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Biochemical and Biophysical Research Communications 322 (2004) 197–202
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Increased cytotoxicity of soluble Fas ligand by fusing isoleucine zipper motif☆

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Received 16 July 2004
Available online 5 August 2004

Abstract

Fas (CD95) ligand (FasL) has the ability to induce apoptosis in Fas-expressing glioma cells by binding to Fas. Several molecular species have been designed to be soluble Fas ligands for therapeutic purposes. We successfully constructed a chimeric soluble FasL by fusing an isoleucine zipper motif for self-oligomerization and a FLAG sequence to the extracellular domain of the human Fas ligand (FIZ-shFasL). The cytotoxic effect of FIZ-shFasL on Jurkat cells was equivalent to that of membrane-bound FasL and approximately 10-fold stronger than that of agonistic anti-Fas antibody (CH-11). Flow cytometric analysis demonstrated that the differential Fas expression of human brain tumor cell lines partially correlated with levels of apoptosis through FIZ-shFasL. The upper limit of FIZ-shFasL for safe systemic administration to rat is estimated as below 2 μg/ml in plasma concentration. FIZ-shFasL could be applicable as a therapeutic agent for cancer.

Keywords: Soluble Fas ligand; Isoleucine zipper motif; Glioma; Apoptosis; Fas; Bcl-2

Apoptosis is one of the key mechanisms by which anticancer therapies exert their cytotoxic effects [1]. Fas (also called CD95 or APO-1) is a type I transmembrane cell surface protein belonging to the tumor necrosis factor (TNF)/nerve growth factor receptor superfamily [2–4]. The Fas ligand (FasL), a member of the TNF family, specifically binds to Fas and causes recruitment of adapter molecules that, in turn, activate caspases and other proapoptotic signals that eventually lead to cell death [1,5–7].

Several studies have demonstrated that Fas is expressed on the surface of malignant glioma cells and that the IgM subtype of anti-Fas monoclonal antibody (CH-11) or IgG3 subtype of anti-Fas monoclonal antibody can induce apoptosis in Fas-expressing glioblastoma cell lines, suggesting a possible apoptosis induction role for therapy in malignant glioma cases [8–10].

We have previously constructed recombinant membrane-bound FasL (mFasL) using a baculoviral vector and verified the cytotoxic activity of mFasL against glioblastoma cells that express Fas [11]. However, it is not practical to produce mFasL in a large scale. On the other

☆ This work was supported by Grant #12671367 from the Ministry of Education, Culture, Sports, Science and Technology.
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doi:10.1016/j.bbrc.2004.07.098

hand, the soluble form of FasL (sFasL) that is generated by proteolytic cleavage of the mFasL is easy to manipulate. However, it has less cytotoxic activity than mFasL.

Several reports suggest that the oligomerization of Fas and aggregated forms of FasL is required to trigger DISC (death-inducing signaling complex) formation, which are essential for transducing the apoptotic signal [12–14].

We have attempted to solve these problems with a novel recombinant human sFasL along with an isoleucine zipper motif (FIZ-shFasL) to form an aggregated structure. We have studied its biological activity in vitro as well as any adverse effects noted upon intravenous injection of FIZ-shFasL in rats.

Materials and methods

Cells. Five human glioblastoma cell lines (A172, U-87 MG, U-138 MG, U-373 MG, and T98G), one human medulloblastoma cell line (DAOY), and one rhabdomyosarcoma cell line (TE671) were used in this study. A172 was obtained from the Health Science Research Resources Bank (Osaka, Japan). All except A172 were obtained from the American Type Culture Collection (Rockville, MD). All cells were cultured in Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Nalgene, Canterbury, Australia) and 100 U/ml penicillin–streptomycin (Schering-Plough, Osaka, Japan) in a water-saturated 5% CO_2 atmosphere at 37 °C.

Jurkat T lymphocytic leukemia cells obtained from American Type Culture Collection were kept in continuous logarithmic growth by passaging them at a concentration of 2.5×10^5 cells/ml every other day in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY). The cell cultures were maintained in a humidified incubator at 37 °C under 5% CO_2.

Construction of expression plasmids for human FasL. To construct the expression plasmid for human sFasL (shFasL), the DNA fragment coding for human Fas signal sequence (aa 1–16) was fused to the 5′ terminus of the cDNA fragment coding for the extracellular domain of human FasL (aa 103–281) by PCR. The PCR product was ligated to the expression plasmid pEF-BOS having human EF-1α promoter [15]. To construct the other expression plasmids for the shFasL derivatives, the DNA fragments encoding FLAG [16] and/or isoleucine zipper [17] sequences were inserted between the coding fragments of the Fas signal sequence and the shFasL on the expression plasmid of shFasL. The predicted products of these expression plasmids are F-shFasL, IZ-shFasL, and FIZ-shFasL (Fig. 1).

Production and purification of FasL derivatives. The expression plasmids for various FasL derivatives were used for production in mammalian cells. COS-1 cells were transfected with the expression plasmids using the DEAE–dextran method [18] or using FuGENE6 Transfection Reagent (Roche). Ninety-six hours later, the cultured media of the transfected cells were centrifuged at 450*g* for 10 min and the supernatants were filtered by a 0.45 μm filter (Millipak 60 Filter Unit, Millipore) to remove cell debris.

The supernatant containing FIZ-shFasL was applied to a Sepharose column (NHS-activated Sepharose 4 Fast Flow, Amersham Biosciences) conjugated with anti-human FasL monoclonal antibody. The column was washed with PBS and 1 mol/L NaCl in PBS, and then eluted with 50 mmol/L glycine–NaOH (pH 11) containing 1 mol/L NaCl. The fractions were immediately neutralized by adding 1/4 volume of 1 mol/L Tris–HCl buffer (pH 8). The concentrations of the various FasL derivatives were determined by human FasL specific ELISA as previously described [19].

Cytotoxicity assay. Cytotoxic activity of human Fas ligand for various tumor cell lines was determined by the MTT method, as described elsewhere [20]. Adherent type cells (1×10^4/well) were inoculated in 96-well plates 1 day before the Fas ligand was added and suspension type cells (2×10^4/well) were inoculated on the same day as FasL addition. After incubation for 16 h at 37 °C with Fas ligand, 1/10 volume of Premix WST-1 Cell Proliferation assay system (Takara, Siga, Japan) was added to each well and incubated for 30 min or 2 h. Then, the absorbance was measured at 450–630 nm using an automated ELISA-reader. The absorbance of well for medium alone (no cells in well) was determined as background absorbance, and that of well without FasL was determined as control absorbance. The percent of specific cytotoxic activity of FasL was calculated as follows: [experimental absor-

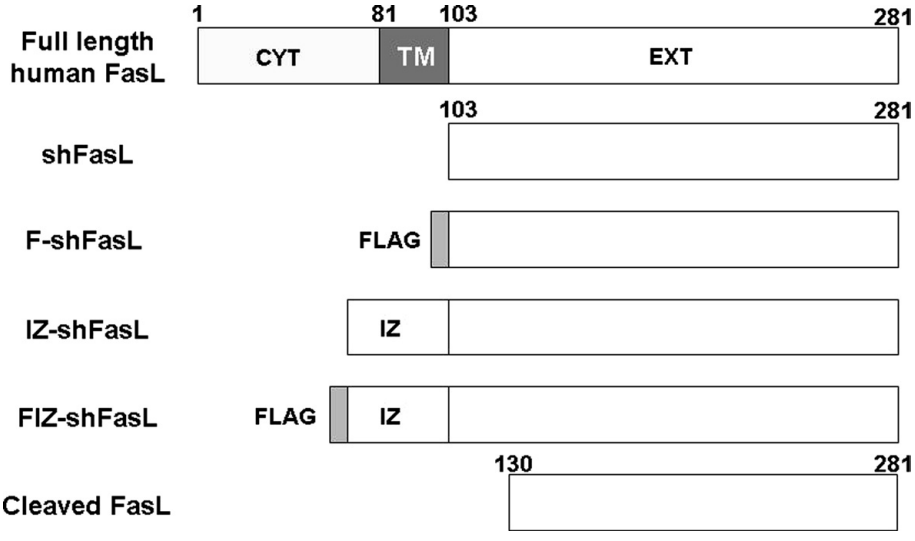

Fig. 1. Schematic structures of the various human FasL molecules The structures of full length human FasL and shFasL derivatives are schematically shown. CYT, TM, EXT, and IZ represent cytoplasmic domain, transmembrane, extracellular domain, and isoleucine zipper, respectively. The shFasL, F-shFasL, IZ-shFasL, and FIZ-shFasL are artificial molecules, as described in the Materials and methods. Cleaved FasL represents the physiologically cleaved form of shFasL. Numbers indicate amino acid positions in the respective proteins.

bance − background absorbance]/[control absorbance − background absorbance] × 100 (%). These cytotoxic activities were completely inhibited by adding the mouse anti-human FasL neutralizing antibody.

All experiments were carried out in duplicate and conducted two times or more.

Flow cytometric analysis of Fas and Bcl-2 expression. To determine Fas expression, cells (1×10^6) were detached using trypsin, washed twice in cold buffer consisting of PBS, 1% BSA, and 0.02% sodium azide, and pelleted. The cells were suspended in 10 μg/ml of either FITC-conjugated anti-mouse monoclonal antibody (mAb) against human Fas (Caltag Lab., Burlingame, CA) or an isotype-matched control antibody in 100 μl PBS, and incubated for 30 min at 4 °C with occasional shaking. The cells were washed twice, resuspended in 0.5 ml PBS containing 0.02% sodium azide, and analyzed by FACScan cytometer (Becton–Dickinson, San Jose, CA). Only viable cells established using forward and side scatter parameters were used for analysis. Background fluorescence intensity was determined using a non-binding primary control mAb and corrected mean fluorescence intensities were calculated by subtracting the mean values of cells stained with the irrelevant primary monoclonal antibody from the mean values of cells stained with specific primary molecular antibody. For detecting Bcl-2 expression, we used FITC-conjugated anti-mouse monoclonal antibody against human Bcl-2 (Caltag Lab., Burlingame, CA) as a primary antibody. After incubating cells with the primary antibody for 15 min and washing twice with PBS, fixation and permeabilization of cells was performed using FIX & PERM cell permeabilization kits (Caltag Lab., Burlingame, CA) following manufacturer's instruction.

Pharmacokinetics and toxicological studies in the administration of Fas ligand fusion protein in vivo. Six- to eight-week-old female Wistar Hanover rats were purchased from Japan CLEA. After acclimatization, the healthy animals were selected and used for experiments. Animals were individually housed in stainless steel cages and maintained on a 12-h lighting cycle at an environmental temperature of 21–25 °C with food and water ad libitum.

Purified FIZ-shFasL fusion protein was dissolved in PBS (Sigma) containing 0.1% human serum albumin as a protein carrier. FIZ-shFasL was injected into the tail vein (1 ml/kg in each dose). Blood samples were collected via jugular vein or abdominal aorta. For hematological examinations, blood samples were mixed with anticoagulant EDTA-2K and analyzed by automated hematology analyzer (F-800, Sysmex, Tokyo, Japan). For blood biochemistry, blood samples were mixed with anticoagulant heparin sodium and then centrifuged by 1000*g* for 10 min at room temperature. The blood biochemical parameters were measured by VetScan automatic analyzer (AVAXIS, CA, USA). FIZ-shFasL in plasma sample was determined by human Fas ligand specific ELISA (21) and analyzed on pharmacokinetic parameter of $C_{2\,\mathrm{min}}$ (intravenous injection) and $T_{1/2}$.

Individual body weight and food consumption were measured. And the day after the FasL administration, animals were euthanized by pentobarbital injection and necropsied. Brain, pituitary, thyroid glands (including parathyroid), submaxillary glands, thymus, lungs, heart, kidneys, spleen, adrenal glands, and testis were excised and weighed.

Results

Cytotoxic activity of various Fas ligand derivatives on Jurkat cells

The cytotoxic activities of shFasL and their fusion proteins were tested on Jurkat cell (Fig. 2). The extracellular domain of FasL (shFasL) exerted a weak effect on Jurkat cells. The FLAG-fused form (F-shFasL) had no effect on Jurkat cell. On the other hand, the fusion protein (IZ-shFasL) between isoleucine zipper motif which can self-oligomerize and shFasL showed about 10 times higher activity than that of shFasL. Surprisingly, additional fusion of FLAG sequence on IZ-shFasL (FIZ-shFasL) increases the potency of killing activity to the Jurkat cell. This activity was stronger than that of the Fas agonistic antibody clone CH-11. FLAG sequence decreased the activity of extracellular domain of shFasL, but for IZ-shFasL this motif has additional effect on inducing apoptosis. These cytotoxic activities of FasL derivatives were neutralized with FasL specific neutralizing antibody (data not shown).

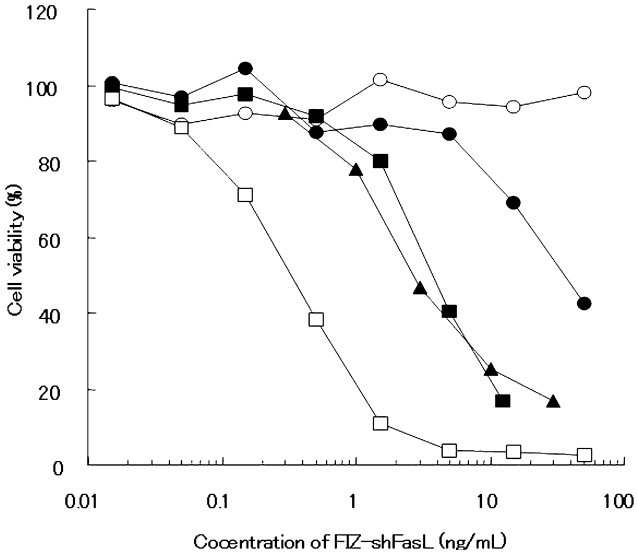

Fig. 2. Cytotoxic activities of human FasL derivatives against Jurkat cells The cytotoxic activities of various human FasL derivatives [shFasL (●), F-shFasL (○), IZ-shFasL (■), and FIZ-shFasL (□)], and Fas agonistic antibody [CH-11 (▲)] against Jurkat cells were assayed by the MTT method, as described in the Materials and methods. After incubation with FasL for 16 h, cell viabilities were determined with WST-1 reagents. The cell viabilities are expressed as percentages of the viability observed without cytotoxic agents.

Apoptosis inducing effect on human brain tumor cell lines

The cytotoxic effect of FIZ-shFasL was assessed on human brain tumor derived cell lines. The response to FIZ-shFasL treatment was quite different among cell lines. The high susceptibility of U-87 MG, U-138 MG, and T98G to FIZ-shFasL was observed. DAOY also died at high concentrations of FIZ-shFasL. On the other hand A172, TE671, and U-373 MG showed no response to treatment of FIZ-shFasL (Fig. 3).

Relationship between Fas and Bcl-2 expression levels and sensitivity to FIZ-shFasL

To address the differential sensitivity to FIZ-shFasL-mediated cell death, cell lines were analyzed for Fas and

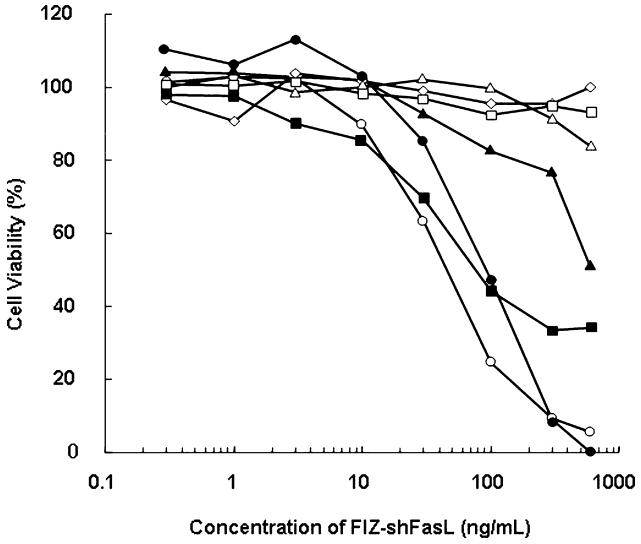

Fig. 3. Anti-tumor effect of FIZ-shFasL on various human brain tumor cell lines U-87 MG (○), U-138 MG (●), U-373 MG (□), T98G (■), A172 (△), DAOY (▲) and TE671 (◇). Each cell was incubated for 16 h at 37 °C with the indicated concentration of FIZ-shFasL. Cell viability was determined using the WST-1 assay as described in Materials and methods, and is expressed as percentages of the viability in the absence of FIZ-shFasL. All assays were performed in triplicate.

Bcl-2 expression using flow cytometry. The corrected mean fluorescence intensities of Fas and Bcl-2 are summarized in Table 1. Percentage of cells that were positive for Fas varied between 1.2% and 73.7% (mean 29.9%). All cells except A172 express high levels of Bcl-2. In U-87 MG, T98G, DAOY, and A172 cells, there was relationship between Fas expression and FIZ-shFasL sensitivity. It should be noted that U-138 MG cells demonstrate high levels of FIZ-shFasL-mediated apoptosis despite expressing lower level of Fas than U-87 MG and T98G cells. Although expressing high level of Fas, U-373 MG showed resistance to FIZ-shFasL-mediated apoptosis. Conversely, U-138 MG showed high sensitivity to FIZ-shFasL while expression of Fas was relatively low.

Toxicology and pharmacokinetic analysis of FIZ-shFasL

Fas agonistic agents (e.g., agonistic antibody) cause severe systemic damage [21]. Those toxicological parameters that were significantly changed following a single intravenous administration of FIZ-shFasL are summarized in Table 2. At dosage level of 1.0 mg/kg FIZ-shFasL injection, increases in GOT and GPT were found. There were no increases in GOT and GPT, but a slightly decreased weight of the thymus was found at the dose of 0.3 mg/kg. At the lower dosage up to 0.2 mg/kg, no significant change was observed for any parameters we studied. The plasma concentration of FIZ-shFasL after its intravenous injection was determined. After injection of 0.1 and 0.3 mg/kg of FIZ-shFasL, plasma $C_{2\,min}$ was 591 and 1849 ng/ml, respectively, and the plasma $T_{1/2}$ β was 10.4 and 15.7 min, respectively. Under the anesthesia condition plasma $C_{2\,min}$ was 304 ng/ml and $T_{1/2}$ β was 19.2 min at 0.1 mg/kg intravenous injection. Anesthesia decreases the blood flow then affects the pharmacokinetics of FIZ-shFasL. Toxic effect was found at dose of 0.3 mg/kg and $C_{2\,min}$ of this dosage was approximately 2 μg/ml. These data suggest that if plasma concentration of FIZ-shFasL were lower than 2 μg/ml, there would be no serious toxicity observed.

Discussion

Numerous research efforts have been directed at enhancing apoptosis in glioma cells by utilizing the Fas/FasL pathway, including studies involving glycosylated human FasL [22], membrane-bound recombinant FasL [11], Fas gene transfer [10], TNF α gene transfer [23], FADD gene transfer [24], and FasL gene transfer [25,26].

In terms of drug production, sFasL is a promising candidate for use as an apoptosis-inducing molecule because it can be easily produced. Accordingly, several molecular species have been designed as soluble FasL for therapeutic applications. Most of them are, however, impractical because of either their low biological activity or difficult production.

Coiled-coil proteins have a characteristic seven-residue repeat $(a, b, c, d, e, f, g)_n$, with hydrophobic residues at positions *a* and *d* and polar residues generally elsewhere [17]. When isoleucine is placed at both the *a* and *d* positions, peptides containing these sequences

Table 1
Summary of flow cytometric analysis and FIZ-shFasL sensitivity

Cell lines	Origin	Cell viability when treated with 0.6 μg/ml FIZ-shFasL (%)	CD95[a]	Bcl-2[a]
U-138 MG	Glioblastoma	0	21.7	76.2
U-87 MG	Glioblastoma	6	60.0	71.3
T98G	Glioblastoma	34	28.5	88.8
DAOY	Medulloblastoma	51	13.0	91.8
A172	Glioblastoma	84	11.5	45.2
U-373 MG	Glioblastoma	93	73.7	96.2
TE671	Rhabdomyosarcoma	100	1.2	99.5

[a] Numbers represent corrected mean fluorescence intensities.

Table 2
Toxicity summary for FasL fusion protein FIZ-shFasL in rats

		Control	FIZ-shFasL (mg/kg)			
			0.1	0.2	0.3	1.0
	n:	2	3	2	1	1
Body weight gain (g)						
Day 1		4.1	2.6	ND	0.5	−8.9
Food consumption (g)						
Day 1		16.3	16.7	ND	16.2	8.1
Hematology						
PLT ($\times 10^4\,\mu l^{-1}$)		80	88.2	ND	96.6	58.3
Biochemistry						
GOT		62.5	61.7	56.5	68	1326
GPT		22	25	22	27	1432
Absolute organ weight						
Thymus		0.42	0.45	0.43	0.29	0.3

FIZ-shFasL was dissolved in PBS containing 0.1% HSA as a protein carrier with indicated concentrations and administrated in tail vein. A day after the FIZ-shFasL administration body weight and food consumption were measured and all rats were sacrificed for sampling of blood and tissues. Hematology and blood biochemical analysis were described in the Materials and methods. ND, not determined.

adopt trimeric conformations spontaneously in the buffer solution [27]. We have constructed fusion proteins containing this isoleucine zipper motif and sFasL (FIZ-shFasL) in order to increase the cytotoxic activity of the recombinant proteins. In our results, FIZ-shFasL demonstrated approximately a 10-fold higher degree of cytotoxic activity compared to proapoptotic anti-Fas antibody (CH-11) to Jurkat cells. In addition, FIZ-shFasL successfully induced apoptotic cell death of the brain tumor cell lines in vitro. These results indicate a possible therapeutic application of FIZ-shFasL in treating Fas-expressing malignant human brain tumors. However, a few of the glioma-cultured cells such as A172 and U-373 MG demonstrated resistance to the cytotoxic effect of FIZ-shFasL. The efficacy of FIZ-shFasL as a therapeutic agent may depend on the sensitivity of tumor cells to Fas-mediated apoptosis. However, the detailed molecular mechanism that underlies FasL resistance remains obscure.

Our present results on flow cytometry show that the high expression levels of Fas are partially correlated with increased sensitivity to FIZ-shFasL. On the other hand, the sensitivity toward FIZ-shFasL-induced apoptosis was not due to differential expression levels of Bcl-2. We cannot explain the mechanism of the resistance of U-373 MG expressing high level of Fas. Although many cancer cell lines express Fas on the membrane, not all lines are sensitive to Fas-mediated apoptosis [27,28]. The most obvious mechanism of Fas resistance of tumor cells could be associated with the loss of functional Fas. Mutations within the CD95 gene were reported in nasal NK/T cell lymphomas and testicular germ cell tumors [28,29]. A soluble Fas generated by several alternative mRNA splicing can bind FasL and block Fas-mediated apoptosis [30]. Overexpression of DcR3 which competes with Fas for FasL binding has been found in gastrointestinal tumors [31,32]. Interference with the cytosolic domain of Fas and block of FADD function is another mechanism of resistance. FAP-1 expression in pancreatic adenocarcinoma cells [33] and FLIP overexpression in melanoma [34] are reported. Further study to elucidate the molecular mechanism of FIZ-shFasL resistance will be needed to select the patients who have potential sensitivity for FIZ-shFasL treatment in the future.

It is well known that the administration of an agonistic anti-Fas molecular antibody into mice causes fulminant hepatitis and kills the mice within 3–6 h [21]. In this study, systemic administration of FIZ-shFasL also caused serious damage to liver. The upper limit of FIZ-shFasL for safe systemic administration to rat is estimated as below 2 μg/ml in plasma concentration. Since plasma concentration varied highly according to general conditions, a local administration of FIZ-shFasL, for example intrathecal injection for brain tumors, should be considered to use FIZ-shFasL as a therapeutic agent.

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