Medical Science

AIMS Medical Science, 9(4): 467–485.
DOI: 10.3934/medsci.2022025
Received: 12 August 2022
Revised: 20 September 2022
Accepted: 20 September 2022
Published: 26 September 2022

http://www.aimspress.com/journal/medicalScience

Review

Human Fas ligand extracellular domain: current status of biochemical characterization, engineered-derivatives production, and medical applications

Michiro Muraki*

Independent researcher, 1-16-8, Hitachino-nishi, Ushiku, Ibaraki 300-1206, Japan

* **Correspondence:** Email: michiromuraki@gmail.com.

Abstract: Human Fas ligand extracellular domain (hFasLECD) is a soluble protein agent with significant relevance to medicine because of the immunologically essential functions of parental membrane-bound Fas ligand (CD95L, CD178) in the human body. This paper aimed to overview the current status of the studies focused on hFasLECD, ranging from its fundamental molecular properties and biological activity to its potential as a novel, practical cell death-inducing therapeutics or a diseases biomarker. The main part of this review was divided into four sections, the background of biological functions of human Fas ligand, the fundamental biochemical characteristics of hFasLECD, the production of engineered hFasLECD derivatives, and the potential of hFasLECD for medical applications. The translation of hFasLECD into an established agent in medicine is in progress, based on the clinical use-oriented research works in the relevant fields of basic science and engineering.

Keywords: human Fas ligand (CD95L, CD178); extracellular domain; biological functions; biochemical characteristics; recombinant expression; engineered derivatives; medical applications

Abbreviations: hFasL: Human Fas ligand; hFasLECD: Extracellular domain of hFasL; NK: Natural killer; sFasL: Soluble hFasL; scFv: Single chain variable fragment; T: Thymus-derived; TNF: Tumor necrosis factor

1. Background of biological functions

1.1. Location of Fas ligand gene in the human genome

Human Fas ligand (hFasL, CD95L, CD178) is a major member of the tumor necrosis factor (TNF) superfamily ligand proteins. This protein was originally mapped at the 1q23 loci of the human genome [1]. Currently, the encoding gene: FASLG is identified in the q24.3 loci of chromosomal 1 in the assembly of Genome Reference Consortium Human Build 38 [2].

1.2. Basic biological function of hFasL in the human body

hFasL protein transduces cytotoxic signals via apoptosis to the target cells by binding to its cognate Fas receptor on the cell surface. The major physiological roles of this apoptotic cell-death induction process include homeostasis of the thymus-derived (T) lymphocytes through activation-induced cell death [3], elimination of virus-infected and tumorous cells by cytotoxic T and natural killer (NK) lymphocytes [4], and killing of inflammatory cells in immune privilege sites such as the eye, testis, and brain [5]. The physiological apoptosis-signaling function by hFasL is considered to be implemented through the activated intracellular cascade of caspase proteases in the target cells. The impairment of this process can lead to the onset of many types of serious diseases including cancers and autoimmune diseases [4]. On the other hand, non-physiological functions of hFasL, including the unwanted proliferation of T-cells and inflammatory destruction of the immune privileged organs, may become unmasked under the circumstances of preventing proper apoptosis induction [6]. These nonapoptotic functions of FasL are considered to be mediated through the activation of the signaling pathways involving transcription factor family proteins, represented by nuclear factor-kappa B, mitogen-activated protein kinase, and phosphatidyl-inositol kinase/protein kinase B, which can interrupt the Fas-mediated apoptosis by the negative regulators such as caspase-8 inhibitory proteins (FLIPs).

2. Fundamental biochemical characteristics

2.1. Generation of hFasLECD in the human body

The soluble domain of hFasL (sFasL), which structurally corresponds to the extracellular domain of hFasL (hFasLECD), is known to be predominantly generated by the cleavage of the stalk region of hFasL (Figure 1a) with an extracellular matrix metalloproteinase called MMP7/matrilysin in the human body [7]. This shedding process drastically reduces the cell death-inducing activity of hFasL, and the resulting sFasL becomes a competitive inhibitor to the original hFasL. Consequently, sFasL was initially considered to work as a blocking agent in the killing of the target cells in general [8] However, to date, various non-apoptotic biological functions of sFasL also have been suggested to bring about the deterioration of human diseases' severity by many researchers, which include metastasis in triple-negative breast cancer [9], promotion of inflammation in autoantibody-induced arthritis [10], and perturbation in macrophages responses to *Toxoplasma gondii* infection [11].

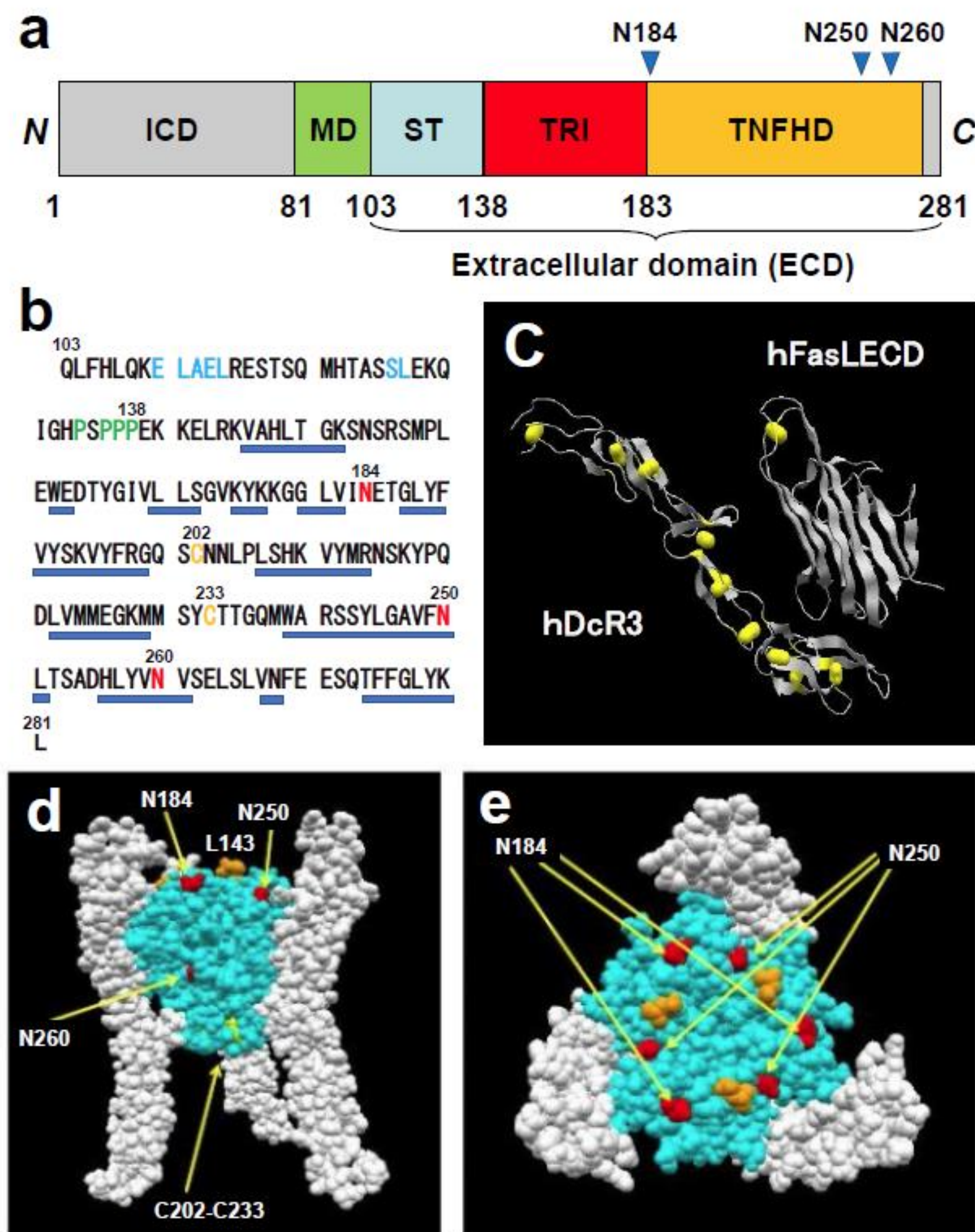

Figure 1. Primary and three-dimensional structures of hFasL and hFasLECD. a. Structural domains and regions in hFasL. ICD: intracellular domain; MD: membrane-spanning domain; ECD: extracellular domain; ST: stalk region; TRI: trimerization region; TNFHD: TNF homology domain region. Terminal amino acid residues in each region and putative N-glycosylation sites were numbered. b. Primary structure of hFasLECD. Two matrix

metalloprotease cleavage sequences in the ST region (pale blue), successive proline residues in the ST region (green), three asparagine residues at the N-glycosylation sites (red), two cysteine residues forming a disulfide-bridge (orange), and secondary β-sheet structure forming regions (blue bars) were highlighted. c. Three-dimensional (3D) main chain structure of a single asymmetric unit of hFasLECD- hDcR3 complex (PDB ID: 4msv) [12]. The secondary β-sheet structure forming regions (gray ribbons) and the disulfide bridges forming cysteine residues (yellow spheres) were highlighted. d and e. 3D structures of a trimeric unit of the hFasLECD- hDcR3 complex in space-filling models (d: a side view; e: a vertical view). The positions of the asparagine residues in the N-glycosylation sites (N184, N250, and N260, red), the disulfide-bridge forming cysteine residues (C202-C233, yellow), and the N-terminal residue in this X-ray structure model (L143, orange) were indicated. This figure was created by transforming and remixing the materials in previous papers [13,14]. © Michiro Muraki 2014 and 2018, licensed under Creative Commons Attribution 4.0 International License.

2.2. Primary and three-dimensional structures

The primary structure of hFasL can be divided into three domains by the positional relationship to the cell membrane, namely the intracellular (ICD), membrane-spanning (MD), and extracellular domains (ECD) (Figure 1a) [13]. The extracellular domain part can be further separated into several regions, designated as the stalk region (ST), the trimerization region (TRI), and the TNF homology domain region (TNFHD) according to the biological functions and the roles in the subunit assembly of the hFasLECD trimer. The amino acid sequence of hFasLECD, highlighting the ST cleavage sites, the putative N-glycosylation sites, the proline residues-rich sequence, the disulfide bridge-forming cysteine residues, and the possible secondary structure parts, is shown in Figure 1b. The X-ray three-dimensional (3D) structural views of hFasLECD in complex with human decoy receptor 3 (hDcR3) together with the annotations included in Figure 1b are shown in Figure 1c–e. In contrast to hDcR3, hFasLECD contains many extended β-sheet type secondary structure regions, but only a single disulfide bridge buried rather inside the molecule. Some fundamental biochemical parameters are compared among the two represented types of hFasLECD (with or without the ST region) and the whole hFasL molecule (Table 1). All of them contain more abundant basic amino acid residues (lysine and arginine) than acidic residues (aspartate and glutamate) as characterized by the theoretical pI values around 9. Among these three molecules, the hFasLECD part composed of the amino acid residues 139–281 was predicted to be most stable by an instability index analysis. Unfortunately, the X-ray 3D-structural information on the complex between hFasLECD and human Fas receptor is not currently available yet.

Table 1. Some biochemical parameters of human Fas ligand extracellular domain (hFasLECD) and whole human Fas ligand (hFasL)[a].

Biochemical parameters	hFasLECD (aa 103–281)	hFasLECD (aa 139–281)	hFasL (aa 1–281)
Number of aa	179	143	281
Molecular weight[b]	20448.48	16412.94	31484.72
Number of Cys	2	2	4
Theoretical pI	8.96	9.15	9.41
Instability index[c]	49.93	39.05	63.33

Note: [a]Calculated using Expasy/ProtParam Tool program [15]. [b]Post-translational modifications are not considered. [C]A value <40 is predicted to be stable, and >40 may be unstable.

3. Production of engineered derivatives

3.1. Recombinant expression

Many types of host cells have been examined for the expression of recombinant hFasLECD as a basis for the production of its engineered derivatives to date. The production of the recombinant hFasLECD was initially performed using the mammalian-derived host cells represented by human embryonic kidney 293 (HEK293) cells [16]. One of the obvious merits of using the mammalian host cells would be the identicalness or close resemblance of the carbohydrate chain structures to those in hFasLECD in the human body. However, as a result of the technological advancement in the heterologous expression systems, the secretory production of recombinant hFasLECD derivatives has been also investigated using other eukaryote expression systems of non-mammalian origins, such as amoebas (e.g. *Dictyostelium discoideum*), yeasts (e.g. *Pichia pastoris*), to date [13]. The important merits of using the heterologous expression host cells would include the more powerful and economical production of biologically active recombinant hFasLECD than the cases using the mammalian expression systems. Concerning the structural modifications in hFasLECD, it should be remarked that the glycosylation at the N260 site was essential for the secretion of the product [17] and the deletion of the ST region comprising the sequence with successive proline residues (PSPPP) at its C-terminal greatly improved the productivity in the case of *P. pastoris* expression system [18]. If the post-translational modifications by the carbohydrate chains are not always concerned, the in vivo expression systems using the bacterial hosts (e.g. *Escherichia coli*) and in vitro expression systems (e.g. wheat-germ extracts) may become other choices. The *E. coli* expressed product was refoldable into a biologically active form but prone to aggregate due to the lack of native glycosylation [12].

3.2. Commercial availability of the recombinant proteins

Table 2 summarizes the commercial availability of hFasLECD products from numerous biotech companies. Among the expression systems described above, human cells (e.g., HEK 293 and Chinese hamster ovary cells), yeast cells (e.g., *P. pastoris*), bacterial cells (*E. coli*), as well as the in vitro system using wheat-germ extracts, have been applied for the production of recombinant hFasLECD derivatives including whole hFasL molecule so far. The structural regions of hFasLECD employed for

the expression consisted of Q103-L281, L107-L281, P134-L281, and E139-L281 in the case of biologically functional products characterized by apoptosis-inducing activity. This reflected the importance of the integrity of the carboxyl-terminal region to L281, but the amino-terminal end can be flexible, for the maintenance of the native 3D structure of the expressed products. The compatible expression tags were always located at the N-terminal side of hFasLECD part concerning all biologically functional products carrying information about their quantitative apoptosis-inducing or Fas receptor-binding activity. On the other hand, the whole hFasL (1–281) molecule products containing the MD and ICD regions have mostly been intended for use that does not always require native biological functions (e.g., antibody production and western-blotting control).

3.3. Engineered derivatives by gene-fusions

Despite the therapeutically useful biological functions of parent hFasL, represented by the cell death-inducing activity against pathogenic cells such as malignant tumor cells, a single trimeric hFasLECD unit can show fairly weak apoptosis-inducing activity alone. However, the cell death-inducing activity of the single trimeric hFasLECD unit was greatly enhanced by joining more than two units with the help of additional self-multimerizing domains, including the collagen-like domain of human adiponectin (ACRP30)/human IgG1-Fc domain [19] and the isoleucine-zipper motif [20]. The former derivatives consisting of hexameric hFasLECD are already available as commercial reagents (Table 1) and the ACRP30-conjugated derivative has been suggested to effectively induce the apoptosis of various types of pathogenic cells, including human glioma cells [21]. However, it was also found to be difficult to identify a therapeutic window for the approach using this derivative combined with radiotherapy in the case of colon cancer [22]. The clinical trials of this product, named MegaFasL/APO010, phase I and II studies in a group of multiple myeloma (MM) patients and a phase I study in patients with solid tumors have been already conducted as Clinical Trial.gov ID: NCT03196947 (terminated due to sponsors decision) (2020) and NCT00437736 (completed) (2015), respectively. It was suggested that the resistance mechanism to APO010 in the variants of human MM and Burkitt's lymphoma cell lines could be mediated by the downregulation of Fas receptor expression levels in the target cells [23].

Table 2. Commercially available recombinant hFasLECD products.

Hosts used for expression (strains)	hFasLECD parts (AA sequence regions)	Additional tag parts [sequence[a], (attached end)]; total molecular weights [kDa (criteria)]	Biological activities or biochemical functions (criteria)/suitable applications	Product identifiers
Human cells (HEK293/293T)	M1-L281	31AA (containing MYC and FLAG)-linker peptide (C); 31.3 (Cal)	WB control for anti-FLAG Ab	LS-G99101/75888
Human cells (HEK293)	Q103-L281	FLAG+26AA-linker peptide (N); 32 (Ng, SDS), 35/37–42 (Gl, SDS)	APO (ED_{50}: 1 or 50 ng/ml, A20 B-cells />1 or >10 ng/ml, sensitive cells), approx. 50-fold increase w a CL-Ab; LB (1–100 ng/ml, FasR or nd)	S8689, PF092, FASLG-2424H, FL-101, ALX-522-020
Human cells (HEK293)	Q103-L281	FLAG (N/nd); 32/33 (Gl/nd, SDS)	APO (ED_{50}: 50 ng/ml, A20 B-cells; >10 ng/ml wo an enhancer, 0.1–1/<1 ng/ml or 50–100 higher activity w 0.1–1 mg/ml of the enhancer, Jurkat T-cells)	LS-G3868, ab109359/157085, AG-40B-0001
Human cells (HEK293)	Q103-L281	A tag-linker peptide (N); nd	APO (ED_{50}: 50 ng/ml, A20 B-cells), LB (human, rat and mouse FasR)	PF033
Human cells (nd)	Q103-L281	Human IgG_1-Fc, E99-A330 (C); nd	nd	MBS188537
Human cells (HEK293/293/ Expi293)	P134-L281	His6/Poly-His/His (N); 17.7/18/18.8/19.834 (Cal), 20–30/25–30/25–33/31–36 (Gl, SDS/nd), 65–85 (>90%, SEC), or nd	APO (0.1–1.5 ng/ml or ED_{50}: 1–5 ng/ml w 10 mg/ml mouse anti-polyHis mAb or ED_{50}: <10 ng/ml wo CL, Jurkat T-cells), LB (a linear range of 2–31 or 0.3–5 ng/ml of human FasR-Fc, immobilized 2 mg/ml of this product), ^{13}C and ^{15}N-labeled, or nd	#7443, HY-P72568, FASLG-234H/46H, ab168908, 228-11920-2, CYT-051, #5452, RP02353, OOPA00062, FAL-H5241

Continued on next page

Hosts used for expression (strains)	hFasLECD parts (AA sequence regions)	Additional tag parts [sequence[a], (attached end)]; total molecular weights [kDa (criteria)]	Biological activities or biochemical functions (criteria)/suitable applications	Product identifiers
Human cells (nd)	P134-L281	His6 (N); approx. 20–30 (SDS)	nd	MBS2569069, arb759026
Human cells (HEK293)	E139-L281	FLAG+human ACRP30 headless (AA18-111) (N/nd); 40 (SDS)	APO (<0.2 ng/ml, Jurkat T-cells), LB (human and mouse FasR)	LS-G3967, AG-40B-0130
Human cells (HEK293)	E139-L281	Human IgG_1-Fc (N/nd); 50 (SDS)	APO (<0.2 ng/ml, Jurkat T-cells), LB (human and mouse FasR, stimulation of natural membrane assisted aggregation of FasR)	FASLG-245H, LS-G3966, AG-40B-0132
Human cells (HEK293)	nd	His6/His (N/nd); 17.7 (Cal), 25–32 (Gl, SDS)	APO (ED_{50}: 0.1–1.5 ng/ml w 10 mg/ml anti-polyHis mAb, A20 B-cells) or nd	SPR6448, LS-G5227, 268-11044-1
Chinese hamster cells (CHO)	L107-L281	nd; 19.9 (Cal)	APO (ED_{50}: <10 ng/ml wo CL, Jurkat T-cells)	FASL 15-R
Chinese hamster cells (CHO)	P134-L281	m+His8/His8/His6 (N); 17.9/18 (Cal), 19–32/26–28/29–30 (SDS)	APO (ED_{50}: <10.0 ng/ml, HuT78 and Jurkat T-cells; <10.0 ng/ml wo CL, 0.2–0.5 mg/ml w anti-6xHis CL-Ab or 0.3–1.5 ng/ml w 10 mg/ml mouse anti-polyHis mAb, Jurkat T-cells)	SRP3036, F0427, FASLG-1616H, LS-G331/G4953, ab186673, 268-10756-1, 126-FL/FL-CF, #7140, 589402/589404 /589406, 313-03H
Chinese hamster cells (CHO)	TNF homologous region	His8 (N)/nd; 17.9 (Cal, 175AA)	APO (ED_{50}: <10.0 ng/ml wo a cross-linker, Jurkat T-cells)	FASLG-5412H/5413H

Continued on next page

Hosts used for expression (strains)	hFasLECD parts (AA sequence regions)	Additional tag parts [sequence[a], (attached end)]; total molecular weights [kDa (criteria)]	Biological activities or biochemical functions (criteria)/suitable applications	Product identifiers
Chinese hamster cells (CHO)	nd	His (N)/nd; 17.9 (Cal, 148AA)/18 (Cal, 157AA or nd)/19.9 (Cal, 156AA), 26–28/29–30 (SDS)	APO (ED_{50}: <10.0 ng/ml wo CL or 4.0–16 ng/ml dose-dependent, Jurkat T-cells); sandwich ELISA (as a capture molecule)	FASLG-2703H, LS-G137682, F1106, 064-05881
Pichia pastoris cells (nd)	P134-L281	His (N); 26 (nd)	LB (ED_{50}: 0.06–0.14 mg/ml for cynomolgus FasR-Fc, immobilized 10 mg/ml of this product)	HY-P74158
Yeast cells (nd)	Q103-L281	His6/His (N/nd); 22.4 (Cal)	nd	FASLG-1394H, LS-G22918, MBS9422290
Yeast cells (nd)	Q130-L281	His (N/nd); 19.3 (Cal) or nd	nd	LS-G22917, MBS9422290
Yeast cells (nd)	P134-L281	Poly-His/His (N) or nd; 19.3 (Cal, 168AA) or nd, 26 (SDS) or nd	LB (ED_{50}: 40–200 ng/ml, immobilized 2 mg/ml of human FasR-Fc; ED_{50}: 0.06–0.14 mg/ml for cynomolgus FasR-Fc, 0.22–0.52 mg/ml for rat FasR-Fc, immobilized 10 mg/ml of this product) or nd	FASLG-1395H/ 28165TH, MBS8118600, 10244-H07Y
Escherichia coli cells (nd)	Q103-L281	His6/His (N/nd); 24.4/24.5/(14.3*) (Cal) or nd (pI = 8.9)	Blocking peptide or nd	FASLG-2884H, LS-G20726, MBS1561030/ 9420232/2011269, abx066487*, Ag27009
Escherichia coli cells (nd)	Q103-L281	GST+His/GST+gstsgsg+His6+saglvprgstaigmketaaakferqhmdspdlgtlevlfqgi (N); 52.4 (Cal, pI = 6.8)	SDS, WB, ELISA, IP or nd	FASLG-623H, LS-G11315, MBS2012383, abx066484

Continued one next page

Hosts used for expression (strains)	hFasLECD parts (AA sequence regions)	Additional tag parts [sequence[a], (attached end)]; total molecular weights [kDa (criteria)]	Biological activities or biochemical functions (criteria)/suitable applications	Product identifiers
Escherichia coli cells (nd)	Q103-L281	His+S (N/nd) or m+His6+ssglvprgsgm+S+pdlgtddddkamadigs (N); 14.3 (Cal, pI = 6.8) or nd	SDS, WB, ELISA, IP or nd	FASLG-624H, LS-G11318, MBS2009859
Escherichia coli cells (nd)	Q103-L281	m (N) and e+His6 (C); nd	nd	MBS300512
Escherichia coli cells (nd)	H122-V191	His6+ABP (N); 25 (Cal)	BP for anti-human FasL Ab	NBP2-49160PEP
Escherichia coli cells (nd)	Q130-L281	mgss+His6+ssglvprgshm (N); 19.6 (Cal, 173AA) or nd	SDS or nd	FASLG-7108H, LS-G2109, MBS145887/ 5308642, ab139255, 228-20072-2, CYT-031
Escherichia coli cells (nd)	Q130-L281	His (nd); 21.4 (Cal)	nd	LS-G20725, MBS9420231
Escherichia coli cells (nd)	TNF homologous region	His8 (N); 17.9 (Cal)	nd	SRP6216
Escherichia coli cells (nd)	175AA (positions unspecified)	nd; 47 (nd)	APO (ED_{50}: <10 ng/ml wo a cross-linker, Jurkat T-cells)	sc-4855
Wheat germ extract (*in vitro,* liposome technology/nd)	M1-L281	No; 31.5 (Cal)	AP, FS, CS or AP	FASLG-3855H, H00000356-P01

Continued on next page

Hosts used for expression (strains)	hFasLECD parts (AA sequence regions)	Additional tag parts [sequence[a], (attached end)]; total molecular weights [kDa (criteria)]	Biological activities or biochemical functions (criteria)/suitable applications	Product identifiers
Wheat germ extract (*in vitro*)	M1-L281	GST (N/nd); 56.65 (Cal)	AP, PA, ELISA, WB, or nd	FASLG-3857H, LS-G27496, H00000356-P01
Wheat germ extract (*in vitro*)	S172-L281	GST (N)/nd; 37.84 (Cal)/nd	AP, PA, ELISA, WB or nd	LS-G57795, H00000356-Q01

Note: [a]: Amino acid sequences of the non-abbreviated tag regions were written in small one-letter symbols. All data were obtained from the online data sheets (*: an extraordinary value in the same category). Abbreviations: AA: Amino acids; Ab: Antibodies; ABP: Albumin-binding protein-tag; ACRP30: Adiponectin; AP: Antibody production; APO: Apoptosis-inducing activity; BP: Binding peptide; C: Carboxy-terminal; Cal: Calculated; CL: Cross-linking; CS: Compound screening; ED_{50}: Effective dose 50%; ELISA: Enzyme-linked immunosorbent assay; FasR: Fas receptor; Fc: Fragment crystallizable region; FLAG: Dykddddk-tag; FS: Functional studies; Gl: Glycosylated products; GSH: Reduced glutathione; GST: Glutathione S-transferase-tag; His/Poly-His: Polymeric-histidine-tag; His6: Hhhhhh-tag; His8: Hhhhhhhh-tag; HPLC: High-performance liquid chromatography; IgG_1: Immunoglobulin gamma subclass-1; IP: Immunoprecipitation; LB: Ligand-binding activity; MYC: Eqkliseedl-tag; N: Amino-terminal; nd: not described; Ng: Non-glycosylated products; PA: Protein array; RP: Reversed-phase; S: S-peptide-tag; SDS: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC: Size exclusion chromatography; w: in the presence of; WB: Western-blotting; wo: in the absence of.

From the viewpoint of systemic toxicity, hFasLECD is inferable to be a less toxic cell death-inducing agent compared with an agonistic antibody against human Fas receptor from the experimental result that a recombinant hFasLECD product expressed in *P. pastoris* exhibited at least 20 times higher specific activity and 50 times lower cytotoxicity against the liver in mice, compared to a hamster-derived agonistic antibody against mouse Fas receptor [24]. To further reduce the systemic toxicity for the avoidance of damage to the normal cells composing sensitive organs such as the liver and enhance the objective activity by plausible concentration effects on the surface of the target cells, numerous kinds of hFasLECD derivatives have been developed. These derivatives using the gene-fusion technique were equipped with either the single chain variable fragment (scFv) against the surface antigens on the targeted cells or those with other cell-surface locating proteins that specifically bind to the respective cognate surface antigens on the target cells [13], represented by cytotoxic T-lymphocyte associated antigen (CTLA-4) and CD40 recognizing CD80/CD86 and CD40 ligand, respectively [25]. In either case, the fusion of the additional proteins has been made always at the N-terminal side of the hFasLECD gene, suggesting the importance of the integrity of the C-terminal region of the hFasLECD gene for the recombinant expression again.

3.4. Engineered derivatives by site-specific chemical conjugations

Except for the gene-fusion techniques, site-specific chemical modifications provided another promising way for the addition of functionality to hFasLECD by other molecules [26]. In principle, unlike the gene-fusion methodology, this technique does not limit the functional moieties for conjugation to peptide/protein molecules, while essentially requires excellent specificity in the chemical reactions for conjugations to obtain the objective products in good yields. To date, two types of chemical reactions have been employed for the site-specific chemical conjugations with hFasLECD. One is a direct conjugation using a thiol-ene reaction between the free-cysteine residue introduced to the N-terminal tag-sequence region of hFasLECD derivatives and the maleimide group in the molecules with aimed functions. The other is a two-step cycloaddition-type conjugation using an inverse-electron deficient Diels-Alder reaction between methyltetrazine and *trans*-cyclooctene groups in the second step. The former type of conjugation has been applied to fluorescent dyes [27], a biotin moiety, and the first step reaction in the two-step conjugation. The latter type of conjugation has been used for fluorescent dyes, polyethylene glycol moieties, and other functional protein molecules, such as rabbit IgG-derived Fab' and chicken avidin [28]. The interaction between avidin and biotin is one of the strongest non-covalent molecular bonding discovered to date ($K_d = \sim 10^{-15}$ M). Studies on the preparation of a site-specific biotin group-modified hFasLECD maintaining cell death-inducing activity [29] and the application of a biotinylated hFasLECD derivative for an organization on DNA origami-based nanostructures [30] have been published recently.

4. Potential for medical applications

4.1. Sensitization with exogenous agents

As exemplified by the effectiveness of Fas receptor expression level's up-regulation by gemcitabine administration in the treatment of patients with osteosarcoma lung metastasis, manipulations of Fas ligand-mediated cell death signaling pathway can be a promising way for the treatment of diseases [31]. As mentioned, the proteolytic cleavage of the whole hFasL molecule into soluble hFasLECD fragment greatly reduces the killing activity against the target cells to the level of working as a competitive inhibitor. However, various exogenous agents, including non-cytokine compounds, such as low-molecular-weight anti-cancer drugs (e.g., cisplatin, 5-fluorouracil, camptothecin, and gemcitabine), as well as cytokine agents represented by interferon-γ have been revealed to significantly sensitize the cell death-inducing activity of hFasLECD [32]. This sensitization of soluble hFasLECD fragment is considered to be mediated through either up-regulation of pro-apoptotic molecular machinery and/or down-regulation of anti-apoptotic factors. The former mechanism was typically realized by the up-regulation of cell-surface Fas receptor level, and the latter mechanism encompasses the down-regulation of intracellular levels of apoptosis inhibitory proteins such as cellular-FLIP. Sensitization by exogenous agents is expected to become a more promising way of transforming soluble hFasLECD derivatives into effective clinical pharmaceuticals for treatments of serious diseases by combining them with the protein engineering techniques described in the above sections.

4.2. Delivery and arrangement on the surface of target cells

The targeting approaches by the fusion with specific binders to surface antigens on the target cells using the gene-fusion and site-specific chemical modification techniques possess significant potential for overcoming the dose-limiting cytotoxicity against sensitive organs. In this connection, a pre-targeting method utilizing the fluorescein isothiocyanate (FITC)-modified monoclonal antibody against tumor antigens and a FITC-scFv fused with hFasLECD has also been developed for the selective delivery of hFasLECD to cancer cells [33]. Another promising pre-targeting approach for efficient delivery using chemical modification was a pre-biotinylation of spleen cells [34] and the pancreatic islets [35], followed by the incubation with a streptavidin-hFasLECD fusion protein. This strategy was developed for preventing the rejection of the heart grafts and the allogeneic islets after transplantation by systemic immunomodulation. Also, although the application to hFasLECD is still awaited, it may be possible to apply the arrangement by covalent membrane display in the style of glass-supported lipid bilayers and liposomes on cancer cells, which has been studied on another clinically relevant soluble TNF superfamily ligand protein [36].

4.3. Treatment of diseases in immune-privileged sites

Among the illness of the immune-privileged organs, ocular diseases have been most intensively investigated as the treatment target, for which hFasLECD could be used as the potential therapeutic agent, to date [37]. For example, a commercial sFasL reagent was suggested to ameliorate acute and recurrent herpetic stromal keratitis [38].

4.4. Usefulness as disease biomarkers

In previous studies, soluble hFasLECD levels in the body fluids (e.g., serum/plasma and cerebrospinal fluid) have often been revealed to be useful as a possible clinical biomarker regarding the diagnosis and/or prognosis of treatments for numerous human medical problems, including not only the diseases directly caused by the impairment of the cell-death inducing systems involving Fas ligand but also more general illness related to inflammation [39]. The former mainly consisted of various types of cancers and autoimmune diseases such as autoimmune lymphoproliferative syndrome and systemic lupus erythematosus, and the latter included other autoimmune, allergic, infectious, and many inflammatory diseases specific to the cardiovascular, hematologic, and other organ systems (e.g. the renal, hepatic, respiratory, central nervous, endocrine, dermal, and obstetric systems), miscellaneous problems related to metabolic syndrome, aging, transplantation, and also envenomation and smoking. The number and type of cases showing the significance of sFasL levels are currently increasing, and therefore it is expected that the translation of this biomarker into practical clinics will certainly contribute to effective treatments of a wide variety of human diseases in the future.

4.5. Patents issued

A large number of patents have been issued concerning the applications of Fas receptor-meditated cell death signaling process for solving human medical problems during the last 25 years before 2022 [6]. Among the 127 patents published, approximately 27% of the patents contained either "Fas ligand", "Fasl", "cd95 ligand", or "cd95l", as the keyword in their titles, including some patents with the keywords, "fas ligand fragments" or "soluble fas ligand". This indicated a strong relation of the biological function of hFasL/hFasLECD to the practical strategies for medical treatment. As expected, the targeted types of the diseases in these patents were mainly composed of cancers, autoimmune diseases, and inflammatory diseases, however, other types of diseases were also targeted in many instances. The employed approaches ranged over various strategies, including the development of polypeptides, antibodies, fusion proteins, nucleotide complexes, RNA interfering molecules, expressing cells, reprogrammed viruses, and drug delivery systems.

5. Concluding remarks

Since the identification of hFasL as a key death-inducing component of cytotoxic T-cells and NK-cells against various harmful cells causing serious diseases in the human body, the applications of hFasLECD for solving human medical problems have been extensively sought by many researchers, and new approaches are still emerging currently. From the viewpoint of protein-based biopharmaceuticals, hFasLECD derivatives can be more promising because of their fair biochemical stability and relatively low molecular weight as compared to agonistic monoclonal antibodies against human Fas receptor. The smaller size of hFasLECD derivatives would be beneficial for the agent's access to the target cells' surface. Recently, chimeric antigen receptor (CAR)-T and CAR-NK cells have been paid attention to as effective therapeutic agents against malignant tumors, but their clinical application is still under development and is rather expensive. These therapeutic agents depend significantly on the apoptotic cell-killing activity of hFasL in their action mechanisms [40–42]. The combination of hFasLECD derivatives fused with tumor-targeting domains assisted by sensitization with exogenous agents has the potential to partly play a role as a surrogate for CAR therapy. Also, liquid biopsy is useful for efficient treatments of human diseases as a relatively economical non-invasive diagnostic method [43]. The fair stableness of soluble hFasLECD in many human body fluids and wide applicability to numerous human diseases would be an important advantage as disease biomarkers. Nevertheless, the translation of hFasLECD and its derivatives into clinical medicine is still underway to be established as practical agents for therapeutic and diagnostic purposes. To overcome this irritating situation, we will be required to further strive for basic science and engineering studies aimed at enhancing their suitability in clinical settings.

Acknowledgments

This research was privately conducted after the expiration of the contract period with the National Institute of Advanced Industrial Science and Technology, Japan. The author appreciates technical support from the National Diet Library (Online Search and Request Service) for retrieving some of the reference papers and Ms. Keiko Muraki's secretarial assistance.

Conflict of interest

The author declares that he has no conflict of interest.

References

1. Takahashi T, Tanaka M, Inazawa J, et al. (1994) Human Fas Ligand: gene structure, chromosomal location and species specificity. *Int Immunol* 6: 1567–1574. https://doi.org/10.1093/intimm/6.10.1567
2. Fairley S, Lowy-Gallego E, Perry E, et al. (2020) The international genome sample resource (IGSR) collection of open human genomic variation resources. *Nucleic Acids Res* 48: D941–D947. https://doi.org/10.1093/nar/gkz836
3. Alderson MR, Tough TW, Davis-Smith T, et al. (1995) Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med* 181: 71–77. https://doi.org/10.1084/jem.181.1.71
4. Nagata S (1999) Fas ligand-induced apoptosis. *Annu Rev Genet* 33: 29–55. https://doi.org/10.1146/annurev.genet.33.1.29
5. Green DR, Ferguson TA (2001) The role of Fas ligand in immune privilege. *Nat Rev Mol Cell Biol* 2: 917–924. https://doi.org/10.1038/35103104
6. Risso V, Lafont E, Le Gallo M (2022) Therapeutic approaches targeting CD95L/CD95 signaling in cancer and autoimmune diseases. *Cell Death Dis* 13: 248. https://doi.org/10.1038/s41419-022-04688-x
7. Herrero R, Kajikawa O, Matute-Bello G, et al. (2011) The biological activity of FasL in human and mouse lungs is determined by the structure of its stalk region. *J Clin Invest* 121: 1174–1190. https://doi.org/10.1172/JCI43004
8. Suda T, Hashimoto H, Tanaka M, et al. (1997) Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. *J Exp Med* 186: 2045–2050. https://doi.org/10.1084/jem.186.12.2045
9. Malleter M, Tauzin S, Bessede A, et al. (2013) CD95L cell surface cleavage triggers a prometastatic signaling pathway in triple-negative breast cancer. *Cancer Res* 73: 6711–6721. https://doi.org/10.1158/0008-5472.CAN-13-1794
10. Jeong D, Kim HS, Kim HY, et al. (2021) Soluble Fas ligand drives autoantibody-induced arthritis by binding to DR5/TRAIL-R2. *eLife* 10: e48840. https://doi.org/10.7554/eLife.48840
11. Tiffiney EA, Coombes JL, Legembre P, et al. (2022) Cleaved CD95L perturbs in vitro macrophages responses to Toxoplasma gondii. *Microbes Infect* 24: 104952. https://doi.org/10.1016/j.micinf.2022.104952
12. Liu W, Ramagopal U, Cheng H, et al. (2016) Crystal structure of the complex of human FasL and its decoy receptor DcR3. *Structure* 24: 2016–2023. https://doi.org/10.1016/j.str.2016.09.009
13. Muraki M (2018) Development of expression systems for the production of recombinant human Fas ligand extracellular domain derivatives using Pichia pastoris and preparation of the conjugates by site-specific chemical modifications: A review. *AIMS Bioengineer* 5: 39–62. https://doi.org/10.3934/bioeng.2018.1.39

14. Muraki M (2014) Disulfide-bridged proteins with potential for medical applications: therapeutic relevance, sample preparations and structure-function relationships. *Integr Mol Med* 1: 38–56. https://doi.org/10.15761/IMM.1000109
15. Gasteiger E, Hoogland C, Gattiker A, et al. (2005) Protein identification and analysis tools on ExPASy server. In: Walker JM, The Proteomics Protocols Handbook, Humana Press, 571–607. https://doi.org/10.1385/1-59259-890-0:571
16. Schneider P, Bodmer JL, Holler N, et al. (1997) Characterization of Fas (Apo-1, CD95)-Fas ligand interaction. *J Biol Chem* 272: 18827–18833. https://doi.org/10.1074/jbc.272.30.18827
17. Muraki M (2006) Secretory expression of synthetic human Fas ligand extracellular domain gene in Pichia pastoris: influences of tag addition and N-glycosylation site deletion, and development of a purification method. *Prot Expr Purif* 50: 137–146. https://doi.org/10.1016/j.pep.2006.08.006
18. Muraki M (2008) Improved secretion of human Fas ligand extracellular domain by N-terminal part truncation in Pichia pastoris and preparation of the N-linked carbohydrate chain trimmed derivative. *Prot Expr Purif* 60: 205–213. https://doi.org/10.1016/j.pep.2008.03.027
19. Holler N, Tardivel A, Kovacsovics-Bankowski M, et al. (2003) Two adjacent Trimeric Fas ligands are required for Fas signaling and formation of a death-inducing signaling complex. *Mol Cell Biol* 23: 1428–1440. https://doi.org/10.1128/MCB.23.4.1428-1440.2003
20. Shiraishi T, Suzuyama K, Okamoto H, et al. (2004) Increased cytotoxicity of soluble Fas ligand by fusing isoleucine zipper motif. *Biochem Biophys Res Commun* 322: 197–202. https://doi.org/10.1016/j.bbrc.2004.07.098
21. Eisele G, Roth P, Hasenbach K, et al. (2011) APO010, a synthetic hexameric CD95 ligand, induces human glioma cell death in vitro and in vivo. *Neuro Oncol* 13: 155–164. https://doi.org/10.1093/neuonc/noq176
22. Verbrugge I, Wissink EHJ, Rooswinkel RW, et al. (2009) Combining radiotherapy with APO010 in cancer treatment. *Clin Cancer Res* 15: 2031–2038. https://doi.org/10.1158/1078-0432.CCR-08-2125
23. Jandu H, Nielsen A, Brunner N, et al. (2020) Characterization of resistance to a recombinant hexameric Fas-ligand (APO010) in human cancer cell lines. *Exp Hematol* 87: 33–41. https://doi.org/10.1016/j.exphem.2020.06.005
24. Tanaka M, Suda T, Yatomi T, et al. (1997) Lethal effect of recombinant human Fas ligand in mice pretreated with Propionibacterium acnes. *J Immunol* 158: 2303–2309.
25. Orbach A, Rachmilewitz J, Shani N, et al. (2010) CD40-FasL and CTLA-4-FasL fusion proteins induce apoptosis in malignant cell lines by dual signaling. *Am J Pathol* 177: 3159–3168. https://doi.org/10.2353/ajpath.2010.100301
26. Muraki M (2014) Improved production of recombinant human Fas ligand extracellular domain in Pichia pastoris: yield enhancement using disposable culture-bag and its application to site-specific chemical modifications. *BMC Biotechnol* 14: 19. https://doi.org/10.1186/1472-6750-14-19

27. Muraki M (2016) Preparation of a functional fluorescent human Fas ligand extracellular domain derivative using a three-dimensional structure guided site-specific fluorochrome conjugation. *SpringerPlus* 5: 997. https://doi.org/10.1186/s40064-016-2673-8
28. Muraki M, Hirota K (2017) Site-specific chemical conjugation of human Fas ligand extracellular domain using trans-cyclooctene-methyltetrazine reactions. *BMC Biotechnol* 17: 56. https://doi.org/10.1186/s12896-017-0381-2
29. Muraki M, Hirota K (2019) Site-specific biotin-group conjugate of human Fas ligand extracellular domain: preparation and characterization of cell-death-inducing activity. *Curr Top Pep Prot Res* 20: 17–24.
30. Berger RML, Weck JM, Kempe SM, et al. (2021) Nanoscale FasL organization on DNA origami to decipher apoptosis signal activation in cells. *Small* 17: 2101678. https://doi.org/10.1002/smll.202101678
31. Koshkina N, Yang Y, Kleinerman E (2020) The Fas/FasL signaling pathway: its role in the metastasic process and as a target for treating osteosarcoma lung metastases. *Adv Exp Med Biol* 1258: 177–187. https://doi.org/10.1007/978-3-030-43085-6_12
32. Muraki M (2020) Sensitization to cell death induced by soluble Fas ligand and agonistic antibodies with exogenous agents: A review. *AIMS Med Sci* 7: 122–203. https://doi.org/10.3934/medsci.2020011
33. He Y, van Bommel PE, Samplonius DF, et al. (2017) A versatile pretargeting approach for tumor-selective delivery and activation of TNF superfamily members. *Sci Rep* 7: 13301. https://doi.org/10.1038/s41598-017-13530-w
34. Zhao H, Woodward KB, Shirwan H, et al. (2013) Posttransplantation systemic immunomodulation with SA-FasL-engineered donor splenocytes has robust efficacy in preventing cardiac allograft rejection in mice. *Transplant Proc* 45: 1805–1807. https://doi.org/10.1016/j.transproceed.2013.01.049
35. Skoumal M, Woodward KB, Zhao H, et al. (2019) Localized immune tolerance from FasL-functionalized PLG scaffolds. *Biomaterials* 192: 271–281. https://doi.org/10.1016/j.biomaterials.2018.11.015
36. Nair PM, Flores H, Gogineni A, et al. (2015) Enhancing the antitumor efficacy of a cell-surface death ligand by covalent membrane display. *Proc Natl Acad Sci U S A* 112: 5679–5684. https://doi.org/10.1073/pnas.1418962112
37. Gregory-Ksander M, Marshak-Rothstein A (2021) The FasLane to ocular pathology-metalloproteinase cleavage of membrane-bound FasL determines FasL function. *J Leukoc Biol* 110: 965–977. https://doi.org/10.1002/JLB.3RI1220-834R
38. Rogge M, Yin XT, Godfrey L, et al. (2015) Therapeutic use of soluble Fas ligand ameliorates acute and recurrent herpetic stromal keratitis in mice. *Invest Ophthalmol Vis Sci* 56: 6377–6386. https://doi.org/10.1167/iovs.15-16588

39. Muraki M (2022) Soluble Fas ligand, soluble Fas receptor, and decoy receptor 3 as disease biomarkers for clinical applications: A review. *AIMS Med Sci* 9: 98–267. https://doi.org/10.3934/medsci.2022009
40. Benmebarek MR, Karches CH, Cadilha BL, et al. (2019) Killing mechanisms of chimeric antigen receptor (CAR) T cells. *Int J Mol Sci* 20: 1283. https://doi.org/10.3390/ijms20061283
41. Khawar MB, Sun H (2021) CAR-NK cells: from natural basis to design for kill. *Front Immunol* 12: 707542. https://doi.org/10.3389/fimmu.2021.707542
42. Pang Z, Wang Z, Li F, et al. (2022) Current progress of CAR-NK therapy in cancer treatment. *Cancers* 14: 4318. https://doi.org/10.3390/cancers14174318
43. Wallach-Dayan SB, Petukhov D, Ahdut-HaCohen R, et al. (2021) sFasL-the key to a riddle: Immune responses in aging lung and disease. *Int J Mol Sci* 22: 2177. https://doi.org/10.3390/ijms22042177

AIMS Press

© 2022 the Author(s), licensee AIMS Press. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0)