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Short Communication

Site-specific biotin-group conjugate of human Fas ligand extracellular domain: preparation and characterization of cell-death-inducing activity

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ABSTRACT

Engineering of human Fas ligand extracellular domain (hFasLECD) protein can provide us with an advanced way for treatment or diagnosis of many serious diseases represented by cancers. In this study, we prepared a site-specific biotin group conjugate of hFasLECD and conducted the characterization of its cell-death-inducing activity. Precipitation of complexes using magnetic beads for affinity-based capturing revealed that the conjugate retained the original binding activity to both human Fas receptor extracellular domain and streptavidin. A significant sensitization effect on the cell-death-inducing activity against a colorectal cancer cell line, HT-29 cells, was observed for the pretreatment with human interferon-γ, accompanied by a synergistic effect of 5-fluorouracil. The findings suggested that the prepared hFasLECD conjugate would be applicable to the future development of medically useful devices to detect the counterpart receptors in biological specimens as well as novel cytotoxic agents against diseased cells.

KEYWORDS: human Fas ligand, extracellular domain, biotin, conjugation, cell-death inducing activity, interferon-γ, 5-fluorouracil.

INTRODUCTION

Human Fas ligand (hFasL) is an essential membrane protein for medicine, since this protein can induce the cell-death of many diseased cells, such as cancer cells and virally-infected cells, *via* implementation of apoptosis in the human body [1]. The apoptosis process is triggered by the specific binding of hFasL extracellular domain (hFasLECD) to human Fas receptor extracellular domain (hFasRECD) on the surface of the target cells. Serum concentration levels of soluble hFasRECD (shFasR) and the soluble decoy receptor of hFasL, hDcR3, were found to be up- or down-regulated in many serious chronic diseases including cancers and cardiovascular failures, and they have been suggested as the potential prognostic biomarkers in the diseases [2 - 5]. Hence, a great benefit will be obtained, if we can develop effective molecular agents either for treatment or diagnosis of the serious diseases relating to Fas ligand-mediated apoptosis through the engineering of hFasLECD.

The binding of biotin to avidin/streptavidin is one of the strongest (K_d = ~10^{-15} M) non-covalent interactions ever known. This extremely high affinity is attractive for combining two molecules possessing different functions to each other, and enables wide applications for the development of many innovative derivatives, including the detection probes in bioanalytical devices [6, 7]. The use of this interaction can also be a versatile strategy for adding new functions to high molecular-weight therapeutic proteins, represented by antibodies [8, 9]. For such aims, it is important to introduce the new functional component into the target protein without impairing the original biological function. Chemical modifications of hFasLECD can provide a new

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way for developing medically useful molecular agents. In a previous study, the author presented the possibility of attaching new functions to a recombinant hFasLECD derivative expressed in *Pichia pastoris* by the site-specific chemical modification with a fluorescent dye molecule, guided by its three-dimensional structure [10]. There are two alternative ways for the derivatization intended to use the protein molecule as a component in the biotin-avidin/streptavidin binding. One is the conjugation with an avidin/streptavidin protein and the other is that with a biotin group. Recently, we reported the preparation of a site-specific chemical conjugate between hFasLECD and hen egg-white avidin, which retained both hFasRECD and biotin group binding activity [11]. However, the overall yield of the final product was substantially lower than the case of single-step chemical modification reactions using an excess molar amount of low molecular-weight (MW) compounds, since the conjugation between high MW proteins required two-step chemical conjugation reactions [12]. Also, the purification of the final product needed a rather delicate separation of the conjugated product from the nonconjugated proteins using a high-performance size-exclusion chromatography, due to the MW similarity of the proteins to each other (approx. MW ratio, 1:2) [11]. A biotin group has a much smaller molecular size than avidin/ streptavidin and is fairly stable, which makes this functional group possible to be introduced into hFasLECD by employing an excess amount of commercially available low MW reagent for the chemical modification. The large difference in MW (approx. MW ratio, 1:60) facilitates an efficient removal of the excess reagent from the conjugated protein in the purification step. In this study, we prepared a site-specific biotin group conjugate using a single-step thiol-ene reaction and investigated some basic biological functions of the conjugate. The conjugated product displayed a strong binding activity to hFasRECD and streptavidin, showing both functional components remained intact after the conjugation. The engineered hFasLECD exhibited a significant cell-death-inducing activity against a colorectal cancer cell line, HT-29 cells, in the presence of pretreatments with human interferon-γ, accompanied by a synergistic effect of 5-fluorouracil.

MATERIALS AND METHODS

Materials

Human Fas ligand extracellular domain containing a deletion mutation of the amino-acid residues from 103 to 138 and substitution mutations (Asn184 to Gln, and Asn250 to Gln) [hFasLECD (139-281), N184Q, N250Q], together with an additional N-terminal FLAG-$(Lys)_3$GlyCys$(Gly)_4$ tag sequence (NFK3G1CG4-hFasLECD), was prepared as described in a previous paper [11]. Biotin-PEG3-Maleimide (B-PEG3-Mal) was obtained from Click Chemistry Tools, Co (Catalog No. #1029). Dimethyl sulfoxide, Super-dehydrated (Dry DMSO) and L-Cysteine hydrochloride monohydrate (L-Cys HCl) were purchased from Wako Pure Chemicals Ind. Tris-(2-carboxyethyl)phosphine (TCEP) neutral pH solution was from Thermo Fisher Scientific (TFS). SureBeads Protein G and Dyna Beads Streptavidin (M-270) magnetic beads were supplied from Bio-Rad Laboratories and Invitrogen, respectively. The recombinant hFasRECD-Fc was produced in a baculovirus-*Bombyx mori* larvae expression system as previously described [13]. Protein concentration was determined by a bicinchoninic acid (BCA) protein assay kit from TFS using bovine serum albumin as the standard sample. Human colorectal adenocarcinoma cell line, HT-29 cells (ECACC EC91072201, equivalent to ATCC HTB-38) were obtained from DS-Pharma Biomedical, and maintained in McCoy's 5A (Modified) medium (08457-55, TFH) supplemented with 10% fetal bovine serum (FBS) (10437-028, TFH) and 2% Penicillin-Streptomycin mixed solution [09367-34, Nacalai Tesque (NT)] at 310 K in a 95% humidified air–5% CO_2 incubator. Cell passages were performed at 80% confluence. Animal-derived-free human IFN-γ for cell biology (IFN-γ) and 5-fluorouracil (5-FU) were purchased from Fuji Film Wako Pure Chemicals Ind. A Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS) was from NT (14249-95). The poly-L-lysine-coated 96 well microplates used for thiazolyl blue tetrazolium assay (MTT assay) were from Iwaki. Chemical structures were drawn using ChemBioDraw Ultra, ver. 14.

Conjugation of NFK3G1CG4-hFasLECD with B-PEG3-Mal

The recombinant NFK3G1CG4-hFasLECD was produced using a *Pichia pastoris* secretory expression

system as previously described [11]. The sample employed for the chemical conjugation with B-PEG3-Mal was purified using a cation-exchange chromatography column [Hi-Trap SP HP, GE healthcare (GEH)] preceding the reaction. The concentration of the purified sample in 50 mM sodium acetate plus 300 mM NaCl (pH 5.5), used as the starting material for the conjugation reaction, was 12.6 mg/ml. To the solution (3.4 ml) of NFK3G1CG4-hFasLECD, 34 μl of ethylenediaminetetraacetic acid sodium salt (EDTA Na) solution (pH 8.0) and 68 μl of 0.5 M TCEP (Neutral pH) solution were added, and the reaction mixture was incubated for 1 h at 296 K. The reaction mixture was resolved and buffer-exchanged by a PD-10 size-exclusion chromatography column (GEH) using 25 mM phosphate plus 2 mM EDTA Na buffer (pH 6.4), and the elution fraction (Total 7.0 ml) containing the reduced NFK3G1CG4-hFasLECD free of excess TCEP was diluted with 14.0 ml of the same buffer of pH 6.4. Freshly prepared 20 mM B-PEG3-Mal solution in Dry DMSO (2.0 ml) was added, and incubated for 4 h at 297 K. The almost clear pale-yellow conjugation-reaction mixture was quenched with 525 μl of 1 M L-Cys HCl by incubating for another 1 h at 297 K.

Purification of B-PEG3-Mal conjugated NFK3G1CG4-hFasLECD

The above quenched reaction mixture was centrifuged at 5000 G for 10 min at 277 K to remove small amounts of insoluble materials. Aliquots (2.5 ml each) of the resulted clear pale-yellow solution were subjected to a PD-10 column to remove low-MW substances. The fractions containing the conjugated protein were combined (Total 35.0 ml) and concentrated to 5.0 ml using an Amicon Ultra 15 centrifugation device (MW cutoff: 10 kDa, GEH). The recovered sample was centrifuged again (5000 G, 10 min, 277 K) to remove traces of the remaining insoluble materials before applying to the resolution column (Superdex Increase 200 10/300 GL, GEH) of a high-performance size-exclusion chromatography, using 50 mM Tris HCl plus 150 mM NaCl (pH 7.5) as the elution buffer at the flow rate of 0.75 ml/min. Aliquots (230 μl each) of the sample were applied to the size-exclusion chromatography column. The single main peak fraction in each run was collected and combined as the final purified product (Total 21.0 ml).

Detection of binding activity

Detection of the hFasRECD and streptavidin binding activity of the B-PEG3-Mal-conjugated NFK3G1CG4-hFasLECD was conducted by a receptor-mediated co-immunoprecipitation method and a direct binding method, respectively, using the magnetic beads for each affinity-based capturing. Either the conjugated sample (5.0 μg each) in 0.1 ml of 50 mM Tris HCl plus 0.15 M NaCl buffer (pH 7.5) or the same buffer alone (0.1 ml) as the negative control sample was mixed with hFasRECD-Fc (8.0 μg) plus Protein G-conjugated magnetic beads (1.0 mg) or Streptavidin-conjugated magnetic beads (1.0 mg) in 0.9 ml of 50 mM Tris HCl (pH 7.5) containing 0.15 M NaCl, 1% Nonidet P40 and 0.5% sodium deoxycholate. The beads were rigorously washed twice with the same buffer (0.9 ml) and subsequently with 10 mM Tris HCl (pH 7.5) containing the same concentrations of the detergents (1.0 ml). After removing the supernatants, the bound proteins on the precipitated magnetic beads were solubilized using a non-reducing sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE) sample buffer (41 μl) composed of 125 mM Tris HCl (pH 6.8), 4.3% SDS, 30% Glycerol and 0.01% Bromophenol Blue by incubating at 368 K for 12 min. Five μl each of the resulting supernatants was used as the samples for SDS-PAGE analysis.

Cell-viability measurements

HT-29 cells were seeded at 3 x 10^3 cells/well in 150 μl of the culture medium in 96-well microplates. After allowing the cells to attach and grow for 24 h, 50 μl of the medium containing IFN-γ (0 or 100 IU/ml) and 5-FU (0, 25, 50 or 100 ng/ml) was added, and incubated for another 24 h (Pretreatment step). Then, the medium was replaced with 200 μl of a new culture medium containing either the hFasLECD conjugate (100 ng/ml) or PBS alone diluted with the same medium, and further incubated for 72 h (Treatment step). Ten μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide (MTT) solution (5 mg/ ml in PBS) was added 4 h earlier than the time of absorbance measurement. Finally, the culture medium was removed and the contents of the well were dissolved in DMSO

(200 μl each per well) to measure the absorbance at 535 nm. The cell-viability was estimated as the relative value to the average value of the PBS-alone sample. Four independent experimental data under each treatment condition were obtained for the calculation of the average value and the standard deviation.

RESULTS AND DISCUSSION

Preparation of site-specific biotin-group conjugate of hFasLECD

In Figure 1, the thiol-ene reaction used for the conjugation of B-PEG3-Mal with FLK3G1CG4-hFasLECD is schematically presented. Detailed experimental procedures for the preparation of the site-specific biotin-group conjugate of hFasLECD are as described in the Materials and Methods section. An SDS-PAGE analysis of the samples during the conjugation reaction and purification steps is presented in Figure 2a. The starting material of the NFK3G1CG4-hFasLECD partially formed a disulfide-bridge between the cysteine residues in the N-terminal tag sequence of the subunits (Lane a). This disulfide bridge was cleaved by 10 mM TCEP treatment (Lane b) and the product after the conjugation with B-PEG3-Mal migrated at a little retarded position as compared to the nonconjugated sample, showing a slight increase in the molecular weight (Lane c). The reaction mixture after the conjugation was concentrated by ultrafiltration (Lane d), and further purified by a high-performance size-exclusion chromatography (Lane e). The size-exclusion chromatography profile presented a single major peak at the expected elution position (Figure 2b), which facilitated an efficient recovery of the purified conjugate. Peak elution position of the final product (approx. MW: 63 kDa in the native trimetric state) was 19.14 min, which was much later than that of bovine serum albumin dimer (132 kDa, 16.00 min), near to that of bovine serum albumin monomer (66 kDa, 18.32 min), and considerably earlier than that of horse muscle myoglobin (17.6 kDa, 22.24 min) under the same elution conditions. The final recovery yield was 10.3 mg. Figure 3 shows the results of precipitation experiments using the magnetic beads to detect the hFasRECD and streptavidin binding activity. In both experiments, an evident band corresponding to the hFasLECD conjugate was observed in the SDS-PAGE analysis of the recovered precipitates, which showed the strong affinity of the conjugate toward both hFasRECD and streptavidin. The above results ensured the conjugation of functional biotin groups to FLK3G1CG4-hFasLECD without impairing the hFasRECD binding activity. The biotin-group conjugate of hFasLECD prepared in this study will be readily applicable to the development of

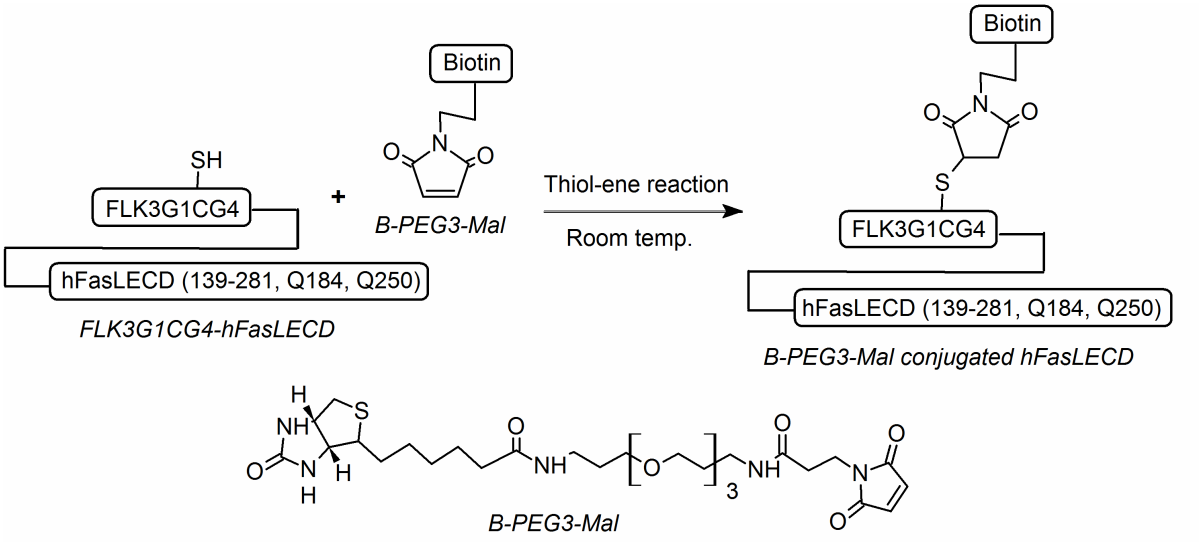

Figure 1. Reaction scheme for the conjugation of B-PEG3-Mal to FLK3G1CG4-hFasLECD. Unabbreviated names of each compound are as described in the text.

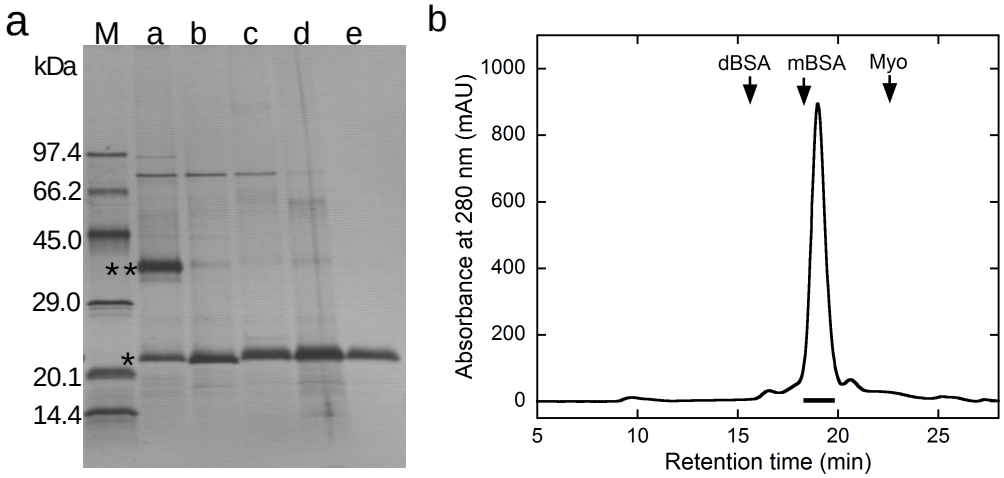

Figure 2. Preparation of B-PEG3-Mal-conjugated hFasLECD. **Panel a**, SDS-PAGE analysis of the conjugation reaction. Lanes: M, molecular-weight size markers; a, starting material; b, after TCEP reduction; c, after the reaction with B-PEG3-Mal; d, after concentration of the reaction mixture by ultrafiltration; e, final product after purification by size-exclusion chromatography. *monomer subunit of NFK3G1CG4-hFasLECD, **disulfide-bridged dimer subunits of NFK3G1CG4-hFasLECD. **Panel b**, elution profile of the concentrated reaction mixture in the high-performance size-exclusion chromatography. The fraction shown in a bar was collected as the final product. Molecular-weight markers: dBSA, bovine serum albumin dimer; mBSA, bovine serum albumin monomer; Myo, horse muscle myoglobin.

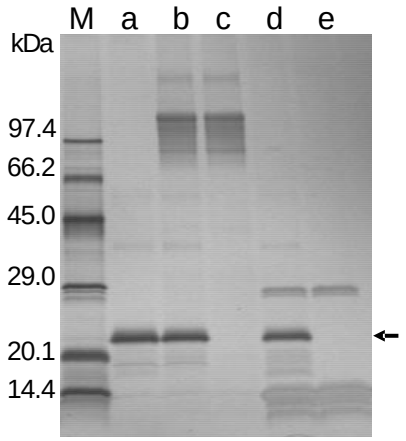

Figure 3. SDS-PAGE analysis of the binding activity of B-PEG3-Mal-conjugated hFasLECD toward hFasRECD and streptavidin. Lanes: M, molecular-weight markers; a, purified B-PEG3-Mal-conjugated hFasLECD; b and c, recovered materials in the co-immunoprecipitaion experiments using hFasRECD-Fc and Protein G conjugated magnetic beads (b, B-PEG3-Mal-conjugated hFasLECD sample; c, buffer alone sample); d and e, recovered materials in the direct binding experiments using streptavidin-conjugated magnetic beads (d, B-PEG3-Mal-conjugated hFasLECD sample; e, buffer alone sample). The arrow indicates the migration position of B-PEG3-Mal-conjugated hFasLECD.

devices for the detection and quantification of its membrane-bound and soluble counterpart receptors (hFasR, shFasR and DcR3) on the cell surfaces or in the biological fluids, since a variety of streptavidin-conjugated fluorescent dye products and analytical tips for bio-sensors are already commercially available.

Evaluation of cell-death-inducing activity

The cell-death-inducing activity of B-PEG3-Mal conjugated hFasLECD was investigated in conjunction with the combined pretreatment with IFN-γ and 5-FU. First, the effect of IFN-γ alone was examined. The viability of HT-29 cells was reduced by the treatment with the hFasLECD conjugate only in the presence of the pretreatment with IFN-γ (Figure 4a). A significant enhancement of the cell-death-inducing activity was observed in the presence of the pretreatment with IFN-γ of higher concentrations than 10 IU/ml, and the strength of the cell death induction was positively correlated with the concentration of IFN-γ. The above results were the same as that found for the Fluorescein- and Avidin-conjugated hFasLECD in the previous study [14]. Then, the effect of 5-FU (25 ng/ml) on the cell viability in the presence or

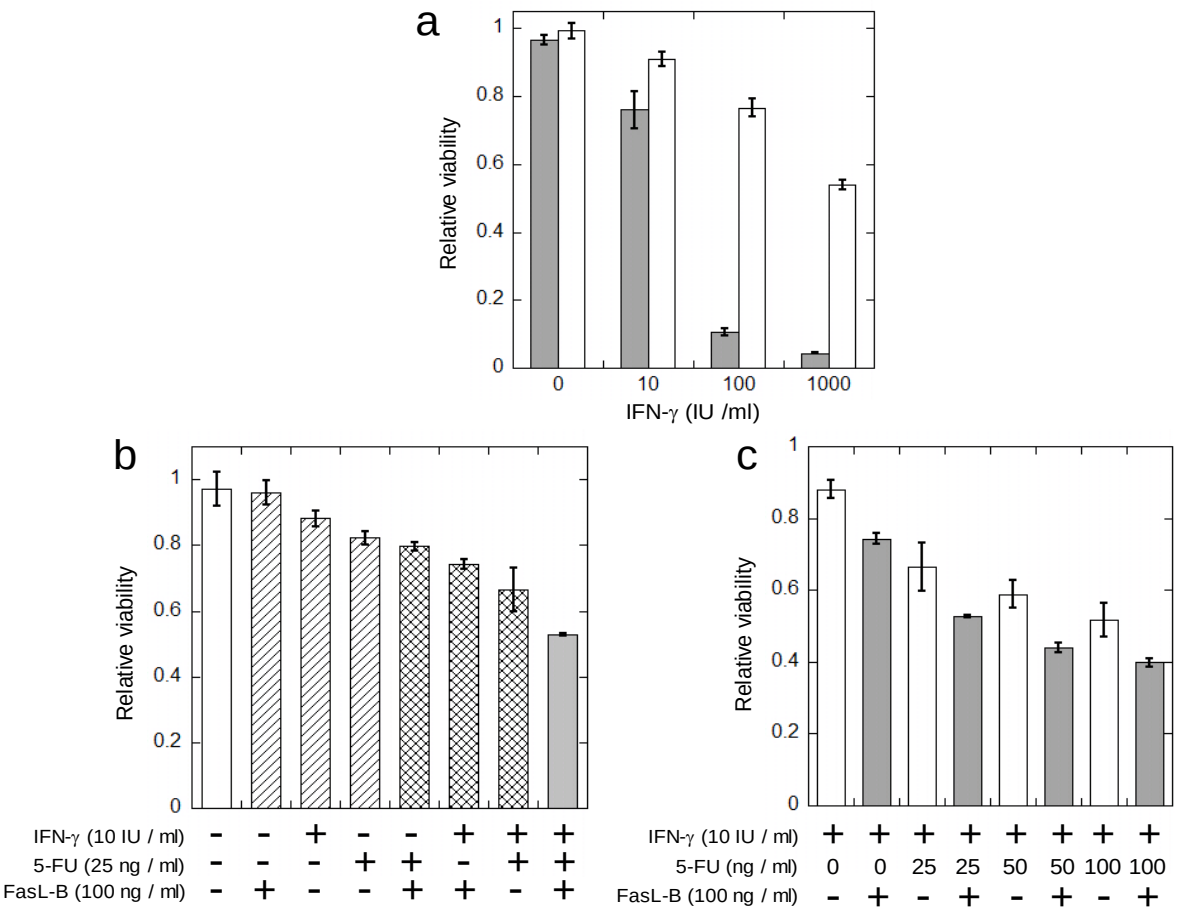

Figure 4. Cell-death-inducing activity. **Panel a**, effect of the pretreatment using IFN-γ alone. Bars: filled, in the presence of the treatment with B-PEG3-Mal-conjugated hFasLECD (100 ng/ml); open, in the absence the treatment. **Panel b**, effect of the combined pretreatment with IFN-γ (10 IU/ml) and 5-FU (25 ng/ml) in the presence or absence of the treatment with B-PEG3-Mal-conjugated hFasLECD (FasL-B, 100 ng/ml). **Panel c**, effect of coexisted 5-FU concentration in the combined pretreatment with IFN-γ (10 IU/ml) in the presence or absence of the treatment with B-PEG3-Mal-conjugated hFasLECD (FasL-B, 100 ng/ml).

absence of IFN-γ during the pretreatment period was examined. In evaluating the synergistic effect of 5-FU on the cell viability, the concentration of IFN-γ in the pretreatment step and that of the hFasLECD conjugate in the treatment step were set to 0 or 10 IU/ml and 0 or 100 ng/ml, respectively. As shown in Figure 4b, the combined pretreatment of 5-FU and IFN-γ followed by the treatment with the hFasLECD conjugate gave the strongest effect on the cell viability among all conditions of the combinations. The effect of the combined pretreatment with IFN-γ and 5-FU was greater than that of the pretreatment with IFN-γ or 5-FU alone. In support of this observation, the regrowth of 5-FU-treated HT-29 cells was reported to be prevented by the combined treatment with IFN-γ [15]. A considerable level of cell-death induction was also observed in the absence of the treatment with the hFasLECD conjugate, especially in the case of the combined pretreatment with IFN-γ and 5-FU. However, irrespective of the concentration of 5-FU (0, 25, 50 or 100 ng/ml), the degree of reduction in the cell viability was always greater in the presence of the treatment with the hFasLECD conjugate, as compared to the cases in the absence of the treatment (Figure 4c). It is known that the treatment with IFN-γ greatly increases the expression level of cell-surface hFasR in HT-29 cells [16], and the level of upregulation was further enhanced by the co-existence of 5-FU and

leucovorin *in vivo* and *in vitro* [17]. Also, the apoptosis of HT-29 cells caused by a FLAG-tagged hFasLECD was reported to be significantly sensitized with 5-FU [18]. Therefore, it was considered that IFN-γ and 5-FU showed a synergistic effect in the pretreatment preceding the treatment with the hFasLECD conjugate in this study. Moreover, it is noteworthy that 5-FU exhibited much greater cell-death-inducing activity by the combination with IFN-γ and the hFasLECD conjugate than the case of the usage of 5-FU alone (Figure 4b), since 5-FU is a clinical chemotherapeutic agent currently used for the treatment of colorectal cancers [19]. Recently, various functionalized nanoparticles have been extensively studied for their potential as delivery vehicles of protein and peptide drugs [20, 21], and methodological advances in the synthesis of nanosized substances, such as liposomes [22], gold nanoparticles [23] and polymers [24], tagged by streptavidin molecules have been reported. Once effective diseased-cell-targeting molecules attached with a biotin group become available, the conjugate prepared in this study will contribute to the development of novel cytotoxic agents for the treatment of diseases in cooperation with such substances and molecules.

CONCLUSION

A site-specific biotin-group conjugate of hFasLECD, retaining the original hFasRECD and streptavidin binding activity, was prepared using a single step thiol-ene reaction. The conjugate showed a strong cell-death-inducing activity against HT-29 cells in the presence of a synergistic pretreatment with IFN-γ and 5-FU. The findings in this study suggested that the conjugate would be applicable to the development of devices and agents, intended for the detection of counterpart receptors in biological specimens and the treatment of diseased cells, respectively.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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