Purification and Characterization of the Fas-ligand that Induces Apoptosis

By Takashi Suda and Shigekazu Nagata

From the Osaka Bioscience Institute, Furuedai, Suita, Osaka 565, Japan

Summary

Fas is a 45-kD cell surface protein belonging to the tumor necrosis factor/nerve growth factor receptor family, and transduces the signal for apoptosis. The cytotoxic T lymphocyte (CTL) hybridoma, PC60-d10S requires the presence of Fas on target cells to induce cytolysis in target cells. This CTL cell line was weakly but specifically stained by a chimeric protein that consisted of the extracellular domain of mouse Fas and the Fc portion of human immunoglobulin G1 (mFas-Fc). Moreover, mFas-Fc inhibited the cytotoxic activity of PC60-d10S. Sublines of d10S that were stained intensively by mFas-Fc were isolated by repetitive fluorescence-activated cell sorter sorting. A cell-surface protein of about 40 kD was specifically precipitated by mFas-Fc from the lysates of these sublines. This protein was homogeneously purified by sequential affinity chromatographies using mFas-Fc and concanavalin A beads. The purified protein exhibited cytotoxic activity against cells expressing Fas but not to the cells which do not express Fas. These results indicated that the 40-kD membrane glycoprotein expressed on PC60-d10S cells is the Fas-ligand that induces the apoptotic signal by binding to Fas.

Homeostasis in mammals is controlled not only by cell proliferation and differentiation but also by cell death. Once a cell is destined to die, a programmed death process occurs in the cell. Apoptosis is a morphologically defined death process often associated with programmed cell death during the normal development of multicellular organisms (1–3). Apoptotic cell death has also been identified in other types of cell death, which include cytolysis induced by TNF/lymphotoxin (LT)[1] or some cytotoxic T cells (4, 5).

The Fas antigen (Fas) is a 45-kD cell-surface protein belonging to the TNF/nerve growth factor (NGF) receptor family that includes two TNF receptors (TNFRβ, type I or p55; TNFRα, type II or p75), the low-affinity NGF receptor, and the CD40, CD27, CD30, and OX40 (6). Some mAbs recognizing Fas induce apoptotic cell death in cells expressing Fas (7, 8). These facts suggest that Fas is a receptor for a death factor (6).

Both lymphoid and nonlymphoid cells, as well as normal and transformed cell lines, are susceptible to the cytolytic activity of agonistic anti-Fas mAbs (anti-Fas or anti-APO-1 antibodies) (8–12). We have shown that various tissues including the thymus, heart, lung, liver, and ovary express the Fas mRNA (13). These results suggest that Fas is involved in many aspects of apoptotic cell death, including those that occur in normal development, homeostasis, and tumor regression.

In recent reports, we and others have suggested that Fas plays important roles especially in the immune system. We have demonstrated that the mouse lymphoproliferation (*lpr*) mutation that causes abnormal T cell development and SLE-like autoimmune disease, is a mutant of Fas gene (14). This discovery, together with the facts that thymocytes from wild-type mice express Fas and are susceptible to anti-Fas mAb (12), suggests that Fas is involved in T cell development in the thymus. Fas is also expressed on human peripheral mature T cells, which acquire susceptibility to anti-Fas mAbs in culture in vitro (11). These results and a report that mature T cells of *lpr* mice have a defect in antigen-stimulated suicide (15), imply a role for Fas in the peripheral tolerance of mature T cells. Recently, Rouvier et al. (16) demonstrated that the CTL hybridoma PC60-d10S (d10S) and allospecific CTL derived from peritoneal exudate lymphocytes require the expression of Fas on target cells to induce their Ca^{2+}-independent cytotoxicity. These results suggested the expression of the Fas-ligand on the surface of d10S cells, and a role for the Fas-ligand as an important effector molecule involved in T cell–mediated cytolysis. To confirm the presence of the Fas-ligand on d10S cells, and if so confirmed, to isolate and characterize it, we here constructed a soluble fusion protein that consisted of the extracellular region of Fas and the Fc region of human IgG1 (mFas-Fc). Using mFas-Fc, we identified and purified a 40-kD membrane glycoprotein that can specifically induce cytolysis in cells expressing Fas.

[1] *Abbreviations used in this paper:* LT, lymphotoxin; PVDF, polyvinyldifluoride; TBS, Tris-buffered saline.

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Materials and Methods

Cell Lines

A CTL hybridoma cell line, PC60-d10S (16) and its sublines were maintained in DMEM (Nissui, Tokyo, Japan) supplemented with 10% FCS, 50 nM 2-ME, and 100 U/ml benzylpenicillin potassium (Meiji Seika Co., Ltd., Tokyo, Japan) plus 100 μg/ml streptomycin sulfate (P+S; Meiji Seika Co.). WR19L and its mouse Fas cDNA transfectant (W4) (12) were maintained in RPMI 1640 medium (Nissui) supplemented with 10% FCS and P+S. BTS-1 (17), a transfectant of BSC-40 carrying a temperature-sensitive SV40 large T antigen gene, was maintained in DMEM (high glucose type; Nikken Biomedical Lab., Tokyo, Japan) supplemented with 10% FCS, an extra 0.6% glutamine, and P+S. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C, except that BTS-1 was maintained at 39.5°C.

Construction of Expression Plasmids for Fusion Proteins

An expression plasmid for mFas-Fc was constructed as follows. A pair of oligonucleotide primers containing a sense sequence in intron 4 (GATTTTCAACCACTCAGTCG) and an antisense sequence in intron 5 of the mouse Fas gene (GCTGGATCCTTTG-TATGAAATTGAGTAAT) were synthesized. The latter oligonucleotide contains a BamHI site at its 5′ end. A 383-bp DNA fragment containing exon 5, and its 5′ and 3′ flanking regions was amplified by PCR from a plasmid harboring the mouse Fas chromosomal gene (Watanabe-Fukunaga, R., and S. Nagata, unpublished results) using the above primers. The PCR product was digested at the PstI site in exon 5 and at the BamHI site in the antisense primer. The 128-bp PstI-BamHI fragment containing the 3′ end of exon 5 and part of intron 5 was used to replace the PstI-BamHI DNA fragment of pMF1 (13) to generate the plasmid pMFX. The plasmid pMH4 carries exons for the human IgG1 H chain constant region (18). The 1.7-kb HaeII DNA fragment of the plasmid pMH4 was subcloned into the XbaI site of pBluescript KS(+) (Stratagene, La Jolla, CA). The 1.4-kb HincII-ApaI DNA fragment containing the exons for the hinge, CH2, and CH3 domains was inserted into the XbaI site of pMFX to produce pFAS-Fc. The 2.3-kb KpnI-NotI DNA fragment of pFAS-Fc was ligated into the mammalian expression vector pEF-BOS (19), and the resulting plasmid was designated pFAS-FcII. To construct an expression plasmid (hTNFRβ-Fc) for a chimeric protein consisting of the extracellular region of human TNFRβ and the Fc region of human IgG1 (nTNFRβ-Fc), the 0.7-kb HincII-HindIII fragment of pFAS-FcII carrying the extracellular region of Fas was replaced with the 650 bp of the KpnI-HindIII fragment of p55TNFr-HG1 that contains the cDNA sequence for the extracellular region of the human TNFRβ, followed by an artificial splice donor sequence (20).

Production of mFas-Fc and hTNFRβ-Fc

COS cells were transfected using the DEAE-dextran method as described (21). The transfected cells were successively incubated for 24 h in medium containing 10% FCS, then for 48 and 72 h in serum-free medium. The serum-free supernatant was combined, centrifuged, and passed through a 0.45-μm filter to remove cell debris. To produce the chimeric molecules in BTS-1 cells (17), stable transformants carrying the expression plasmids were established by transfecting BTS-1 by means of electroporation (22). In brief, 10^7 cells were transfected with 50 μg of the plasmid DNA digested with ApaLI for pFAS-FcII or SacI for pTNFRβ-Fc, and 5 μg of XhoI-digested pSTneoB. After selection in DMEM containing 10% FCS and 300 μg/ml of G-418 for 10 d, individual G-418–resistant colonies were isolated and grown at 39.5°C. To identify the clones that produce the chimeric molecules, aliquots of cells were cultured at 33°C for 3 d, and the chimeric proteins secreted into the medium were assayed by ELISA using purified goat anti–human IgG-Fc antibodies (Cappel, Durham, NC) as capture antibodies, and horseradish peroxidase–conjugated goat anti–human IgG-Fc antibodies (Jackson ImmunoResearch, West Grove, PA) as detection antibodies. One transformant that efficiently produces the chimeric molecule was selected for each mFas-Fc and hTNFRβ-Fc, and expanded at 39.5°C. To generate culture supernatants containing chimeric proteins, the transformants were plated on 10-cm dishes at ~50% confluence and cultured for 1 wk at 33°C. The chimeric molecules were purified on a protein A–Sepharose CL4B column (Pharmacia, Uppsala, Sweden), and their protein concentration was estimated by measuring the OD at 280 nm based on the assumption that OD of 1.4 corresponds to 1 mg/ml.

Flow Cytometric Analysis and Cell Sorting

The mFas-Fc was biotinylated using sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin; Pierce, Rockford, IL) according to the manufacturer's protocol. To prepare FITC-conjugated hTNFRβ-Fc, 1 mg of protein was mixed with 20 μg of FITC in 1 ml of 50 mM sodium carbonate buffer (pH 9.5). After an incubation at room temperature for 4 h, free FITC was removed by Sephadex G-25M column chromatography. For flow cytometry, ~10^6 cells were first incubated on ice for 10 min in 50 μl of staining buffer (PBS containing 2% FCS and 0.02% NaN_3) containing 5 μg/ml rat anti–mouse FcγII receptor antibody (PharMingen, San Diego, CA). 50 μl of biotinylated mFas-Fc (10 μg/ml) was added to the mixture, and incubated on ice for 30 min. After washing with staining buffer, the cells were stained on ice for 30 min with PE-conjugated streptavidin (25-fold dilution; Becton Dickinson & Co., Mountain View, CA) in 100 μl of staining buffer. Cells were then washed with staining buffer and analyzed using a FACScan® (Becton Dickinson).

A subpopulation of d10S cells that stained intensely with mFas-Fc was selected by repetitive FACS® sorting. In brief, 1–3 × 10^7 d10S cells were stained with FITC-conjugated hTNFRβ-Fc and biotinylated mFas-Fc followed by PE-streptavidin as described above (except that the staining buffer did not contain NaN_3), and sorted using a FACStar® (Becton Dickinson). Cells providing the highest levels of PE-fluorescence signal (top 0.3–0.5%), but without significant FITC-hTNFRβ-Fc staining, were collected and expanded in DMEM containing 10% FCS and 50 nM 2-ME.

Cytotoxicity Assay

Cytotoxicity was assayed essentially as described previously (16). WR19L or W4 cells (10^6 cells) were incubated for 2 h at 37°C with 20 μCi of [^{51}Cr] sodium chromate (Amersham, Bucks, UK) in 100 μl of RPMI 1640 containing 10% FCS. After washing with medium, these cells were used as targets. The ^{51}Cr-labeled target cells (10^4 cells/well) were mixed with d10S or its derivatives at various ratios in round-bottomed microtiter plates in a total volume of 200 μl. The plates were centrifuged at 80 *g* for 2 min, and incubated for 4 h at 37°C. The plates were then centrifuged at 250 *g* for 5 min, and 100 μl aliquots of the supernatants were assayed for radioactivity using a γ-counter. The spontaneous release of

^{51}Cr was determined by incubating the target cells with medium alone, whereas the maximum release was determined by adding Triton X-100 to a final concentration of 0.1%. The percent specific lysis was calculated as follows: [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)].

Biotinylation of Cell Surface Proteins

Cell surface proteins were biotinylated as described previously (23). Briefly, cells (10^7 cells/ml) were suspended in 10 mM sodium borate (pH 8.5), and 150 mM NaCl containing 50 μg/ml of NHS-LC-biotin. After an incubation at room temperature for 15 min, the reaction was stopped by adding a final concentration of 10 mM NH_4Cl, then washed three times with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 8.0, 150 mM NaCl).

Preparation of mFas-Fc Affinity Column

4 mg of the purified mFas-Fc in 4 ml PBS (pH 7.4) was mixed with 2 ml of protein A–Sepharose CL4B beads and allowed to bind for 1 h at 4°C. The beads were washed three times with TBS, then once with 200 mM sodium borate (pH 9.0) to remove free proteins. The bound mFas-Fc was covalently conjugated to the beads by an incubation in 200 mM sodium borate (pH 9.0) containing dimethylpimerimidate (DMP) for 45 min at room temperature.

Purification of Fas-Ligand

Large Scale Culture. The Fas-ligand was purified from the sorted sublines of PC60-d10S (sorted 8–12 times). The cells were cultured in 10 roller bottles (Corning, Inc., Corning, NY) containing 500 ml/bottle of 10% FCS-DMEM supplemented with 50 nM 2-ME and 20 mM Hepes (pH 7.4), and placed on a roller (ROLLACELL; New Brunswick Scientific, NJ) in a conventional atmosphere at 37°C. When the cell concentration reached ~2×10^5 cells/ml, 10 ng/ml of PMA and 500 ng/ml of ionomycin were added to the culture and incubated for another 4 h. The cells were then harvested by centrifugation at 250 *g* for 20 min, and washed three times with PBS and once with TBS. The cells were pelleted and stored at −80°C until they were used to prepare the membrane fraction. One batch of cells (~10^9 cells) was surface biotinylated as described above.

Solubilization of the Membrane Fraction. The frozen cell pellet (7.5 ml, ~10^9 cells) was disrupted in four volumes (30 ml) of 0.3 M sucrose containing 1 mM (*p*-aminophenyl) methanesulfonyl fluoride hydrochloride (APMSF), 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.02% NaN_3 using an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany) at the blue position for 2 min on ice, and centrifuged at 1,000 *g* for 20 min at 4°C to remove nuclei and unbroken cells. The supernatant was centrifuged at 100,000 *g* for 90 min at 4°C to obtain the membrane-enriched fraction. The pellet was dissolved in 40 ml of lysis buffer (TBS containing 1% NP-40, 1 mM APMSF, 1 μg/ml pepstatin, and 1 μg/ml leupeptin), and rocked overnight at 4°C. The solubilized membrane fraction was obtained by centrifugation at 100,000 *g* for 60 min at 4°C, and stored at −80°C. Surface biotinylated cells were processed similarly and stored at −80°C as 10-ml aliquots.

Affinity Purification Using mFas-Fc Column and Con A Beads. As a tracer for the Fas-ligand, 10 ml of the solubilized membrane proteins from the biotinylated cells were added to 100 ml of the pooled solubilized crude membrane fraction. The mixture was applied to a 1.4 ml mFas-Fc affinity column equilibrated with TBS containing 1% NP-40, and the flow-through fraction was applied once more to the same column. The column was washed with 50 ml of TBS containing 1% NP-40, then with 50 ml of TBS containing 0.1% NP-40. The Fas-ligand bound to the column was eluted by 50 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl and 0.1% NP-40. Fractions of 1 ml were collected, and 10 μl of each was resolved by SDS-PAGE, then transferred to a polyvinyldifluoride (PVDF) membrane. Biotinylated proteins were stained with horseradish peroxidase–conjugated streptavidin and detected using the ECL (enhanced chemiluminescence) system (Amersham) according to the manufacturer's protocol. Fractions containing the 40-kD biotinylated Fas-ligand were pooled and incubated with 10 μl of Con A–agarose beads (E·Y Laboratories, San Mateo, CA) overnight at 4°C. After washing four times with TBS containing 0.1% NP-40, the Fas-ligand was eluted from the beads in 200 μl of PBS containing 0.1% NP-40 and 2 M α-methylmannoside.

Results and Discussion

Production and Purification of mFas-Fc and hTNFRβ-Fc. Recently, several groups have successfully used chimeric molecules consisting of the extracellular region of the receptor and the Fc portion of human IgG1 to identify the ligand for orphan receptors (24, 25). To identify the Fas-ligand, a similar chimeric molecule was constructed by fusing the extracellular region of mouse Fas to the Fc region of human IgG1. As a control, a soluble form of human TNF receptor (hTNFRβ-Fc) was constructed by a similar method. The mFas-Fc and hTNFRβ-Fc chimeric molecules were transiently produced in COS cells, or in a stable BTS-1 transformant. These chimeric molecules were purified by protein A affinity chromatography. When the purified mFas-Fc and hTNFRβ-Fc were analyzed by SDS-PAGE, major bands at 55 and 60 kD were observed, respectively, under reducing conditions, and 110- and 140-kD bands were apparent under nonreducing conditions (Fig. 1). These results indicated that both mFas-Fc and hTNFRβ-Fc existed mainly as dimers.

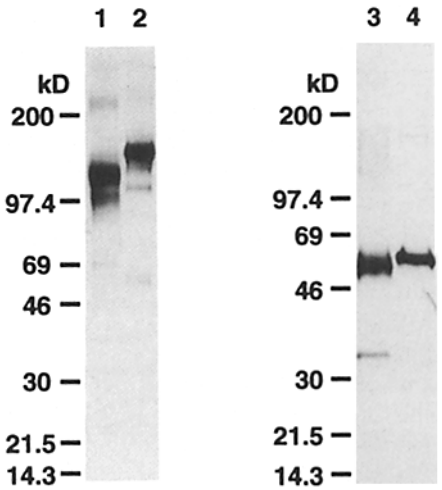

Figure 1. Analysis of the purified mFas-Fc and hTNFRβ-Fc by SDS-PAGE. 2 μg of the purified mFas-Fc (lanes *1* and *3*) or hTNFRβ-Fc (lanes *2* and *4*) were separated on a gradient PAGE (4–20%) in the presence of 0.1% SDS under nonreducing (lanes *1* and *2*) or reducing conditions (lanes *3* and *4*), and stained by Coomassie brilliant blue. Molecular weight markers (*kD*) (Rainbow markers, Amersham) are shown on the left.

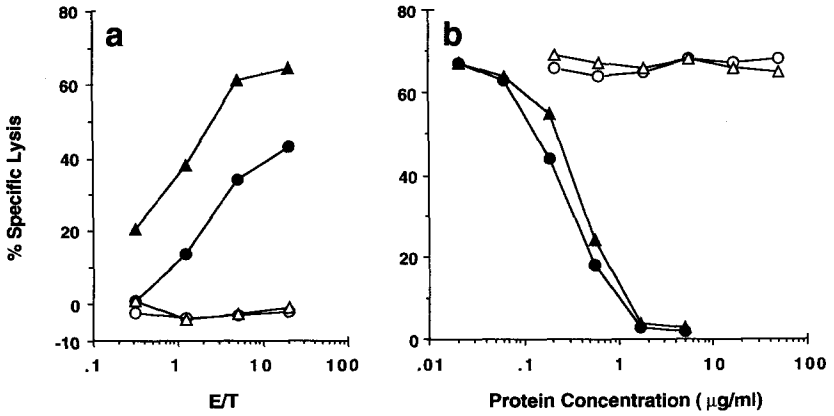

Figure 2. The Fas-dependent cytotoxic activity of d10S cells and its inhibition by mFas-Fc. (*a*) The cytotoxic activity of d10S cells against W4 (●, ▲) or WR19L cells (○, △) was assayed as described in Materials and Methods in the presence (▲, △) or absence (●, ○) of PMA (10 ng/ml) and ionomycin (3 μg/ml). (*b*) Indicated doses of mFas-Fc (●), biotinylated mFas-Fc (▲), hTNFRβ-Fc (△), human IgG (Zymed Laboratories, S. San Francisco, CA) (○) were added to the assay mixture containing 2×10^5 d10S cells and 10^4 W4 cells (E/T = 20).

Expression of the Fas-ligand on the Cell Surface of d10S. Previously, Rouvier et al. (16) reported that the cytotoxic T cell line d10S requires expression of Fas on target cells for their cytolysis. These results were confirmed using W4, a cell line transfected with mouse Fas cDNA and its parental cell line, WR19L. As shown in Fig. 2 *a*, d10S specifically induced cytolysis in W4 but not in WR19L cells. This cytotoxic activity of d10S cells was inhibited by mFas-Fc in a dose-dependent manner, whereas neither the purified human IgG nor hTNFRβ-Fc affected the cytotoxicity of d10S cells (Fig. 2 *b*). A simple explanation of these results is that mFas-Fc bound to the Fas-ligand expressed on d10S cells, and inhibited the interaction between the Fas on W4 cells and the Fas-ligand.

To examine whether d10S cells actually express the Fas-ligand, d10S cells were analyzed by flow cytometry using the biotinylated mFas-Fc. The mFas-Fc was biotinylated as described in Materials and Methods, and the biotinylated mFas-Fc retained activity similar to the unbiotinylated mFas-Fc in the cytotoxicity inhibition assay (Fig. 2 *b*). As shown in Fig. 3 *a*, d10S cells were weakly but significantly stained with the biotinylated mFas-Fc followed by PE-conjugated streptavidin. The binding of the biotinylated mFas-Fc to d10S cells was specific because it was blocked by unlabeled mFas-Fc but not by hTNFRβ-Fc (data not shown). As reported previously (16), the cytotoxic activity of d10S cells was enhanced by stimulation with PMA and ionomycin (Fig. 2 *a*). Correspondingly, flow cytometry indicated that the expression levels of the Fas-ligand on d10S cells were enhanced by culturing the cells for 3 h in the presence of PMA and ionomycin (Fig. 3 *a*).

Establishment of d10S Sublines Expressing High Levels of the Fas-ligand. To characterize the Fas-ligand biochemically, we first established sublines of d10S expressing the higher levels of the Fas-ligand. The d10S cells were stained with the biotinylated mFas-Fc followed by PE-conjugated streptavidin, and sorted using a FACStar®. To exclude the possibility of enriching the Fcγ receptor–positive population, cells were also stained with FITC-conjugated hTNFRβ-Fc. A cell population (~0.3–0.5% of the total), which stained intensely with mFas-Fc but not with hTNFRβ-Fc, was sorted. These cells were expanded in culture and then sorted again. This cycle of sorting and expansion was repeated 16 times. The staining intensity of sorted cells by mFas-Fc was gradually increased, and after the 16th sorting, clones expressing the highest levels of Fas-ligand were isolated by limiting dilution. One cell line, d10S16-2, exhibited about a 120-fold–higher mean staining intensity compared with the original d10S cells (Fig. 3). Correspondingly, d10S16-2 cells were about 100 times more efficient to induce 50% specific lysis of W4 cells compared with the original d10S cells based on the E/T ratio (Fig. 4 *a*). Stimulation of d10S16-2 cells with PMA and ionomycin resulted in about a twofold increase of mean staining intensity with mFas-Fc (Fig. 3 *b*), but little enhancement of the cytotoxic activity of d10S16-2 was observed by this treatment (Fig. 4 *a*).

Neither Rouvier et al. (16) nor we could detect any cytotoxic activity in the culture supernatant of the original d10S cells (Fig. 4 *b*). However, when the medium conditioned with d10S16-2 cells for 24 h was assayed for the cytotoxic activity, it showed a significant activity against W4 cells but not against WR19L cells (Fig. 4 *b*). Addition of PMA and ionomycin during the last 4 h of the culture resulted in an approximately threefold increase of the cytotoxic activity in the culture supernatant. These observations indicate that the Fas-ligand can

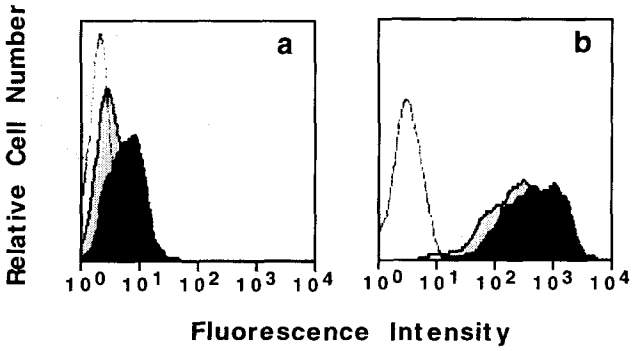

Figure 3. Flow cytometric analysis for Fas-ligand expression on original d10S and d10S16-2 cells. The d10S (*a*) and d10S16-2 cells (*b*) were stained with biotinylated mFas-Fc followed by PE-streptavidin before (*gray area*) or after (*dark area*) stimulation with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 4 h, and analyzed by flow cytometry. The profiles of cells stained with steptavidin-PE alone are also shown (*unfilled area*).

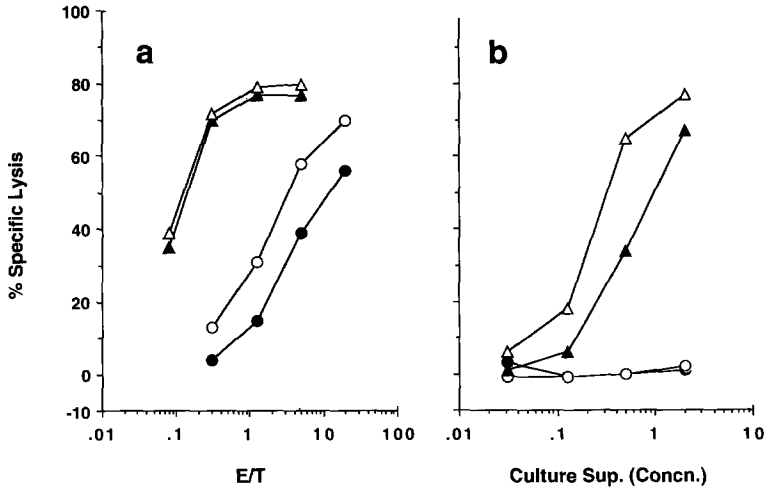

Figure 4. Cytotoxic activity of original d10S and d10S16-2. The d10S (●, ○) or d10S16-2 cells (▲, △) (2.5×10^5 cells/ml) were cultured for a total of 24 h in 10% FCS-DMEM. Cells were cultured in the presence (○, △) or absence (●, ▲) of PMA (10 ng/ml) and ionomycin (500 ng/ml) during the last 4 h of the culture period. The culture supernatant was concentrated eightfold, and passed through a nitrocellulose filter. The cytotoxic activity of the cells (*a*) or the culture supernatants (*b*) was assayed as described in Materials and Methods. Onefold concentration (*b*) corresponds to the neat culture supernatant.

be released into extracellular fluid as an active death factor probably by shedding, when expressed at high levels in the cells.

Purification of the Fas-ligand. The Fas-ligand expressed in the sorted subline of d10S cells was then characterized by immunoprecipitation using mFas-Fc. In Fig. 5, the d10S-12 cells (sorted four times) were surface biotinylated, and immunoprecipitated as described in Materials and Methods. An analysis of the immunoprecipitates by SDS-PAGE showed a broad band(s) at ~40 kD under both reducing and nonreducing conditions (Fig. 5, lanes *2* and *4*). Such bands were not observed when the immunoprecipitation was carried out with hTNFRβ-Fc (Fig. 5, lane *1*) or human IgG (data not shown).

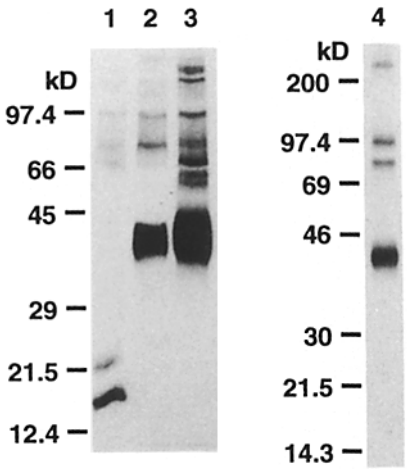

Figure 5. Immunoprecipitation of the Fas-ligand. About 10^7 d10S4 cells (sorted four cycles) were surface biotinylated, then lysed with 1 ml of lysis buffer (see Materials and Methods) on ice for 30 min. After centrifugation at 14,000 rpm for 15 min, the supernatant was precleared by incubation with 50 μl of protein A–Sepharose CL4B beads for 60 min at 4°C. After centrifugation, the supernatant was divided into four portions, and incubated with either 10 μl of hTNFRβ-Fc (lane *1*) or mFas-Fc (lanes *2* and *4*) for 30 min at 4°C followed by incubation with 10 μl of protein A–Sepharose CL4B beads overnight at 4°C, or directly incubated with mFas-Fc–conjugated protein A–Sepharose beads (lane *3*). The beads were extensively washed with 1% NP40-TBS, then boiled in 20 μl of sample buffer for SDS-PAGE. The samples were analyzed on gradient (10–20%) SDS-PAGE under reducing (lanes *1–3*) or nonreducing conditions (lane *4*). The proteins were transferred to PVDF membranes (Millipore, Bedford, MA), probed with horseradish peroxidase–conjugated streptavidin, and visualized by the ECL system. Biotinylated molecular weight markers (Pierce) and Rainbow markers were used in the left and right panels, respectively, and are shown (*kD*).

To prepare a mFas-Fc affinity column, mFas-Fc was covalently conjugated to protein A–Sepharose beads using DMP. Immunoprecipitation with this conjugate confirmed that it can efficiently bind to the Fas-ligand (Fig. 5, lane *3*). As a starting material for purification of the Fas-ligand, total 5×10^9 cells of d10S sublines (sorted 8–12 times) were obtained from 25 liters of culture. One tenth of the cells was surface biotinylated to monitor the purification steps. The plasma membrane–enriched fraction was prepared by homogenization in a hypotonic buffer and differential centrifugation, and was solubilized with 1% NP40. The Fas-ligand was purified from the lysates by sequential affinity chromatography using mFas-Fc column and Con A–agarose beads as described in Materials and Methods. When the purified proteins eluted from the Con A beads were analyzed by SDS-PAGE under reducing conditions, they showed a single major band at the position of ~40 kD, both by silver staining for the total proteins and by the ECL system for the biotinylated proteins (Fig. 6 *a*). The faint band at the 30-kD position detected only by silver staining is probably Con A that leaked from the beads. Since the silver-stained band shown in Fig. 6 *a* amounted to ~10 ng, the total recovery of the ~40-kD purified glycoprotein (gp 40) from 5×10^9 cells was about 0.4 μg or 10 pmol.

To confirm that the purified gp40 is the Fas-ligand, the cytotoxic activity of the protein was assayed using W4 and WR19L cells. As shown in Fig. 6 *b*, the purified gp40 induced cytolysis in W4 cells in a dose-dependent manner, whereas no cytotoxicity was observed against WR19L. When one unit of the Fas-ligand was defined as the amount of the protein required to induce a half-maximal response (40% cell lysis in Fig. 6 *b*) with W4 cells, the specific activity of the purified Fas-ligand was calculated at ~6.5×10^6 U/mg. This value nearly corresponds to the value reported for the purified TNFα (26).

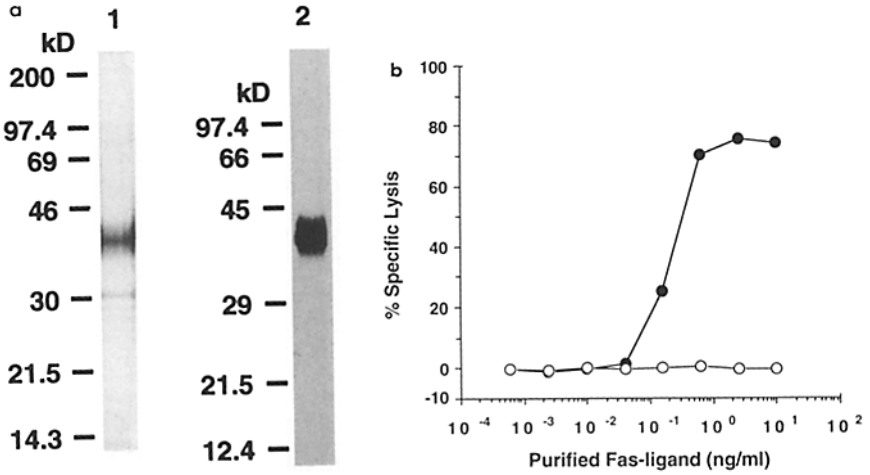

Figure 6. Characterization of the purified Fas-ligand. (*a*) Analysis of the purified Fas-ligand on SDS-PAGE. The purified Fas-ligand was analyzed on a 10–20% gradient PAGE in the presence of 0.1% SDS. Proteins were either stained using a silver staining kit (Wako, Tokyo, Japan) (lane *1*), or visualized using the ECL system after blotting onto a PVDF membrane (lane *2*). Molecular weight markers are Rainbow markers (*left*) and biotinylated molecular weight markers (*right*) and are shown in kD. (*b*) The cytotoxic activity of the purified Fas-ligand. ^{51}Cr-labeled W4 (●) or WR19L cells (○) (10^4 cells/well) were cultured with various doses of the purified Fas-ligand in a round-bottomed 96-well plate for 4 h.

In this report, we have shown that the CTL cell line d10S expresses the Fas-ligand. We developed a simple procedure to purify the Fas-ligand. The purified Fas-ligand (gp40) had a cytolytic activity against the cells expressing Fas. The inhibition of the cytotoxic activity of d10S by the soluble Fas, and the ability of the purified Fas-ligand to induce the cytolysis indicate that interaction of the Fas-ligand with the Fas on the target cells is the only condition necessary for target cell lysis, and that no other molecule expressed on d10S cells is essentially required. The fact that the cytotoxic activity increased in parallel to the increase of the Fas-ligand expression in the sorted d10S cells supports this conclusion.

Various molecules such as perforin, lymphotoxin, and TNF are known to be involved in the CTL-mediated cytolysis (27). Unlike the Fas system, the cytotoxicity of perforin is Ca^{2+} dependent. Although TNF and LT show the Ca^{2+}-independent cytotoxicity, the relative molecular mass of the membrane-bound TNF (26 kD) (28) and the subunit structure of the LT (33 and 25 kD) (29) are different from those of the Fas-ligand. These results rule out the possibility that the Fas-ligand is perforin, TNF, or LT.

The Fas-dependent cytotoxicity has been observed not only in the CTL cell line d10S but also in Ca^{2+}-independent, alloantigen-specific CTL (16). Thus, it is likely that Fas/Fas-ligand system is widely used in common for CTL. We previously demonstrated that administration of agonistic anti-Fas antibodies into mice killed the mice within several hours by inducing severe destruction of hepatocytes as seen in fulminant hepatitis (12). The class I MHC–restricted CTL are suggested to be involved in the hepatitis caused by hepatitis B virus (HBV) (30). Taken together, it is possible that the interaction of CTL specific for the HBV antigen with the HBV-infected hepatocytes induces the expression of the Fas-ligand on their surface, and that the Fas-ligand on the CTL then binds to Fas on hepatocytes, and induces apoptosis in hepatocytes. If so, a soluble form of human Fas or antagonistic antibodies against Fas or Fas-ligand may be used to treat patients during the acute phase of fulminant hepatitis. Further experiments will aim at testing these possibilities.

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Address correspondence to S. Nagata, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita-Shi, Osaka 565, Japan.

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Note Added in Proof: We have recently isolated a Fas-ligand cDNA from the sorted subline of the d10S cells (31).

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