding assay (10) utilizing a commercially available kit (Apoptosis Detection Kit, R&D). Cell death was also evaluated by trypan blue exclusion.

Preparation of recombinant FasL. The 120 bp cDNA fragment of human IL-6 coding signal sequence (amino acid -28-10) was amplified by PCR from pBSF carrying human IL-6 cDNA using ATGAACTCCTTCTCCACA (primer1) as the 5′ primer and ATCTTTGGAATCTTCTCC as the 3′ primer. The 5′ and 3′ primers were tagged with *Xba*I site and anti-sense sequence of human FasL corresponding to nucleotide 307-324, respectively. In a similar way, 540 bp DNA fragment of human FasL coding extracellular domain (nucleotide 307-846) was amplified using GTCGAGAAGGTGGATGTC as the 5′ primer and TTAGAGCTTATATAAG CCGA A (primer2) as the 3′ primer. Human IL-6 sequence (CCTCTTCTAAGGTTTCTA) and *Not*I site were introduced into the 5′ and 3′ primers, respectively. After electrophoresis, these DNA fragments were isolated by QIAEX (QIAGEN) and mixed. Then 660 bp cDNA coding IL-6 signal sequence-FasL fusion protein was amplified by PCR using primer1 and primer2. After *Xba*I and *Not*I digestion, the PCR product of 660 bp was subcloned into *Xba*I and *Not*I site of PVL1393 (PharMingen) and the nucleotide sequence was confirmed by automated sequencer (Applied Bio Systems). Cotransfection of IL-6-FasL/PVL1393 and Baculo Gold into *Spodoptera frugiperda* (SF9) cells was performed according to the manufacture's instruction. After 5 days incubation at 28° C, supernatant was collected and viruses were cloned by plaque assay. Supernatant of infected SF9 cells was collected and recombinant FasL was purified by affinity chromatography on NOK1-conjugated Sepharose column. The concentration of recombinant FasL was determined by sandwich ELISA as described before(4).

Cytotoxic assays. Cytotoxic activity of recombinant soluble FasL and CH-11 against Jurkat and JCaM cells was tested by the alamar blue method as described (4). Human Fas cDNA transfectant of mouse T lymphoma cell line (hFas/WR19L) (11), provided by S. Yonehara (Kyoto University), was used as indicator cells.

Statistical analysis. Statistical significance was assessed by Student's t test and considered significant at $p<0.05$.

RESULTS AND DISCUSSION

To examine whether the functional Fas-FasL interaction is involved in the anti-CD3-induced apoptosis in Jurkat cells, we added a neutralizing anti-FasL mAb (NOK-3) to the anti-CD3-stimulated Jurkat cells. As shown in Table I, the addition of NOK-3 resulted in the significant inhibition of anti-CD3-induced apoptosis of Jurkat while the addition of control Ig or neutralizing anti-TNFα antibody failed to block this. These observations were consistent with previous reports utilizing soluble Fas (1-3) and confirmed that anti-CD3 -induced apoptosis of Jurkat is mediated by the Fas-FasL interaction. We also confirmed our previous finding that both Jurkat and JCaM express Fas antigen and are sensitive to undergo apoptosis upon treatment with an agonistic anti-Fas mAb (CH-11) (Fig 1). Jurkat cells undergo activation-induced apoptosis when stimulated by anti-CD3 mAb, in contrast, JCaM is fairly resistant to death by this treatment (Fig 1). We have previously shown that this resistance of JCaM to anti-CD3-induced apoptosis was associated with a lack of FasL mRNA induction (6). Taken together, these findings indicate that p56*lck* is required for anti-CD3-induced FasL expression thereby contributing to the anti-CD3-induced cell death in Jurkat cells.

These unique characters of Jurkat and its mutant JCaM enabled us to examine which form of FasL, namely mFasL or sFasL, could mediate death signal in anti-CD3-induced apoptosis of Jurkat as a model system of AICD in T lymphocytes. To address this question, we first cultured Jurkat and JCaM in the separated chamber in the presence of anti-CD3 and then examined for apoptosis induction in JCaM to assess whether the sFasL released from anti-CD3-stimulated Jurkat can induce apoptosis in JCaM. As shown in Fig 2 (panel 2), JCaM did not undergo apoptosis when cultured separately with Jurkat cells in the presence of anti-CD3. Secondly, Jurkat cells were pre-labeled by PKH26-GL fluorescent compound and then co-cultured with non-labelled JCaM in the absence or presence of anti-CD3. It was determined in preliminary experiments that PKH-26 dye transfer did not occur between stained-cells and unstained-cells during co-culture. After co-culture for 48 h, PKH-26-negative cells (composed of JCaM) were sorted and then examined for

TABLE I

Effect of Anti-FasL mAb (NOK-3) and Anti-TNFα Antibody on the Anti-CD3-Induced Cell Death in Jurkat Cells

Stimulus	Addition	(μg/ml)	% Dead cell[a]	% Apoptotic nuclei[b]
None	None		12 ± 5.6	16 ± 13.5
Anti-CD3	None		49 ± 11.0	44 ± 14.7
↓	Control Ig	(30)	46 ± 16.8	47 ± 16.5
↓	NOK-3	(1.0)	35 ± 8.6* ($p < 0.05$)	39 ± 24.4
↓	NOK-3	(3.0)	32 ± 7.9* ($p < 0.05$)	28 ± 19.0
↓	NOK-3	(10)	23 ± 8.7* ($p < 0.001$)	19 ± 4.3* ($p < 0.01$)
↓	NOK-3	(30)	21 ± 4.8* ($p < 0.001$)	12 ± 3.0* ($p < 0.001$)
↓	Anti-TNFα	(30)	53 ± 8.6	44 ± 16.2

Note. Jurkat cells were stimulated with anti-CD3 mAb for 48 h in the presence or absence of indicated antibodies. Values denote mean ± SD of five experiments.

[a] Dead cells were determined by trypan blue exclusion.

[b] % apoptotic nuclei were determined in PI stained samples by flow cytometry.

* Statistically different from anti-CD3-stimulated samples (underlined) by Student's t-test. P-values are indicated in parentheses.

apoptosis by PI staining (Fig 3: panels 3, 4). After physical contact with anti-CD3-activated Jurkat, JCaM did undergo apoptosis. Although we cannot rule out the possibility that some apoptotic Jurkat cells might contain reduced PKH-26 dye and thus were sorted as a PKH-negative population, the contamination with Jurkat appeared to be negligible since the percentage of cells undergoing apoptosis in the sorted population was almost comparable with that of anti-CD3-stimulated Jurkat cells which had been cultured separately in the same experiment (Fig 3, panels 3, 4 vs. panel 5). These findings strongly suggest that membrane-bound but not soluble form of FasL could trigger apoptosis in JCaM.

To explore the role of mFasL *vs.* sFasL in the induction of T cell apoptosis more precisely, we estimated the apoptosis-inducing activity of human FasL transfectant (hFasL/L5178Y) against Jurkat and JCaM cells. hFasL/L5178Y has been shown to constitutively express and release FasL into the culture supernatant (4). As shown in Fig 2 (panel 3), JCaM still did not undergo apoptosis in cultures where JCaM were sepa-

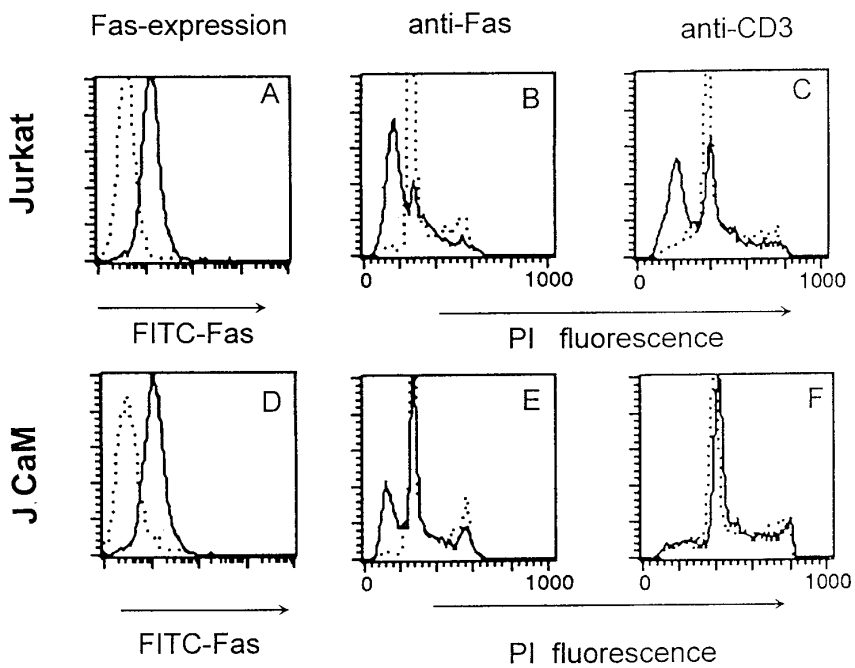

FIG. 1. JCaM are sensitive to anti-Fas but are resistant to anti-CD3-induced apoptosis. Fas antigen expression (A, D) and PI fluorescence histogram of anti-Fas- (B, E) or anti-CD3 (C, F)-treated Jurkat (A-C) or JCaM (D-F) cells are indicated. Cells stained with isotype-matched controls (A, D) or PI staining of untreated controls (B, C, E, F) are indicated by dashed lines.

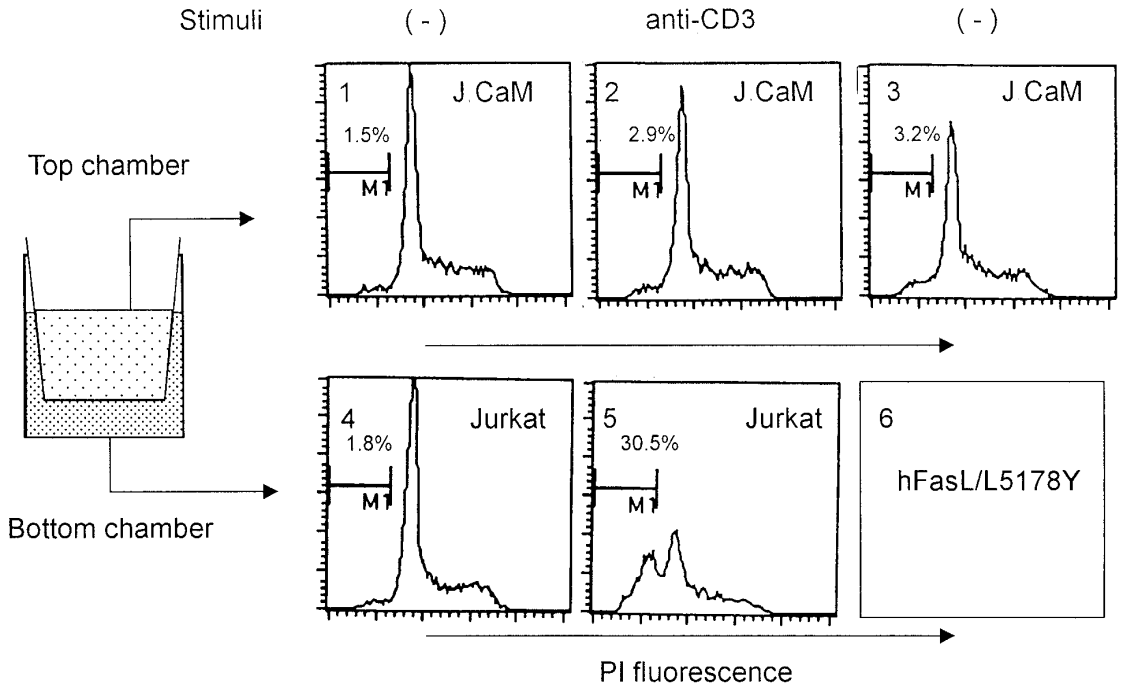

FIG. 2. JCaM failed to undergo apoptosis when cultured separately with anti-CD3-stimulated Jurkat or with FasL transfectants. JCaM cells were placed in the upper chamber (panels 1-3) separated by a membrane from the lower chamber where Jurkat (panels 4, 5) or hFasL/L5178Y (panel 6) were placed. Cells were cultured for 48 h in the presence (panels 2, 5) or absence (panels 1, 4 and 3, 6) of anti-CD3 mAb. Cells were stained with PI and were analyzed by flow cytometry. Values denote the percentage of sub-Go/G1 nuclei marked by bars (M1). Data represent three independent experiments.

rated from hFasL/L5178Y by the membrane. Next, we provided physical contact of Jurkat or JCaM with hFasL/L5178Y and then examined them for their apoptosis induction. To assess apoptosis in Jurkat or JCaM separately from co-cultured hFasL/L5178Y, we used annexin V-FITC binding that detects phosphatidylserine externalization on the apoptotic plasma membrane after gating out the FasL-PE-positive hFasL/L5178Y cells. As shown in Fig 4, significant apoptosis could be demonstrated both in Jurkat and JCaM after the contact with hFasL/L5178Y. Moreover, addition of a metalloproteinase inhibitor KB8301, which resulted in mFasL accumulation on the surface of hFasL/L5178Y (Fig 4B), resulted in increased apoptosis in Jurkat and JCaM cells. Addition of KB8301 alone did not affect the cellular viability but it resulted in increased anti-CD3-induced apoptosis in Jurkat (data not shown). Finally, we compared cytotoxity of anti-Fas mAb (CH-11) and recombinant sFasL against Jurkat or JCaM cells. As shown in Fig 5, while control indicator cells Fas/WR19L, hFas transfectants of mouse cell line, showed almost equal sensitivity to both reagents, Jurkat and JCaM were found to be sensitive to death by CH-11 but were strikingly resistant to death by recombinant sFasL.

Treatment with soluble anti-Fas mAb of IgM subclass (such as CH-11) has been widely used to induce apoptosis in Fas-sensitive cells. This apoptosis inducing ability of CH-11 is probably due to its ability to cross-link the Fas molecules and supports the notion of the requirement of trimerization of Fas molecules for efficient transmission of a cell death signal (12). It has previously been shown that mouse fibroblast transfectants expressing Fas can be killed by sFasL produced in *Propionibacterium acnes* (13). Consistent with this, mouse T cell transfectants (WR19L) expressing human Fas were also found to be sensitive to sFasL produced in insect cells (Fig 5). However, human Jurkat and JCaM were fairly resistant to this treatment. At the present time, we are not sure as to the explanation of these different sensitivities between cell lines, but the following possibilities may account for this. First, although Jurkat/JCaM constitutively express endogenous Fas, forced expression of Fas in transfectants may lead to a much abundant expression of this antigen. Second, species difference of target cells may also account for this. It is possible that the death signal transduction machinery downstream to Fas may differ between human and mouse cells and thus give rise to different sensitivities. Regarding the different sensitivity to death by agonist antibody and soluble FasL, two studies have demonstrated the presence of certain cel-

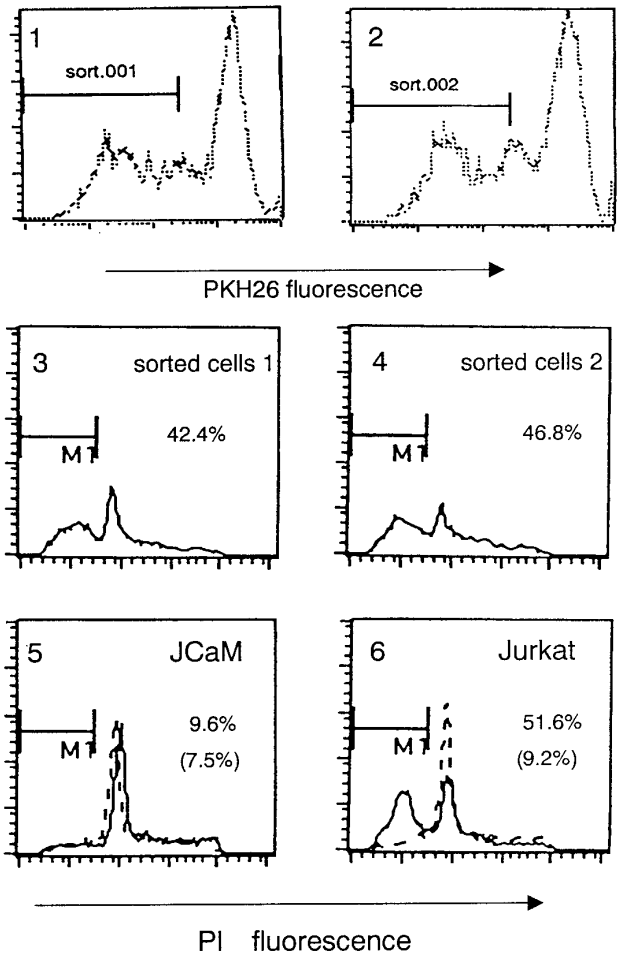

FIG. 3. JCaM underwent apoptosis upon physical contact with Jurkat in the presence of anti-CD3. Unstained JCaM (4×10^6) were co-cultured with PKH26-labelled Jurkat cells (4×10^6) for 48 h in the presence of anti-CD3. Panels 1 and 2 indicate the PKH2 histogram of duplicated samples and PKH26-negative cells (horizontal bar) that were sorted, respectively. Sorted cells were then stained with PI and were analyzed by flow cytometry (panels 3, 4). Panels 5 and 6 indicate the PI histogram of JCaM and Jurkat cells separately stimulated by anti-CD3, respectively. Results of unstimulated control cells were indicated by dashed lines. Values denote the percentage of sub-Go/G1 nuclei marked by bar (M1). Values in parenthesis are from unstimulated controls. Data represent three independent experiments.

lular conditions in which cells are sensitive to death by anti-Fas antibody but not by soluble FasL (14, 15). Probably membrane-anchored ligand could also more efficiently cross-link target Fas molecules than the soluble form. The effect of a metalloproteinase inhibitor, which led to augmented mFasL retention on effector cells and apoptosis induction in target cells, supports this notion. Such a different biologic activity mediated by soluble vs. membrane-bound form of ligand is also noted in TNF-α (16).

Jurkat is extremely sensitive to anti-Fas-induced apoptosis as compared to non-transformed T cells and has been utilized for the estimation of bioactive FasL as indicator cells (17). This sensitivity is partly ascribed to its constitutive expression of Fas antigen and to lack of FAP-1 (Fas-associated phosphatase) which is known to attenuate the death signal transduction through Fas (18). In contrast, although normal peripheral blood T cells express substantial amounts of Fas and upregulates its expression within 24 h of TCR stimulation, they only became sensitive to anti-Fas-mediated

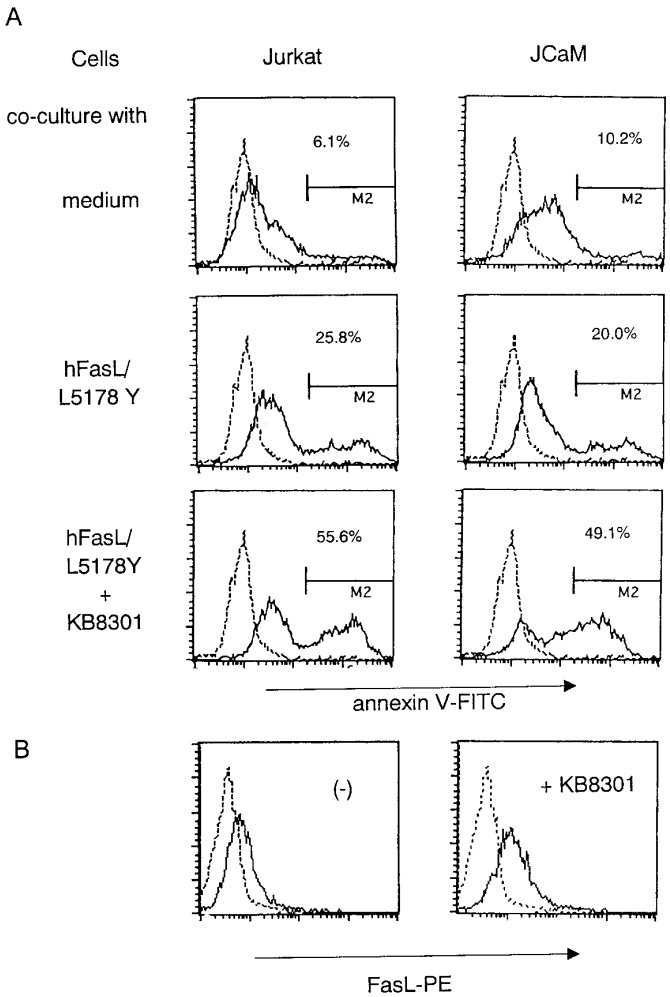

FIG. 4. Both Jurkat and JCaM underwent apoptosis upon physical contact with FasL transfectant and addition of a metalloproteinase inhibitor augmented this process. (A) Jurkat or JCaM cells were cultured alone (top) or with hFasL/L5184Y in the absence (middle) or presence of 10 μM KB8301 (bottom) for 14 h. Cells were harvested and then doubly stained with annexin V-FITC and with anti-FasL-PE. Histograms show the annexin-FITC binding in PE-negative population. Histograms of unstained controls are indicated by dashed line. Values denote the percentage of annexin V-positive cells (marked M2). (B) FasL expression on the surface of hFasL/L5184Y in the absence (left) or presence (right) of 10 μM KB8301. Iso-type control staining is indicated by dashed lines. Data represent three independent experiments.

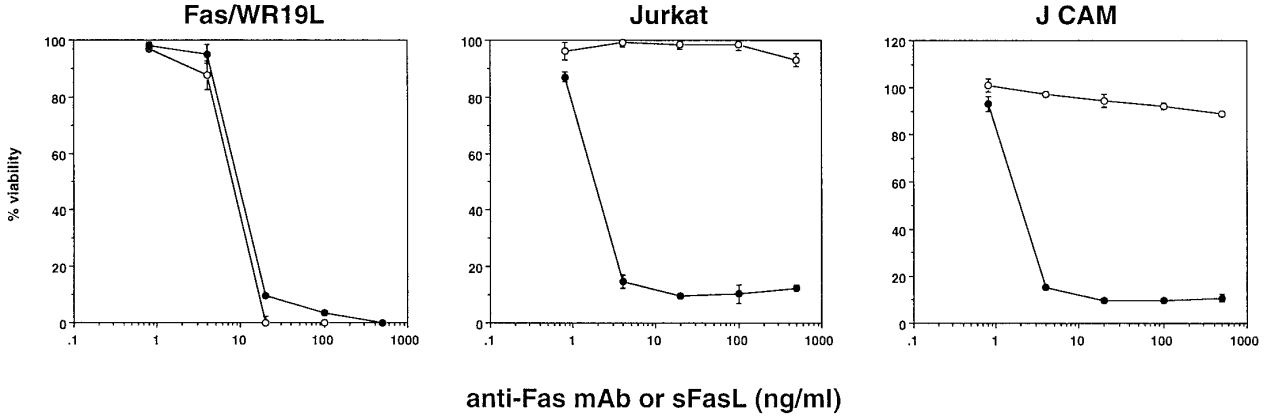

FIG. 5. Jurkat and JCaM cells are susceptible to death by anti-Fas but are resistant to death by recombinant soluble FasL. Cytotoxic activity of recombinant FasL (open circle) and anti-Fas mAb (CH-11; closed circle) was tested against hFas/WR19L, Jurkat, or JCaM cells by alamar blue method. Data represent means ± SD of triplicates.

apoptosis several days later (19, 20). Our present findings thus implicate that, in physiologic conditions, cell-cell contact may be required for AICD. Our data also indicate that there are substantial differences in the biological activity of mFasL, sFasL, and agonistic anti-Fas antibody against different target cells, which may be critical for defining the physiological role of the Fas/FasL system in immune regulation.

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