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Expression of the functional soluble form of human Fas ligand in activated lymphocytes

Masato Tanaka, Takashi Suda, Tomohiro Takahashi and Shigekazu Nagata

Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565, Japan

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Fas is a type I membrane protein which mediates apoptosis. Fas ligand (FasL) is a 40 kDa type II membrane protein expressed in cytotoxic T cells upon activation that belongs to the tumor necrosis factor (TNF) family. Here, we found abundant cytotoxic activity against Fas-expressing cells in the supernatant of COS cells transfected with human FasL cDNA but not with murine FasL cDNA. Using a specific polyclonal antibody against a peptide in the extracellular region of human FasL, a protein of 26 kDa was detected in the supernatant of the COS cells. The signal sequence of granulocyte colony-stimulating factor was attached to the extracellular region of human FasL. COS cells transfected with the cDNA coding for the chimeric protein efficiently secreted the active soluble form of human FasL (sFasL). Chemical crosslinking and gel filtration analysis suggested that human sFasL exists as a trimer. Human peripheral T cells activated with phorbol myristic acetate and ionomycin also produced functional sFasL, suggesting that human sFasL works as a pathological agent in systemic tissue injury.

Key words: apoptosis/cytokine/cytotoxicity/Fas/Fas ligand

Introduction

Fas, also called APO-1, is a type I membrane protein of 45 kDa which is expressed in various tissues and cells including the thymus, liver, ovary and lung (Itoh *et al.*, 1991; Oehm *et al.*, 1992; Watanabe-Fukunaga *et al.*, 1992b). Fas is a member of the tumor necrosis factor (TNF)/nerve growth factor receptor family (Beutler and van Huffel, 1994; Nagata, 1994; Smith *et al.*, 1994), and mediates apoptosis when crosslinked with agonistic anti-Fas or anti-APO-1 antibody (Trauth *et al.*, 1989; Yonehara *et al.*, 1989), or Fas ligand (FasL; Suda *et al.*, 1993). FasL is a type II membrane protein of M_r 40 kDa and is a member of the TNF family which includes TNFα, α- and β-chains of lymphotoxin (LT), CD40 ligand and CD30 ligand (Suda *et al.*, 1993). The amino acid sequences of human and murine FasL are 76.9% identical, and they are not species-specific (Takahashi *et al.*, 1994a,b). The expression of FasL mRNA is most prominent in cells of the T cell lineage, except for those of the testis (Suda *et al.*, 1993). The activation of mature T cells with phorbol myristic acetate (PMA) and ionomycin, concanavalin A (Con A) or anti-CD3, induces FasL gene expression (Vignaux *et al.*, 1995; Suda *et al.*, 1995).

Molecular and genetic analyses of the Fas and FasL genes indicated that murine spontaneous, autosomal and recessive *lpr* and *gld* mutations are mutations of Fas and FasL, respectively (Watanabe-Fukunaga *et al.*, 1992a; Nagata and Suda, 1995; Takahashi *et al.*, 1994a). Since *lpr* and *gld* mice develop lymphadenopathy and splenomegaly, and suffer from autoimmune disease by producing auto-antibodies including anti-DNA and rheumatoid factors (Cohen and Eisenberg, 1991), it is clear that the Fas system is involved in the apoptotic process of T cell development. Recent studies of *lpr* and *gld* mice suggested that the Fas system plays an important role in the clonal deletion of autoreactive T cells in the periphery or activation-induced suicide of T cells (Russell and Wang, 1993; Russell *et al.*, 1993; Singer and Abbas, 1994; Watanabe *et al.*, 1995). In addition to the development of T cells, FasL works as an effector molecule of cytotoxic T cells (Kägi *et al.*, 1994; Kojima *et al.*, 1994; Lowin *et al.*, 1994). Accordingly, various T cell clones express FasL upon activation, and then kill the target cells in a Fas-dependent manner (Rouvier *et al.*, 1993; Hanabuchi *et al.*, 1994; Stalder *et al.*, 1994; Vignaux *et al.*, 1995; Suda *et al.*, 1995).

TNFα, a prototype of the TNF family, is produced as a membrane-bound form (Kriegler *et al.*, 1988; Kinkhabwala *et al.*, 1990; Perez *et al.*, 1990) and is processed to an active soluble form by proteolytic shedding. Both forms of TNFα mediate a range of inflammatory and cellular immune responses, including tumor regression, septic shock and cachexia (Beutler and Cerami, 1989; Fiers, 1991). Biochemical and biophysical analyses of TNFα revealed that the soluble form of TNFα is a homotrimer (Smith and Baglioni, 1987; Eck and Sprang, 1989; Jones *et al.*, 1989), and that it binds to the receptor at a ratio of 3:3 (Banner *et al.*, 1993).

Here we have identified the active soluble form of human FasL (sFasL) in the supernatant of COS cells transfected with the full-length FasL cDNA, as well as in the supernatant of activated human peripheral T cells. Functional sFasL was also produced in COS cells using a cDNA, in which the signal sequence was attached to the N-terminus of the extracellular domain of FasL. Chemical crosslinking, followed by Western blotting using anti-FasL antibody, as well as gel filtration indicated that sFasL has a trimeric structure.

Results

The soluble form of human FasL in COS cells transfected with FasL cDNA

Monkey COS cells were transfected with pEF-BOS vector alone or vector carrying the full-length cDNA for murine FasL or human FasL. After 72 h, the supernatants were assayed for cytotoxic activity against murine WR19L or

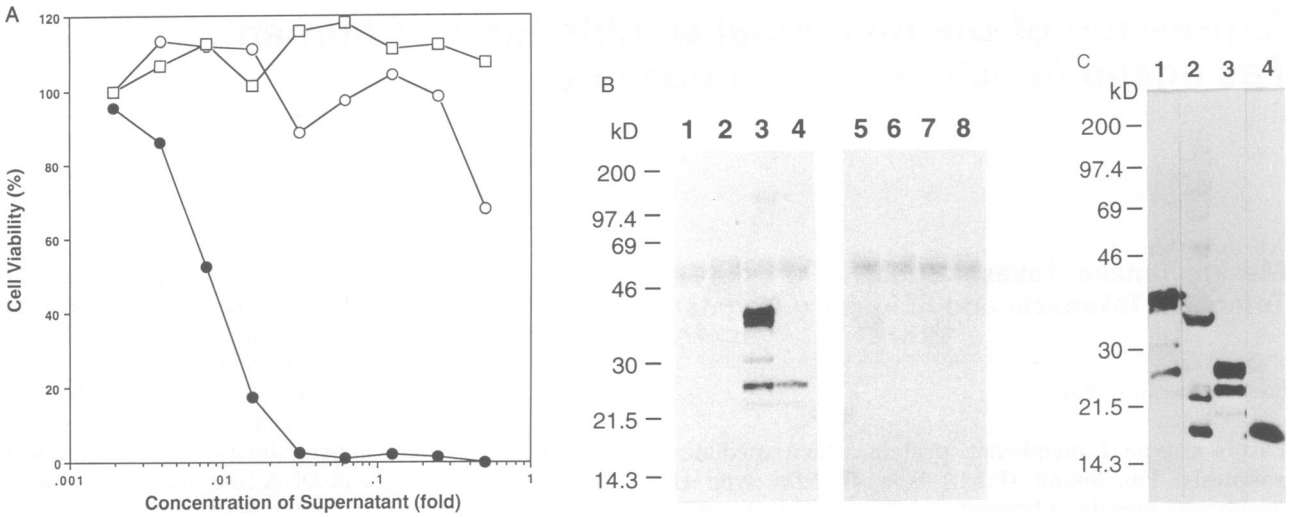

Fig. 1. The production of active human sFasL in COS cells transfected with human FasL cDNA. (**A**) Cytotoxic activity of the COS cell supernatants. COS cells were transfected with pEF-BOS vector (□), pEF-BOS carrying the full-length human FasL cDNA (●) or murine FasL cDNA (○). At 72 h after transfection, the cytotoxic activity of the supernatants was determined by means of the MTT assay using W4 cells, as described in Materials and methods. The cell viability is expressed as the percentage of that observed without the COS cell supernatant. (**B**) Production of human FasL protein in COS cells. COS cells were transfected with pEF-BOS (lanes 1, 2, 5 and 6) or pEF-BOS carrying the full-length human FasL cDNA (lanes 3, 4, 7 and 8). At 72 h after transfection, the cell lysates (lanes 1, 3, 5 and 7) or supernatants (lanes 2, 4, 6 and 8) were immunoprecipitated with mFas-Fc. The immunoprecipitates were heated at 95°C for 2 min in Laemmli's sample buffer containing 0.1 M β-mercaptoethanol, and resolved by electrophoresis on a 10–20% gradient polyacrylamide gel. Human FasL proteins were then detected by Western blotting using anti-human FasL antibody in the absence (lanes 1–4) or presence (lanes 5–8) of 30 μg/ml of the peptide CPSPPPEKKELRKVAH. As size markers, molecular weight standards (Rainbow™, Coloured Protein Marker, Pharmacia) were electrophoresed in parallel and the sizes of the standard proteins are shown in kDa. (**C**) Effects of endoglycosidase on human FasL. COS cells were transfected with pEF-BOS carrying the full-length human FasL cDNA. At 72 h after transfection, the cell lysates (lanes 1 and 2) or supernatants (lanes 3 and 4) were immunoprecipitated with mFas-Fc. The immunoprecipitates were incubated for 5 min at 90°C in PBS containing 0.5% SDS and 0.1 M β-mercaptoethanol. After centrifugation, the supernatants were diluted 3.5-fold with 50 mM phosphate buffer (pH 6.0) and incubated for 16 h at 37°C with (lanes 2 and 4) or without (lanes 1 and 3) 4 mU endoglycosidase H (Genzyme). The samples were then incubated for 5 min at 90°C in Laemmli's sample buffer containing 0.1 M β-mercaptoethanol and analyzed by Western blotting using the anti-human FasL antibody as described above.

its transformant W4, expressing murine Fas. As reported for rat FasL (Suda *et al.*, 1993), the supernatant of COS cells transfected with murine FasL expression plasmid contained a little cytotoxic activity against the Fas-expressing cells (<20 U/ml; Figure 1A). On the other hand, transfection with the human FasL expression plasmid gave rise to a large amount of FasL activity in the supernatant (~1200 U/ml). The cytotoxic activity was specific to the Fas-expressing cells, since none was observed against WR19L cells which do not express Fas.

To confirm that the cytotoxic substance in the supernatant of the COS cells transfected with human FasL cDNA is sFasL, and to analyze its molecular properties, a polyclonal antibody against human FasL was prepared by immunizing rabbits with a peptide of human FasL. The peptide carries the sequence from 134 to 148 of human FasL, which corresponds to the N-terminal region of the soluble TNFα (Suda *et al.*, 1993; Takahashi *et al.*, 1994b). The peptide of human TNFα corresponding to this region has high antigenicity (Socher *et al.*, 1987). The antibody was affinity-purified from the serum of the immunized rabbits using peptide-conjugated resin. The antibody specifically recognized human but not murine FasL (data not shown).

The supernatant and the cell lysates of the transfected COS cells were immunoprecipitated with a chimeric protein consisting of the extracellular region of murine Fas and the Fc region of human immunoglobulin (mFas-Fc; Suda and Nagata, 1994). The immunoprecipitates were resolved by electrophoresis on a polyacrylamide gel, and analyzed by Western blotting using the anti-human FasL antibody. As shown in Figure 1B, both the cell lysates and the supernatant of the COS cells transfected with the human FasL cDNA gave intensive bands recognized by anti-human FasL antibody. All bands recognized by the antibody are specific because these bands disappeared on the inclusion of 30 μg/ml of the human FasL peptide in the solution for Western blotting. The protein of M_r 40 kDa in the lysates seems to be human FasL expressed on the membrane. The lower bands have M_rs identical to those found in the supernatant, indicating that they are probably sFasL. Such a soluble form in the cells was reported previously in the TNF system (Kriegler *et al.*, 1988). The supernatant of the COS cells contained two bands of M_r 26 and 23 kDa which are significantly larger than the calculated M_r for the extracellular region of human FasL. To examine whether human FasL is glycosylated, the immunoprecipitates were treated with endoglycosidase H and analyzed by Western blotting. As shown in Figure 1C, the sizes of human FasL in the lysates were reduced to 33, 22 and 17 kDa. Whereas, a single protein of 17 kDa was detected in the immunoprecipitates of the supernatant, the molecular weights of 33 and 17 kDa agree reasonably with the calculated M_r for the membrane-bound (M_r 31 486) and soluble forms (M_r 17 695) of human FasL. The 22 kDa protein may be sFasL containing the residual sugar moiety. These results indicate that the *N*-glycosylated active sFasL can be produced in COS cells transfected with human FasL cDNA.

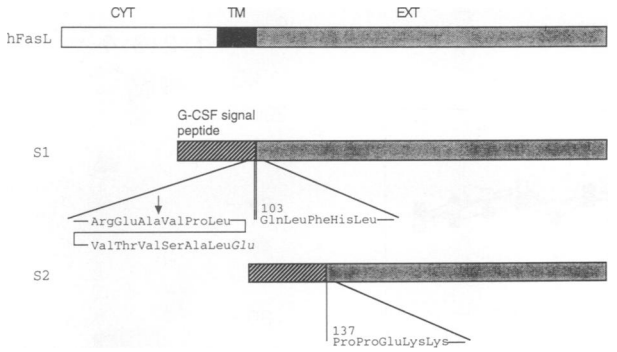

Fig. 2. Schematic diagrams of the human FasL construct carrying the G-CSF signal sequence. The structures of human FasL and its chimeric proteins containing the murine G-CSF signal sequence are shown schematically. CYT, TM and EXT represent the cytoplasmic, transmembrane and extracellular regions of human FasL, respectively. In the S1 construct, the G-CSF signal sequence replaces the cytoplasmic and transmembrane regions of human FasL, whereas the signal sequence was attached to Pro137 of human FasL in the S2 construct. The amino acid sequence at the junction between the murine G-CSF signal sequence and human FasL is shown. The arrow indicates the cleavage site of the murine G-CSF precursor protein.

Production of human sFasL using a signal sequence

The attachment of the signal sequence to the N-terminus of the extracellular region of human TNFα exclusively produces the soluble form of TNFα (Perez *et al.*, 1990). To confirm that human sFasL is functional, two expression plasmids for sFasL were constructed using the signal sequence of murine granulocyte colony-stimulating factor (G-CSF; Tsuchiya *et al.*, 1986). In the first construct (S1 construct), the signal sequence of G-CSF was attached immediately downstream of the transmembrane region of human FasL (Figure 2). In the second construct (S2 construct), the signal sequence was joined to the 34 amino acids downstream of the transmembrane region, the position corresponding to where soluble TNF begins (Pennica *et al.*, 1984). Both constructs, as well as the expression plasmid for the full-length human FasL cDNA, were transfected into COS cells, and the supernatants were harvested 72 h later. As shown in Figure 3A and B, the supernatants of COS cells transfected with S1, S2 and the full-length human FasL cDNA contained ~800, 3800 and 1200 U/ml of cytotoxic activity against W4 cells but not against WR19L cells. The supernatants were then analyzed directly by Western blotting using anti-human FasL antibody (Figure 3C). The COS cells transfected with the S1 construct produced a protein of M_r 33 kDa recognized by the antibody, whereas the supernatants of COS cells transfected with either the S2 construct or full-length cDNA showed bands of similar M_r. The difference in the M_r between the S1 and S2 constructs can be explained by the 34 extra amino acids at the N-terminus of the S1 construct and glycosylation of the putative *N*-glycosylation site in this extra sequence. The proteins produced by the S1 and S2 constructs are supposed to carry nine amino acids from mature G-CSF and one from the linker sequence, in addition to the sequence of human FasL. Considering these points, the similar M_r of the soluble proteins produced by the full-length cDNA and the S2 construct suggested that the membrane-bound FasL coded by the full-length cDNA was cleaved at around amino acid 127. This position is slightly upstream of where the corresponding membrane-bound TNFα is cleaved (Perez *et al.*, 1990).

The soluble form of TNF functions as a trimer (Smith and Baglioni, 1987). To examine whether sFasL also exists as a trimer, the supernatant of COS cells transfected with the full-length FasL cDNA was concentrated and treated with the chemical crosslinker, disuccinimidyl suberate (DSS). After mixing with 0.5% SDS, the samples were boiled for 5 min under reducing conditions, resolved by electrophoresis at room temperature and analyzed by Western blotting using the anti-FasL antibody. As shown in Figure 4, the treatment with the crosslinker produced an intensive band at M_r 50 kDa and faint bands at M_r ~80 kDa, which may correspond to a dimer and trimer of human FasL. It is known that the oligomeric structures of some proteins are conserved if electrophoresis is carried out under milder conditions (Fukunaga *et al.*, 1990). Therefore, the concentrated COS cell supernatants were mixed at 4°C with 0.5% SDS under non-reducing conditions and resolved by electrophoresis at 4°C. Western blotting using the anti-FasL antibody indicated that sFasL, even without crosslinking, has M_rs of 26, 50 and 82 kDa under these conditions (Figure 4). The intensity of the two upper bands was slightly increased by the chemical crosslinker, and no forms larger than the trimer were detected, suggesting that human sFasL is a trimer.

To confirm that human sFasL is a trimer, the supernatant of COS cells transfected with the full-length FasL cDNA was concentrated and analyzed by gel filtration. As shown in Figure 5A, the FasL activity was eluted at the position corresponding to a M_r of ~70 kDa. When each fraction was analyzed by Western blotting using the anti-FasL antibody, the FasL protein was found in fractions which showed the FasL activity, and no FasL protein was detected in the fractions where a monomer of sFasL (M_r 26 kDa) should be eluted (Figure 5B). These results indicated that most human sFasL exists as a trimer, and this trimer has cytotoxic activity.

Production of the soluble form of Fas ligand by activated lymphocytes

The activation of mature T cells induces the expression of the FasL gene, and the cells express the functional FasL on their surface (Suda and Nagata, 1994; Vignaux *et al.*, 1995; Suda *et al.*, 1995). To examine whether human lymphocytes produce sFasL, Con A blasts were prepared from human peripheral blood lymphocytes (PBL) and expanded in the presence of interleukin (IL)-2. The cells were then activated with PMA and ionomycin for 24 h. The supernatant was concentrated and its cytotoxic activity was examined, with WR19L and W4 cells as target cells. As shown in Figure 6A, the supernatant of the activated T cells showed cytotoxic activity (30 U/ml) against the Fas-expressing W4 cells, but not WR19L cells. This activity was inhibited by soluble murine Fas (mFas-Fc) in a dose-dependent manner. On the other hand, the soluble form of the TNF receptor (TNFRβ-Fc) did not affect the cytotoxic activity of the supernatant (Figure 6B). To confirm that the activated human lymphocytes produce sFasL, the supernatant of the activated PBL was immunoprecipitated with mFas-Fc. Western blotting of the immunoprecipitates revealed a small amount of sFasL

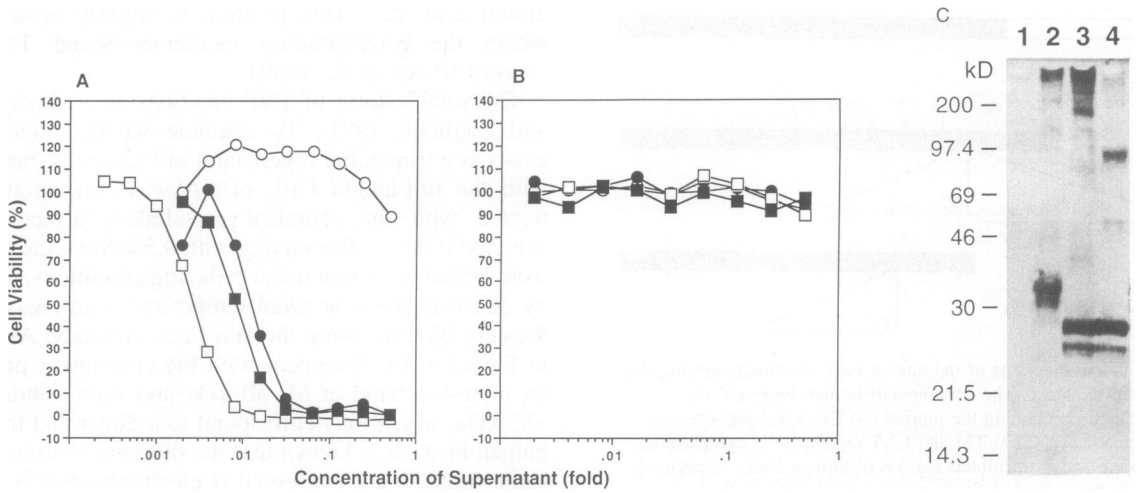

Fig. 3. Production of human sFasL using the G-CSF signal sequence. (**A** and **B**) Cytotoxic activity of the supernatants of COS cells transfected with the human FasL expression plasmids. COS cells were transfected with pEF-BOS-SIG (○), S1 construct (●), S2 construct (□) or pEF-BOS carrying the full-length human FasL cDNA (■). At 72 h after transfection, the cytotoxic activity of supernatants was assayed using W4 (A) or WR19L (B) as the target cells. (**C**) Western blotting. 16 μl of the supernatant of COS cells transfected with pEF-BOS-SIG (lane 1), S1 construct (lane 2), S2 construct (lane 3) or pEF-BOS carrying the full-length human FasL cDNA (lane 4) were heated in Laemmli's sample buffer without β-mercaptoethanol at 90°C for 2 min, and resolved by electrophoresis on a 10–20% gradient polyacrylamide gel. After blotting to a PVDF membrane, human sFasL was detected using anti-human FasL antibody as described in Materials and methods. The sizes of molecular weight standards are shown in kDa on the left.

in the medium conditioned for 24 h with Con A blasts of human PBL (Figure 6C). Activation of PBL with PMA and ionomycin greatly increased the amount of sFasL in the medium. The M_r of the sFasL produced by the activated PBL was similar to that found in the supernatant of COS cells transfected with the full-length FasL cDNA. These results indicated that human peripheral T cells can produce functional sFasL upon activation.

Discussion

TNFα is expressed initially in activated T cells and macrophages as a membrane-bound form which is then proteolytically processed into a soluble cytokine that retains its biological activity (Kriegler *et al.*, 1988; Kinkhabwala *et al.*, 1990). In this report we described that human FasL, a member of the TNF family, also becomes a soluble form. Human sFasL produced in COS cells by transfection with the full-length human FasL cDNA, as well as that produced in activated human PBL, was active; it specifically induced cytolysis of Fas-expressing cells. Various groups have reported that metalloproteases are responsible for the processing of the membrane-bound TNFα (Gearing *et al.*, 1994; McGeehan *et al.*, 1994; Mohler *et al.*, 1994). Although the region of human FasL around amino acid 127, where the proteolytic cleavage should occur, does not show significant similarity to the cleavage site of TNFα (Pennica *et al.*, 1984; Perez *et al.*, 1990), a similar protease(s) may be involved in the processing of membrane-type FasL. In contrast to the supernatant of COS cells transfected with human FasL cDNA, very little Fas-dependent cytotoxic activity was detected in the supernatant of COS cells transfected with murine FasL cDNA. This finding agrees with the findings that activation of rat×mouse T cell hybridoma PC60-d10S with PMA and ionomycin produced little sFasL (Rouvier *et al.*, 1993; Suda and Nagata, 1994). We raised a specific polyclonal antibody against murine FasL

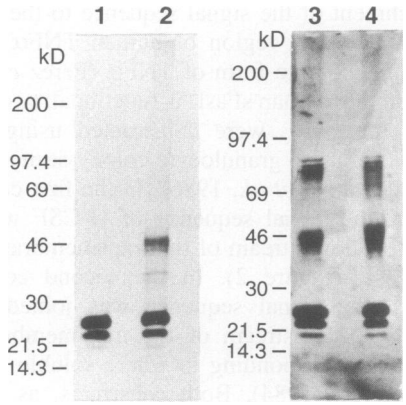

Fig. 4. Chemical crosslinking of human sFasL. COS cells were transfected with pEF-BOS vector carrying the full-length human FasL cDNA. At 72 h after transfection, the supernatant was concentrated ~40-fold and an aliquot was left on ice for 10 min without (lanes 1 and 3) or with 0.15 mM DSS (lanes 2 and 4). In lanes 1 and 2, samples were heated at 90°C for 5 min in Laemmli's sample buffer containing 0.1 M β-mercaptoethanol. Samples were resolved by electrophoresis at room temperature on a 4–20% gradient polyacrylamide gel. In lanes 3 and 4 samples were treated at 4°C for 10 min in Laemmli's sample buffer without β-mercaptoethanol and resolved by electrophoresis at 4°C on a 4–20% polyacrylamide gel. After electrophoresis, proteins were analyzed by Western blotting using the anti-FasL antibody as described in Materials and methods.

(M.Tanaka, T.Takahashi and S.Nagata, unpublished results). Western blotting of the supernatant of COS cells transfected with murine FasL cDNA revealed a similar amount of murine sFasL which has a M_r similar to that of human sFasL found in the COS cell supernatant. These results indicated that murine FasL is also proteolytically processed to the soluble form, but is non-functional or very unstable. In this regard, it is worth noting that an inactive form of murine TNFα is produced by inappropriate processing in the murine macrophage cell line RAW264.7 (Cseh and Beutler, 1989). Normally, mature

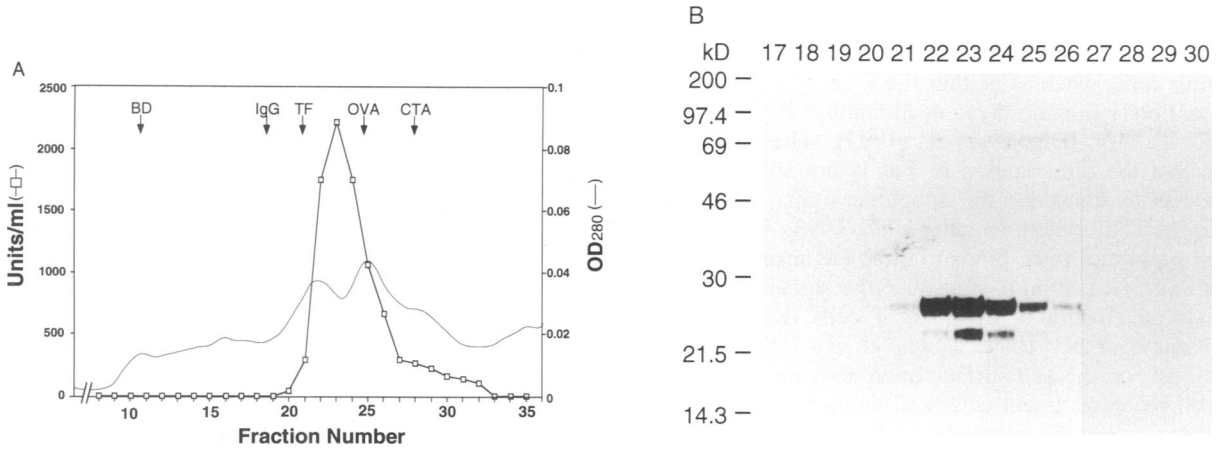

Fig. 5. Gel filtration of human sFasL. COS cells were transfected with pEF-BOS carrying the full-length of human FasL cDNA. At 72 h after transfection, the supernatant was concentrated 20-fold and 80 µl aliquots were loaded onto a Superdex^{TM} 200 HR10/30 column (Pharmacia) attached to the FPLC system (Pharmacia). The column was developed with PBS containing 0.1 mg/ml BSA at a flow rate of 0.5 ml/min; 0.5 ml fractions were collected. (**A**) FasL activity. The cytotoxic activity (□) in the fractions was determined by MTT assay as described in Materials and methods. The profile of the OD at 280 nm is shown by a thin line. The positions of molecular weight marker proteins are indicated by arrows. BD, blue dextran 2000; IgG, human immunoglobulin (160 kDa); TF, transferrin (81 kDa); OVA, ovalbumin (43 kDa); CTA, chymotrypsinogen A (25 kDa). (**B**) The FasL protein. 16 µl aliquots of each fraction were analyzed by Western blotting with the anti-FasL antibody, as described in Materials and methods.

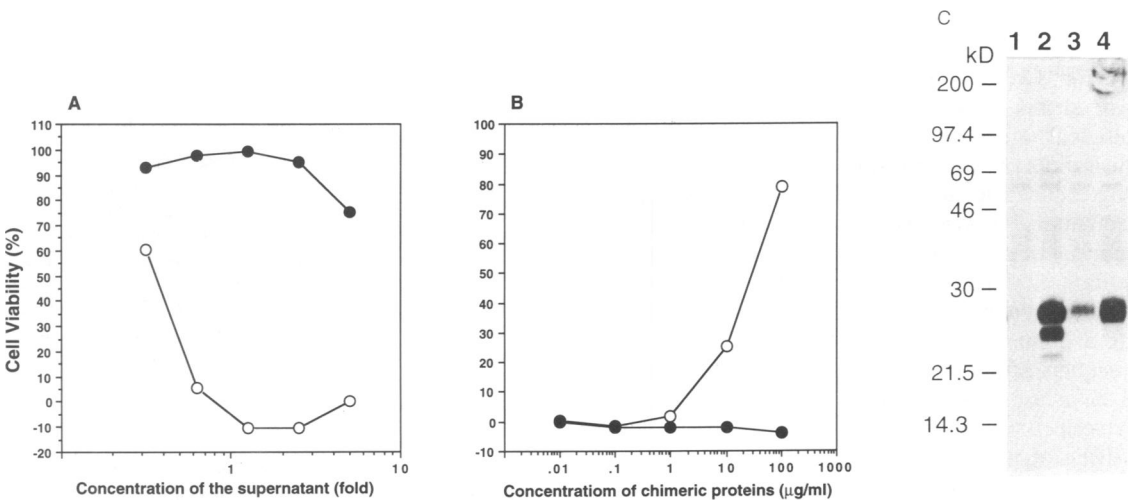

Fig. 6. Production of active human sFasL from activated human PBLs. (**A**) Cytotoxic activity in the supernatant of activated human PBL. Con A blasts of human PBL were incubated at 37°C for 24 h with 10 ng/ml PMA and 500 ng/ml ionomycin. After centrifugation, the cytotoxic activity of the supernatant was determined by the MTT assay using W4 (○) or WR19L cells (●) as the target cells. (**B**) Effect of mFas-Fc on the cytotoxic activity of the supernatant of activated human PBL. The cytotoxic activity of the 1.25-fold concentrated supernatant of activated human PBL was determined by the MTT assay using W4 cells in the presence of indicated concentrations of mFas-Fc (○) or hTNFRβ-Fc (●). (**C**) Detection of sFasL in the supernatant of activated human PBL. Con A blasts of human PBL were incubated with (lane 4) or without (lane 3) PMA and ionomycin, as described above. 1 ml of the supernatant was immunoprecipitated with mFas-Fc and the immunoprecipitate was analyzed by Western blotting with anti-human FasL antibody, as described in Materials and methods. As controls, COS cells were transfected with pEF-BOS (lane 1) or pEF-BOS carrying human FasL cDNA (lane 2). 40 µl aliquots of the supernatant of the transfected COS cells were analyzed by immunoprecipitation and Western blotting as described above. The sizes of the molecular weight standard proteins are indicated as kDa on the left.

T cells express FasL upon activation (Suda *et al.*, 1995). Whether or not murine mature T cells produce non-functional sFasL remains to be studied.

Active TNFα has a homotrimeric structure (Smith and Baglioni, 1987). The X-ray diffraction analysis of human TNFα and TNFβ (LTα) indicates that these cytokines are compact trimers with a 'bell-shaped appearance' (Eck and Sprang, 1989; Jones *et al.*, 1989; Eck *et al.*, 1992). Since the primary structure of the extracellular region of FasL is highly homologous to that of TNFα and TNFβ, especially in the regions where the TNFs are known to form a β-strand, we postulated that FasL forms a homotrimer (Suda *et al.*, 1993). This assumption was confirmed here by gel filtration and chemical crosslinking analyses of human sFasL. Since LT exists as a heterotrimer consisting of one LTα and two LTβ molecules on the cell surface (Androlewicz *et al.*, 1992), the membrane-bound form of human FasL may also be a trimer. Several monoclonal antibodies against Fas have agonistic activities. The original anti-Fas (CH-11; Yonehara *et al.*, 1989) and 2D1 antibodies (Takahashi *et al.*, 1993) are of the immunoglobulin M type, while the anti-APO-1 antibody is an

immunoglobulin G_3 (Trauth *et al.*, 1989) which tends to aggregate. These antibodies induce apoptosis in Fas-expressing cells, whereas neither the $F(ab')_2$ fragment of APO-1 antibody nor the divalent immunoglobulin G has cytolytic activity (Dhein *et al.*, 1992). These results indicate that the dimerization of Fas is not sufficient to activate Fas to transduce the apoptotic signal. Thus, as found in the TNF system (Banner *et al.*, 1993), it is likely that FasL, a homotrimer, binds to three Fas receptors and induces its trimerization to transduce the signal.

FasL is an effector of cytotoxic T cells (Kägi *et al.*, 1994; Kojima *et al.*, 1994; Lowin *et al.*, 1994) which express FasL on the cell surface upon activation through the T cell receptor. Local cell–cell contact is critical to T cell antigen-specific immunosurveillance, and FasL on the cell surface plays a role, at least in part, in killing the target cells such as those infected with virus or those that are cancerous. Fas is constitutively expressed in various tissues, including the thymus, liver, lung and heart (Watanabe-Fukunaga *et al.*, 1992b; Leithauser *et al.*, 1993). The high expression level of Fas receptor in hepatocytes transformed with hepatitis C virus (Hiramatsu *et al.*, 1994), T cells transformed with human immunodeficiency virus or human T cell leukemia virus (Kobayashi *et al.*, 1990; Debatin *et al.*, 1993, 1994), or various cancer cells (Leithauser *et al.*, 1993) substantiates the above notion. On the other hand, the finding of active human sFasL in this report suggests that sFasL functions as a pathological agent to cause systemic tissue injury. The administration of the anti-mouse Fas antibody into mice quickly induced hepatic failure and killed the mice within several hours (Ogasawara *et al.*, 1993). It is possible that the abnormal or excessive activation of T cells causes the production of sFasL, which may have deleterious effects in humans. We produced a large quantity of human sFasL using a human FasL expression plasmid carrying the signal sequence of G-CSF. The recombinant human sFasL will be a useful tool with which to examine its pathological effects in an animal model system. In any event, it will be of interest to examine whether sFasL is present in the circulation of patients suffering from various diseases such as fulminant hepatitis or acquired immune deficiency syndrome.

Materials and methods

Plasmid construction

The human and murine FasL expression vectors (pEX-hFL1 and pEFMFLW4F) carrying the full-length FasL cDNA have been described previously (Takahashi *et al.*, 1994b). The secreted form of human FasL was produced using the signal sequence of murine G-CSF. The 151 bp DNA fragment (nucleotides 34–184) of murine G-CSF cDNA coding for the signal sequence of G-CSF (amino acids −30 to +9) was amplified by PCR from the pMG2 plasmid carrying murine G-CSF cDNA (Tsuchiya *et al.*, 1986). The fragment was inserted into the *Xba*I site of the mammalian expression plasmid pEF-BOS (Mizushima and Nagata, 1990) to produce pEF-BOS-SIG. Two DNA fragments carrying the extracellular region of human FasL (S1, amino acids 103–281; and S2, amino acids 137–281) were amplified by PCR using primers containing a *Xba*I site. The resultant DNA fragments (610 bp for S1, 508 bp for S2) were inserted into pEF-BOS-SIG in the correct orientation.

Transfection of COS cells and culture of human peripheral blood lymphocytes

Monkey COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). COS cells (2×10^6 cells) on 10 cm dishes were transfected with 5 μg of plasmid DNA using DEAE–dextran and cultured in medium containing 10% FCS. At 24 h after transfection, the medium was changed to DMEM containing 1% FCS and incubated for 72 h at 37°C. To analyze FasL in the cells, the transfected cells were lysed by incubation on ice for 30 min in TBS [50 mM Tris–HCl (pH 8.0) and 150 mM NaCl] containing 1% NP-40, 1 mM (*p*-amino-phenyl)methanesulfonyl fluoride hydrochloride, 1 μg/ml pepstatin and 1 μg/ml leupeptin. After centrifugation at 15 000 r.p.m. for 20 min, the supernatant was collected for immunoprecipitation.

Human peripheral blood was obtained from a healthy adult volunteer (T.Suda) and centrifuged on Nycodeny (Nyco Prep™ 1.077) to enrich the lymphocytes. The PBLs were activated with 5 μg/ml Con A and 20 ng/ml human IL-2 (provided by Ajinomoto Co., Tokyo, Japan) for 48 h in AIM-V medium (Gibco), and then grown for 14 days in AIM-V medium containing 20 ng/ml human IL-2. The cells were then stimulated with 10 ng/ml PMA and 500 ng/ml ionomycin for 24 h in AIM-V medium.

Preparation of anti-human FasL antibody

The peptide (CPSPPPEKKELRKVAH) carrying the sequence in the extracellular region of human FasL (amino acids 134–148) was custom-synthesized and conjugated to bovine serum albumin (BSA) with *m*-maleimidbenzol-*N*-hydroxysuccinimide ester (MBS; Pierce) in Multiple Peptide Systems (San Diego, CA). Rabbits (New Zealand White, 8 weeks old) were immunized with 0.5 mg of the peptide–BSA conjugate in complete Freund's adjuvant. Immunization with the conjugate in incomplete Freund's adjuvant was repeated every 4 weeks for 12 weeks. At 4 weeks after the last subcutaneous injection, 20 μg of the conjugates in PBS were injected intravenously into the rabbits; serum was collected after 3 days. The serum was affinity-purified with AF amino-Toyopearl beads (Tosoh Co.) to which the peptide was attached using MBS.

MTT assay

Murine T cell lymphoma WR19L and its transformant W4 expressing murine Fas (Ogasawara *et al.*, 1993) were maintained in RPMI 1640 medium containing 10% FCS. Samples to be assayed were passed through a 0.45 μm filter, serially diluted 1:2 with the medium and mixed with 7.5×10^4 WR19L or W4 cells in a total volume of 100 μl. After an incubation for 15 h at 37°C, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)[^{2}H]tetrazolium monosodium salt (WST-1; Dojin Laboratories) and 1-methoxy-5-methylphenazinium methylsulfate were added to final concentrations of 5.0 and 0.2 mM respectively, and incubated further for 1 h. After centrifugation, the absorbance of the supernatant was measured at 450 nm using an automated Micro-ELISA reader. One unit of the activity represents the concentration of FasL that gave a half-maximal cytotoxicity against 7.5×10^4 W4 cells in 100 μl.

Immunoprecipitation and Western blotting

Forty microlitre of the COS cell supernatant, 1 ml of the supernatant of PBL or 200 μl of the COS cell lysates from 3×10^5 cells were passed through a 0.45 μm filter and incubated with 10 μg mFas-Fc for 30 min at 4°C. 10 μl protein A–Sepharose CL 4B beads (Pharmacia) were added to the mixture and incubated overnight at 4°C. The beads were washed extensively with TBS containing 0.1% NP-40 and suspended in 20 μl Laemmli's sample buffer.

Samples were electrophoresed on a 10–20% polyacrylamide gel and the proteins were transferred to PVDF membranes (Millipore) at 30 V for 15 h at 4°C. After blocking with Block Ace (Dainihon Seiyaku), the membranes were incubated for 1 h at room temperature with 2000-fold diluted anti-human FasL polyclonal antibody in PBS supplemented with 0.5% BSA and 25% Block Ace. After washing three times with PBS containing 0.1% Tween 20 for 30 min, the membrane was incubated for 30 min at room temperature with 1000-fold diluted peroxidase-conjugated goat anti-rabbit IgG, specific to the $F(ab')_2$ fragment (Jackson Immuno-Research Laboratories). The membranes were washed three times for 30 min with PBS containing 0.1% Tween 20, and the proteins recognized by the antibody were visualized by the ECL system (Amersham).

Chemical crosslinking

The supernatant of COS cells transfected with pEX-hFL1 was concentrated ~40-fold using a Centriprep-10 concentrator (Amicon). After filtration using Spin X™ (Costar), an aliquot (10 μl) was diluted with 30 μl PBS and incubated on ice for 10 min with 0.15 mM DSS (Pierce). The reaction was stopped by adding 2 μl of 1 M Tris–HCl (pH 7.5); 20 μl of the reaction mixture was mixed with Laemmli's sample buffer.

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