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PEG-transferrin conjugated TRAIL (TNF-related apoptosis-inducing ligand) for therapeutic tumor targeting

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Abstract

Transferrin (Tf) is considered an effective tumor-targeting agent, and PEGylation effectively prolongs *in vivo* pharmacokinetics by delaying excretion *via* the renal route. The authors describe the active tumor targeting of long-acting Tf-PEG-TNF-related apoptosis-inducing ligand conjugate (Tf-PEG-TRAIL) for effective cancer therapy. Tf-PEG-TRAIL was prepared using a two-step N-terminal specific PEGylation procedure using different PEGs (Mw: 3.4, 5, 10 kDa). Eventually, only 10 kDa PEG was linked to Tf and TRAIL because TRAIL (66 kDa) and Tf (81 kDa) were too large to link to 3.4 and 5 kDa PEG. The final conjugate Tf-PEG_{10K}-TRAIL was successfully purified and characterized by SDS-PAGE, western blotting. To determine the specific binding of Tf-PEG_{10K}-TRAIL to Tf receptor, competitive receptor binding assays were performed on K 562 cells. The results obtained demonstrate that the affinity of Tf-PEG_{10K}-TRAIL for Tf receptor is similar to that of native Tf. In contrast, PEG_{10K}-TRAIL demonstrated no specificity. Biodistribution patterns and antitumor effects were investigated in C57BL6 mice bearing B16F10 murine melanomas and BALB/c athymic mice bearing HCT116. Tumor accumulation of Tf-PEG_{10K}-TRAIL was 5.2 fold higher (at 2 h) than TRAIL, because Tf-PEG_{10K}-TRAIL has both passive and active tumor targeting ability. Furthermore, the suppression of tumors by Tf-PEG_{10K}-TRAIL was 3.6 and 1.5 fold those of TRAIL and PEG_{10K}-TRAIL, respectively. These results suggest that Tf-PEG_{10K}-TRAIL is a superior pharmacokinetic conjugate that potently targets tumors and that it should be viewed as a potential cancer therapy.

Keywords

TRAIL; Transferrin; PEGylation; Passive tumor targeting; Active tumor targeting

1. Introduction

TNF-related apoptosis-inducing ligand (TRAIL) is a recently identified member of the TNF family, and, like FasL, is a type II membrane protein that can induce apoptosis in a variety of cancer cell types [1–3], but not the apoptosis most normal cells, and therefore, it is viewed as a promising anti-cancer cytokine [4,5]. However, TRAIL has critical

shortcomings in terms of its clinical applications, for example, it is rapidly inactivated, has low solubility and stability, is rapidly cleared by the kidneys, is hepatotoxicity, and possibly can induce immune reactions when delivered systemically [6–8].

In order to solve these TRAIL problems, we previously produced an N-terminal specific PEGylated TRAIL (PEG–TRAIL) [5,9,10], which preserved TRAIL biological activity and profoundly improved its physicochemical stability. Moreover, PEG–TRAIL exhibited a much longer pharmacokinetic profile and better antitumor activity than native TRAIL with less hepatotoxicity. PEGylation is known as one of the most useful drug delivery systems for optimizing peptide or protein therapies and for enhancing therapeutic potency and reducing undesirable effects. PEGylation of proteins increases molecular size and steric hindrance, both of which are derived from the attachment of polyethylene glycol (PEG) to bioactive proteins, which extends plasma half-lives and *in vivo* stabilities. PEGylation also improves physical and thermal stabilities, increases solubility, and reduces immunogenicity, antigenicity, and toxicity [11–14].

Although the conjugation of PEG to a protein drug can have a therapeutically beneficial effect, this phenomenon may be due to passive, nonspecific process based on enhanced permeability and retention. In fact, many PEG–protein drugs lack tumor selectivity and do not have useful therapeutic indices.

Transferrins (Tf) are potentially of use in target-oriented delivery systems, and are biodegradable, nontoxic, and nonimmunogenic [15,16]. Furthermore, they can deliver a wide range of drugs, including anticancer drugs, peptides, proteins, and even genes, into primarily proliferating malignant cells due to high expressions of Tf receptors on tumor cell surfaces [17–22].

In order to enhance targeting efficiencies, many attempts have been made to develop site-specific, target-oriented delivery systems that are taken up by cell surface receptors. Several studies have demonstrated that the Tf uptake pathway is highly effective both *in vitro* and *in vivo*. In particular, Tf–doxorubicin conjugate has been reported to be much more cytotoxic than unconjugated doxorubicin to a number of tumor cells *in vivo* [23,24]. These studies, however, although chemical coupling is easily conducted, many non-specific polymeric products are likely to be formed, and these are poorly defined with respect to the chemical link between drugs and carriers.

In the present study, we designed a TRAIL conjugate with both passive and active tumor targeting ability. A two-step N-terminal specific PEGylation method was performed in acidic aqueous conditions by using di-aldehyde PEG of different sizes (Mw: 3.4, 5, 10 kDa) was used to prepare Tf–PEG–TRAIL (Fig. 1). The products obtained were characterized by SDS-PAGE, western blotting. Furthermore, we examined the *in vitro* bioactivity of Tf–PEG_{10K}–TRAIL, its receptor binding, and its *in vivo* biodistribution. Finally, we examined its tumoricidal activities in HCT 116 tumor bearing BALB/c athymic mice.

2. Materials and methods

2.1. Materials and animals

TRAIL was purified as previously described [5,9,10]. Di-aldehyde PEG (3.4, 5, and 10 kDa) was purchased from Laysan Bio (Arab, AL, USA). Human transferrin (iron-free, Tf) of Mw 76–81 kDa was purchased from Sigma (St. Louis, MO). HiPrep 16/60 Sephacryl S-200 prepacked gel filtration columns, which were used in protein purification and separation, were obtained from Amersham Biosciences (Stockholm). All other reagents used were of the highest quality commercially available. The human colon cancer cell (HCT116) was

purchased from the Korean Cell Line Bank (Seoul). Animals were obtained from the Hanlim Experimental Animal Laboratory (Seoul), and cared for according to the National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 80-23, revised 1996). Animals were housed in groups of 6–8 under a 12-h light/dark cycle (lights on at 6 a.m.), allowed food and water *ad libitum*, and acclimatized for 2 weeks. This study was approved by the Ethical Committee on Animal Experimentation at Sungkyunkwan University.

2.2. Preparation and characterization of PEG conjugated Tf (PEG–Tf)

Site-specific PEGylation of the N-terminus of Tf was conducted with dialdehyde PEG (Mw 3.4, 5, 10 kDa) in the presence of 20 mM sodium cyanoborohydride (NaCNBH₃) in 50 mM acetate buffer at pH 5.0. Reaction conditions, such as, PEG/Tf molar ratio and reaction time, were optimized by size exclusion chromatography. After PEGylation, reaction mixtures were separated by gel-filtration chromatography using a Sephacryl S-200 column, equilibrated with 50 mM acetate buffer (pH 5.0). PEG–Tf containing fractions were then collected and concentrated using an Amicon Ultra-4 centrifugal filter (cutoff 30 kDa, Millipore, Bedford, MA). 8% SDS-PAGE and MALDI-TOF mass spectrometry was performed using equal amounts of protein using the Mini-Protein II system (BioRad, Hercules, CA, USA) to confirm Tf PEGylation. Tf and PEG–Tf were determined using BCA Protein Assay Reagent (Pierce). Molecular weights of Tf and PEG–Tf were estimated relative to molecular weight markers. MALDI-TOF MS was performed using a Voyager-RP Biospectrometry Workstation (PerSeptive Biosystems, MA, USA).

2.3. Preparation and characterization of Tf–PEG–TRAIL conjugates

To attach TRAIL to PEG–Tf we used the aldehyde–PEG–Tf (Mw 3.4, 5, 10 kDa) adducts described above. The synthesis was conducted as follows. Aldehyde–PEG–Tf and TRAIL were reacted (molar ratio 5:1) in the presence of 20 mM sodium cyanoborohydride (NaCNBH₃) in 50 mM acetate buffer at pH 5.0. Finally, the TRAIL coupled PEG–Tf mixture was separated by gel-filtration chromatography using a Sephacryl S-200 column equilibrated with 50 mM acetate buffer (pH 5.0). In order to confirm the formation of Tf–PEG–TRAIL, we used SDS-PAGE, western blotting (using Anti-Transferrin antibody, Abcam, USA).

2.4. Radioiodine (¹²⁵I) labeling of Tf–PEG_{10K}–TRAIL

Radioiodinated samples of TRAIL, PEG_{10K}–TRAIL, and Tf–PEG_{10K}–TRAIL were prepared using a modification of the IODO-GEN method previously described [4,9]. Briefly, 100 µL of IODO-GEN (Pierce, Rockford, IL) solution in methylene chloride (1.0 mg/mL) was dispensed into a fresh tube, and evaporated under a nitrogen stream. Aliquots (100 µL) of samples (0.5 mg/mL) and 50 µCi of Na¹²⁵I (Perkin-Elmer, Boston, MA) diluted to 100 mM with PBS (pH 7.4) were then added. The reaction was allowed to proceed for 2 min, and supernatants were then loaded onto a Superose™ 12 HR 10/30 column connected to a flow-through radioisotope detector (Ramona 200, Raytest, Straubenhardt, Germany). ¹²⁵I-labeled protein fractions were collected, and stored at 4 °C until required.

2.5. In vitro cytotoxicity and Tf receptor binding of Tf–PEG_{10K}–TRAIL

The cytotoxicities of TRAIL, PEG_{10K}–TRAIL and Tf–PEG_{10K}–TRAIL were investigated using HCT116, which were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) containing 1% penicillin/streptomycin (Gibco). For cytotoxicity assays, cells were seeded in 96-well plates at 1×10⁴ cells/well and pre-incubated for 24 h. Media were then replaced with fresh serum-free DMEM and pre-determined amounts of TRAIL, PEG_{10K}–TRAIL, or Tf–

PEG_{10K}-TRAIL were added to final concentrations of 0–1000 ng/mL (based on the TRAIL protein). The *in vitro* cytotoxicities of TRAIL, PEG_{10K}-TRAIL, and Tf-PEG_{10K}-TRAIL were determined using an MTT assay after incubation for 24 h.

To assess the targeting potential of Tf-PEG_{10K}-TRAIL, iron incorporation into Tf, as well as into Tf-PEG_{10K}-TRAIL, was carried out by addition of 1.25 μ L of 10 mM iron (III) citrate buffer (containing 200 mM citrate and adjusted to pH 7.8 with sodium bicarbonate) per milligram of Tf. To remove the excess iron (III), the Tf-Fe₂ or Tf-PEG_{10K}-TRAIL-Fe₂ was treated with HBS in the centrifugal filter device (cutoff 10 kDa, Millipore) and centrifuged twice at 7000 $\times$ g at 4 °C for 10 min. The receptor binding of Tf-PEG_{10K}-TRAIL was determined using a previously reported method [25]. Briefly, to assess the specific binding of Tf-PEG_{10K}-TRAIL to Tf receptor, competitive binding assays were performed. K562 cells (1.5×10^6 /mL in BSA/RPMI) were incubated at 4 °C for 2 h while shaking, with the addition of 3.1×10^{-9} M ¹²⁵I-Tf-Fe₂ and different concentrations (from 10^{-11} to 10^{-6} M) of unlabeled ligands (Tf-Fe₂, Tf-PEG_{10K}-TRAIL-Fe₂, or PEG_{10K}-TRAIL). After centrifuging and washing, ¹²⁵I radioactivity was measured to obtain the specifically bound tracer activity (*B*) at each competitor concentration and the maximum binding, carried out without unlabeled competitor (*B*₀). The corresponding competition curves were obtained by plotting *B/B*₀ versus the log concentration of each competitor.

2.6. In vivo pharmacokinetics and biodistribution of Tf-PEG_{10K}-TRAIL

Pharmacokinetic profiles and biodistribution of TRAIL analogs were investigated in C57BL6 mice and Tf-positive B16F10 tumor-bearing mice using optical imaging and γ -counting, respectively. To evaluate *in vivo* pharmacokinetics, TRAIL or Tf-PEG_{10K}-TRAIL was labeled with Cy5.5 dye. Labeling was performed by mixing sample solutions (100 μ g/mL in PBS, pH 7.4) with NHS-activated Cy5.5 and allowed to react overnight at 4 °C. Reaction mixtures were concentrated by ultrafiltration and finally purified by gel filtration chromatography. To avoid strong background signals from the abdomen region, Cy5.5-labeled TRAIL or Tf-PEG_{10K}-TRAIL were injected subcutaneously in C57BL6 mice. At predetermined times, mice were visualized using Image Station 4000 MM (Kodak) at an emission wavelength of 700 nm.

The biodistribution of Tf-PEG_{10K}-TRAIL was determined in Tf-positive B16F10 tumor-bearing mice as described previously [4,9]. Because of the immunodeficiency, we prepared a tumor model using B16F10 murine melanoma instead of human cancer HCT116 cells. First, C57BL6 mice (*n*=4) bearing B16F10 murine melanoma cells (Korean Cell Line Bank (KCLB), Seoul, Korea) (150 mm^3) were prepared. Radioiodinated TRAIL, PEG_{10K}-TRAIL or Tf-PEG_{10K}-TRAIL was administered by i.p. injection (approximately 10 μ g; 13,000,000 cpm). Mice were sacrificed at predetermined times. Liver, spleen, lung, kidney and tumor were quickly excised and rinsed three times with saline. The radioactivities of whole organs were determined by γ -counting (Cobra, Packard Instruments Co., Groningen, The Netherlands).

2.7. Anti-tumor Tf-PEG_{10K}-TRAIL therapy

The antitumor effects of Tf-PEG_{10K}-TRAIL were investigated in HCT116 tumor bearing mice (*n*=6). Briefly, freshly harvested HCT116 cells (3×10^6 cells/mouse) were inoculated s.c. into BALB/c athymic mice, and 5 days later mice were treated with TRAIL, PEG_{10K}-TRAIL, or Tf-PEG_{10K}-TRAIL (1 mg of TRAIL/mouse, i.p.) every 5 days. Tumor volumes were monitored for 24 days after tumor cell administration. Tumor volumes were calculated using longitudinal (*L*) and transverse (*W*) diameters using $V = (L \times W^2)/2$, and tumor growth inhibition (TGI) percent values were calculated using the formula $\text{TGI \%} = (1 - \text{TV}_{\text{sample}} / \text{TV}_{\text{control}}) \times 100$, where TV is tumor volume.

In vivo tumor cell apoptosis was also investigated in HCT116 tumor-bearing BALB/c athymic mice. Briefly, at 24 days after HCT116 administration, tumor tissues were recovered from euthanized animals. Sections (5 μ m) were then cut from 10% neutral buffered, formalin-fixed, paraffin-embedded tissue blocks. Apoptotic cell death in tumor tissues was visualized by performing TdT-mediated dUTP nick end labeling (TUNEL) assays using a commercial kit (*In situ* cell death detection kit, Roche, Applied Science, Mannheim).

2.8. Statistical analysis

Data are expressed as means $\pm$ SDs, and the Student's *t*-test was used throughout.

3. Results

3.1. Characterization of PEG_{10K}-Tf

Tf was modified by N-terminal specific PEGylation with di-butylaldehyde PEG of 3.4, 5, or 10K. After reaction, conjugates were separated from reaction mixtures by size exclusion chromatography. The optimal PEG-Tf ratios at 3.4, 5, and 10K were 1:5, 1:5 and 1:7.5, respectively (Figs. 2A, S1). After purification, the PEG-Tf analogs were characterized by SDS-PAGE (8%) and MALDI-TOF MS (Figs. 2B, C, S1). To confirm their molecular weights, they were subjected to MALDI-TOF MS analysis. The *M_w* values of the PEG-Tf analogs were 81,590.1, 84,749.1, 87,000.6, 91,446.2 Da, respectively.

3.2. Characterization of Tf-PEG_{10K}-TRAIL

Tf-PEG-TRAIL comprises PEG_{10K} coupled to both Tf and trimeric TRAIL as shown in Fig. 3. Size exclusion chromatogram clearly demonstrated that Tf-PEG-TRAIL comprises both Tf-PEG and trimeric TRAIL (Fig. S2). After purification of Tf-PEG-TRAIL, the exact structure was further confirmed by SDS-PAGE and western blot assays as we described previously [3,9]. As described in Fig. 3A and B, due to denaturation during Tf-PEG_{10K}-TRAIL preparation, TRAIL produced only one band at 22 kDa (monomer) by SDS-PAGE. However, Tf-PEG_{10K}-TRAIL produced two distinct bands corresponding to TRAIL monomer and Tf-PEG_{10K}-monoTRAIL (around 112 kDa). In the case of western blotting, Tf antibody detected Tf and Tf-PEG_{10K}-monoTRAIL. The analysis of SDS-PAGE and western blot pattern revealed that PEG_{10K}-Tf was successfully conjugated to TRAIL with high percent of yield (31.8 $\pm$ 3.7%). But PEG_{3.4K}-Tf and PEG_{5K}-Tf were not successfully conjugated to TRAIL because it exhibited a low percent of yield (5.2 $\pm$ 0.5, 8.9 $\pm$ 2.1%) (Fig. S2).

3.3. In vitro cytotoxicity and Tf receptor binding by Tf-PEG_{10K}-TRAIL

The cytotoxicity of Tf-PEG_{10K}-TRAIL was assessed and compared to those of PEG_{10K}-TRAIL and TRAIL (Fig. 4A). The inhibitory concentration (IC₅₀) of Tf-PEG_{10K}-TRAIL for HCT116 growth was 274.6 ng/mL, which was lower to that of both PEG_{10K}-TRAIL (69.8 ng/mL) and free TRAIL (11.2 ng/mL).

To determine the specific binding of Tf-PEG_{10K}-TRAIL to Tf receptor, competitive receptor binding assays were performed on K 562 cells (ATCC, Rockville, MD, USA) with varying amounts of unlabeled ligands (Tf-Fe₂, Tf-PEG_{10K}-TRAIL-Fe₂, PEG_{10K}-TRAIL) and constant amount of ¹²⁵I-Tf-Fe₂. The binding of Tf and its conjugates to Tf receptors showed dose-dependent responses at the concentrations administered to K 562 cell (Fig. 4B). Furthermore, the receptor binding affinities (estimated using EC₅₀ values) of Tf-PEG_{10K}-TRAIL was not significantly different from that of unconjugated Tf. In contrast to the binding of Tf-PEG_{10K}-TRAIL, no significant binding was demonstrated in keeping with the observations on K 562 cells, even at highest concentrations of PEG_{10K}-TRAIL.

3.4. In vivo pharmacokinetics and biodistribution of Tf-PEG_{10K}-TRAIL

Fig. 5A describes time-dependent NIR fluorescence imaging of C57BL6 mice followed by s.c. injection of Cy5.5-labeled TRAIL and Tf-PEG_{10K}-TRAIL to the back of the neck region. Tf-PEG_{10K}-TRAIL continued to fluoresce for 72 h whereas native TRAIL was rapidly eliminated from the body. Once we confirmed the prolonged circulation of Tf-PEG_{10K}-TRAIL, radioiodinated TRAIL analogs were i.p. injected in C57BL6 tumor-bearing mice. As demonstrated in Fig. 5B, it was found that accumulated concentrations of Tf-PEG_{10K}-TRAIL in tumor tissues were significantly higher compared to that of TRAIL or PEG_{10K}-TRAIL, irrespective of time of measurement. At 2 h after administration, TRAIL was gradually eliminated from tumors, whereas the tumor accumulation of Tf-PEG_{10K}-TRAIL was markedly increased at 2 h after administration by 5.2-fold over TRAIL. Furthermore, at 12 h after dosing, the tumor accumulation of Tf-PEG_{10K}-TRAIL remained at a high level. In addition, the accumulation of TRAILs in kidney was rapidly eliminated in the biodistribution study compared to NIR imaging because of the rapid absorption of i.p. injected TRAILs into the body in contrast to the slow absorption of s.c. injected TRAIL.

3.5. Anti-tumor therapy and Tf-PEG_{10K}-TRAIL

The antitumor effects of free TRAIL, PEG_{10K}-TRAIL, and Tf-PEG_{10K}-TRAIL were investigated in HCT116 tumor bearing mice. Five days after inoculation, mice were treated with TRAIL, PEG_{10K}-TRAIL, or Tf-PEG_{10K}-TRAIL (1 mg of TRAIL/mouse, i.p.) every 5 days and tumor volumes were monitored. PEG_{10K}-TRAIL and Tf-PEG_{10K}-TRAIL were found to suppress mean tumor growth, with mean TGI values (24 days) of 46.2 and 67.1%, respectively (Fig. 6A). In particular, Tf-PEG_{10K}-TRAIL retained antitumor activity at five days after a single administration. Furthermore, whereas TUNEL assays of tumor tissues from TRAIL and PEG_{10K}-TRAIL treated mice showed some signs of cell death, TUNEL assays of tumor tissues from Tf-PEG_{10K}-TRAIL treated mice showed obvious tumor cell apoptosis (Fig. 6B).

4. Discussion

Tf has received considerable interest in terms of receptor-mediated delivery due to its site-specific targeting of ligand-specific sites and its biodegradable, nontoxic, and nonimmunogenic character [22]. PEG conjugation is one of the most successful of these strategies, and several PEGylated proteins are now used clinically. PEG has been used widely to prolong the activities of rapidly eliminated drugs, to impart enhanced permeability and retention (EPR), to increase passive targeting by anticancer drugs, and to act as a spacer in numerous delivery systems [13,14].

Previously, we synthesized an N-terminal specific PEGylated TRAIL (PEG_{5K}-TRAIL, PEG Mw: 5000) with enhanced physicochemical, pharmacokinetic, and anti-tumor characteristics [3,5,10]. PEG_{5K}-TRAIL showed improved physiological stability and had a better pharmacokinetic profile than TRAIL, and its activity loss *versus* TRAIL was minimal. Nevertheless, although our PEG_{5K}-TRAIL showed better anti-tumor activity than TRAIL, PEGylation was passive targeting, only based on the enhanced permeability and retention (EPR) effect. So, in the present study, we suggest a Tf-TRAIL conjugate *via* 10 kDa di-aldehyde PEG linkage for effective tumor targeting and therapy. The combination of active targeting, based on the use of Tf, and passive targeting, based on the PEGylation due to EPR effect, may provide a tumor-selective targeting strategy.

Tf-PEG-TRAIL was successfully prepared using di-aldehyde PEG by two-step N-terminal specific PEGylation. In the first step, Tf was PEGylated with di-aldehyde PEG (3.4, 5, or 10 kDa). After purification, PEG-Tf was successfully characterized by SDS-PAGE and

MALDI-TOF MS. In the second step, Tf-PEG-TRAIL conjugate was finally prepared with aldehyde group of PEG-Tf. PEG_{10K} was found to successfully couple Tf to TRAIL, but PEG 3.4 and 5 kDa were unsuitable, presumably because TRAIL (66 kDa) and Tf (81 kDa) were too large. Furthermore, PEG 3.4 and 5 kDa showed lower Tf-PEG-TRAIL yields than PEG 10 kDa. For these reasons, we chose PEG 10 kDa as a linker (Fig. S2).

After preparing ¹²⁵I-PEG_{10K}-TRAIL and ¹²⁵I-Tf-PEG_{10K}-TRAIL-Fe₂, we examined the receptor binding parameters of the conjugates to tumor cell Tf receptors. It was found that the Tf receptor binding ability of Tf-PEG_{10K}-TRAIL-Fe₂ conjugate was slightly less than that of Tf-Fe₂. Furthermore, the molecular size increase caused by modification of Tf with PEG_{10K}-TRAIL only slightly hindered Tf receptor binding, and Tf-PEG_{10K}-TRAIL-Fe₂/Tf receptor and Tf-Fe₂/Tf receptor complexes exhibited ED₅₀ values of the same order of magnitude.

Increasing molecular size deprives TRAIL of its inherent biological activity *in vitro*. Furthermore, the cytotoxicity of Tf-PEG_{10K}-TRAIL was reduced by PEG and Tf, due to the disruption of TRAIL binding to death receptors. Despite a reduction in cytotoxicity due to PEG and Tf, it retained a meaningful cytotoxic effect (4.1% of the efficacy of native TRAIL for Tf-PEG_{10K}-TRAIL).

However, unlike the biological activity loss observed *in vitro*, PEGylation greatly improved the pharmacokinetic profile of TRAIL *in vivo* by prolonging its survival time (Fig. 5A). Several authors have reported that TRAIL is rapidly eliminated and has a short half-life (~30 min), mainly because of its rapid renal clearance [6–8]. However, in the present study, the increased molecular weight and the molecular shielding afforded by PEG_{10K} linker and Tf dramatically enhanced its pharmacokinetic profile.

The effective delivery of TRAIL to tumor tissues requires passive and active tumor targeting. In the present study, it was found that the therapeutic potent of TRAIL was improved by PEG and Tf conjugation. Because of the rapid clearance of TRAIL from the circulation, far less free TRAIL was taken up by tumor tissues than PEG_{10K}-TRAIL or Tf-PEG_{10K}-TRAIL. Furthermore, the tumor accumulation of Tf-PEG_{10K}-TRAIL was much greater than that of PEG_{10K}-TRAIL, because Tf-PEG_{10K}-TRAIL has both passive and active tumor targeting potency (Fig. 7).

To determine whether Tf-PEG_{10K}-TRAIL is potentially useful as a cancer treatment, we examined its anti-tumor activity in HCT116 colon tumor-bearing BALB/c athymic mice. The results obtained showed that PEG_{10K}-TRAIL and Tf-PEG_{10K}-TRAIL produced the desired biological effects. Furthermore, the antitumor activity of PEG_{10K}-TRAIL and Tf-PEG_{10K}-TRAIL matched their observed pharmacokinetic durations. In particular, Tf-PEG_{10K}-TRAIL was found to have the greatest antitumor effect, which we attributed to its pharmacokinetic distribution and active tumor targeting. In the present study, Tf-PEG_{10K}-TRAIL was found to be the strongest antitumor agent candidate.

5. Conclusions

Tf is a candidate tumor targeting agent due to its Tf receptor specificity, and in the present study, PEGylation effectively prolonged its *in vivo*. The Tf-PEG_{10K}-TRAIL developed during this study has passive and active tumor targeting ability, and was prepared by N-terminal specific PEGylation method. Tf-PEG_{10K}-TRAIL showed enhanced Tf receptor specificity and *in vivo* tumor targeting ability. Furthermore, these improved pharmaceutical characteristics resulted in improved therapeutic effects in an animal colon tumor model. We firmly believe that Tf-PEG_{10K}-TRAIL has therapeutic potential as a cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2012.07.021>.

Fig. 1.
Schematic representation of preparation of Tf-PEG-TRAIL using a two-step N-terminal specific PEGylation.

Fig. 2. Characterization of PEG_{10K}-Tf (A) SEC of 10 kDa PEGylated Tf with various Tf: PEG ratio. (B) Analysis of PEG_{10K}-Tf using SDS-PAGE (8%). (B) Analysis of PEG_{10K}-Tf using MALDI-TOF-MS.

Fig. 3. Characterization of Tf-PEG_{10K}-TRAIL. (A) Schematic representation of denatured condition of Tf-PEG_{10K}-TRAIL. (B) Characterization of Tf-PEG_{10K}-TRAIL using SDS-PAGE and western blotting (anti-Tf antibody).

Fig. 4. *In vitro* biological activity of Tf-PEG_{10K}-TRAIL. (A) Cytotoxic effects of TRAIL, PEG_{10K}-TRAIL and Tf-PEG_{10K}-TRAIL on HCT116 tumor cells. (B) Competitive receptor binding assays of Tf-Fe₂, PEG_{10K}-TRAIL and Tf-PEG_{10K}-TRAIL-Fe₂ on K562 cells.

Fig. 5.

In vivo pharmacokinetics and biodistribution of Tf-PEG_{10K}-TRAIL. (A) Imaging of Cy5.5-labeled TRAIL and Tf-PEG_{10K}-TRAIL after injected s.c. into C57BL6 mice (at an emission wavelength of 700 nm). (B) Biodistribution (liver, spleen, lung, kidney and tumor) of radioiodinated TRAIL, PEG_{10K}-TRAIL and Tf-PEG_{10K}-TRAIL after injected i.p. into C57BL6 mice bearing B16F10 murine melanomas cells (n=4; *p<0.05 versus TRAIL).

Fig. 6.

In vivo antitumor activity of Tf-PEG_{10K}-TRAIL. (A) Tumor growth suppressions by TRAIL, PEG_{10K}-TRAIL and Tf-PEG_{10K}-TRAIL (drugs were injected every five days at 1 mg of TRAIL/mouse, i.p.) (n=6; *p < 0.05 versus control). (B) Histological observations of control, TRAIL, PEG_{10K}-TRAIL and Tf-PEG_{10K}-TRAIL after TUNEL staining of apoptotic cell death in tumor (nuclei are stained blue and apoptotic cells green) (×400).

Fig. 7.
Passive and active tumor targeting action scheme of Tf-PEG_{10K}-TRAIL.