Purification and Characterization of the Fas-ligand that Induces Apoptosis

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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 (TNFR, 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 susceptiblity 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 Ca2+-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 cytosis. 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.

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1 Abbreviations used in this paper: LT, lymphotoxin; PVDF, polyvinylidene fluoride; TBS, Tris-buffered saline.
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 mM 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; Nippon 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% CO₂ 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 (GATTITTCACCCACTCAGTCG) and an antisense sequence in intron 5 of the mouse Fas gene (GCTGGATCCTTTGAGGTTA) 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 5' end of exon 5 and part of intron 5 was used to replace the PstI-BamHI DNA fragment of pFLX. The plasmid pMH4 carries exons for the hinge, CH2, and CH3 domains of human IgG1 constant region (18). The 1.7-kb HaelI DNA fragment of the plasmid pMH4 was subcloned into the XbaI site of pBluescript KS(+) (Stratagene, La Jolla, CA). The 1.4-kb HindIII-Apal DNA fragment containing the exons for the hinge, CH2, and CH3 domains was inserted into the XbaI site of pFLX 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-FclI. 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 (hTNFRβ-Fc), the 0.7-kb HindIII-HindIII fragment of pFAS-FcII carrying the extracellular region of Fas was replaced with the 650 bp of the KpnI-HindIII fragment of pSSTNFR-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 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 5' end of exon 5 and part of intron 5 was used to replace the PstI-BamHI DNA fragment of pFLX. The plasmid pMH4 carries exons for the hinge, CH2, and CH3 domains of human IgG1 constant region (18). The 1.7-kb HaelI DNA fragment of the plasmid pMH4 was subcloned into the XbaI site of pBluescript KS(+) (Stratagene, La Jolla, CA). The 1.4-kb HindIII-Apal DNA fragment containing the exons for the hinge, CH2, and CH3 domains was inserted into the XbaI site of pFLX 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-FclI. 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 (hTNFRβ-Fc), the 0.7-kb HindIII-HindIII fragment of pFAS-FcII carrying the extracellular region of Fas was replaced with the 650 bp of the KpnI-HindIII fragment of pSSTNFR-HG1 that contains the cDNA sequence for the extracellular region of the human TNFRβ, followed by an artificial splice donor sequence (20).

Flow Cytometric Analysis and Cell Sorting

The mFas-Fc was biotinylated using sulfo-NHS-LC-biotin (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, ~10⁶ cells were first incubated on ice for 10 min in 50 μl of staining buffer (PBS containing 2% FCS and 0.02% NaN₃) containing 5 μg/ml rat anti-mouse FcγRI 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 FACSscan® (Becton Dickinson).

A subpopulation of d10S cells that stained intensely with mFas-Fc was selected by repetitive FACS® sorting. In brief, 1–3× 10⁷ 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 NaN₃), 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 (10⁶ cells) were incubated for 2 h at 37°C with 20 μCi of [³¹Cr] 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 [³¹Cr]-labeled target cells (10⁶ 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

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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.
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 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
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).

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 x 10⁹ 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 x 10⁹ 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 cytosis 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 x 10⁶ U/mg. This value nearly corresponds to the value reported for the purified TNFα (26).
In this report, we have shown that the CTL cell line dl0S 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 dl0S by the soluble Fas, and the ability of the purified Fas-ligand to induce the cytosis 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 dl0S cells is essentially required. The fact that the cytotoxic activity increased in parallel to the increase of the Fas-ligand expression in the sorted dl0S 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 dl0S 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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Note Added in Proof: We have recently isolated a Fas-ligand cDNA from the sorted subline of the dl0S cells (31).

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