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Stability and bioactivity of nanocomplex of TNF-related apoptosis-inducing ligand

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ABSTRACT

The tumor necrosis factor-related apoptosis inducing ligand (TRAIL) has gained much attention due to its potent therapeutic effect for cancer and rheumatoid arthritis. In this study, we attempted to develop the injectable formulations which can stabilize TRAIL and thus show prolonged blood circulation in vivo. The positively charged TRAIL was mixed with hyaluronic acid (HA), resulting in the formation of nanocomplexes. The zeta-potentials of nanocomplexes and their mean diameters were significantly dependent on the feed ratio of HA to TRAIL. The increase in the feed ratio of HA reduced the particle size and decreased the value of the zeta-potential. The bioactivity of TRAIL in the complexes was comparable to that of native TRAIL, indicating that the complex formation did not affect the activity of TRAIL. Furthermore, the stability of TRAIL in the complexes was retained for 6 days, during which the bioactivity of native TRAIL disappeared. When native TRAIL was subcutaneously injected into the rats, its plasma concentration was not detectable after 12 h. In contrast, for HA/TRAIL nanocomplexes in 1% HA solution, substantial amount of TRAIL was circulated in blood for up to 5 days. These results imply that HA-based formulations of TRAIL hold the potential as the therapeutics.

1. Introduction

The tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), expressed as a type II transmembrane protein, has received much attention because of its potential as a therapeutic agent (Fesik, 2005; Bremer et al., 2006; Cretney et al., 2007). TRAIL has shown particular promise as an anti-cancer agent, capable of selectively inducing apoptosis through death receptor-mediated signaling pathways in cancer cells without significant cytotoxicity to normal cells (Walczak et al., 1999; Lawrence et al., 2001; Ray and Almasan, 2003). This selective and potent activity of TRAIL is due to its binding affinity to the death receptors that are over-expressed on the cancer cells and induce apoptosis. Although TRAIL is a membrane-bound ligand, its truncated version that contains the extracellular domain is water-soluble and exhibits potent anti-cancer activity both in vitro and in vivo (Ashkenazi et al., 1999). Therefore, recombinant TRAIL (rTRAIL) and its agonistic antibodies have been developed to target the death receptors, and a few of them are currently being evaluated as the anti-cancer drug in Phases I and II clinical trials (Fesik, 2005; Schaefer et al., 2007). The half-life of TRAIL, however, is reported to be less than 30 min, which may require frequent administration for preserving the therapeutic level in blood (Kelley et al., 2001; Xiang et al., 2004).

The clinical use of protein drugs often suffered from their short half-lives and susceptibility to proteolytic enzymes in biological fluids. In an attempt to surmount these problems, much effort has been made to develop effective delivery systems that can improve the stability of protein drugs and prolong their therapeutic effects. In particular, biodegradable microspheres using poly(lactic-*co*-glycolic acid) (PLGA) have been most widely used for the sustained protein delivery systems (Bartus et al., 1998; Wei et al., 2004; Tamber et al., 2005). However, these formulations have encountered some limitations on practical applications: (1) protein can be readily denatured by an acidic local environment, associated with degradation of PLGA microspheres; (2) current methods of preparing microspheres often involve exposure

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of protein drugs to harsh environments such as organic/aqueous interface, high temperature, and physical stresses; and (3) acidic exudates by degradation of PLGA may cause inflammation (Johnson et al., 1996; Putney and Burke, 1998; Fu et al., 2000). In recent years, a lot of approaches have provided the promising candidates for the protein delivery system. For example, the incorporation of basic exipients (e.g., $Mg(OH)_2$ and $MgCO_3$) into the PLGA microsphere could prevent a pH drop during degradation of microspheres, thus improving protein stability (Zhu et al., 2000). The solvent exchange method, implemented by the ultrasonic atomizer system, allowed producing reservoir-type microcapsules without protein aggregation and loss of its biological activity (Yeo and Park, 2004; Park et al., 2006). The complex formation of the protein drugs with the oppositely charged polymer has shown to improve protein stability in the microsphere (Lee et al., 2007b).

In this study, nano-sized complexes were prepared by mixing TRAIL with the negatively charged polysaccharide. Hyaluronic acid (HA), composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, was chosen for ionic complexation of TRAIL because HA have unique and excellent physicochemical properties for drug delivery such as biocompatibility, biodegradability, and nonimmunogenicity (Fraser et al., 1997; Hahn et al., 2004; Liao et al., 2005). The effect of the complex formation between HA and TRAIL on protein stability and biological activities were determined. Furthermore, the injectable HA-based TRAIL formulation was subcutaneously injected into the rats to observe the pharmacokinetics of TRAIL.

2. Materials and methods

2.1. Materials

LZ-h-TRAIL was produced as previously described (Youn et al., 2007). Sodium hyaluronate with a molecular weight of 2340 kDa was supplied from Pacificpharma Corporation (Seoul, Korea). Human TRAIL ELISA kit was purchased from BioSource International Inc. (Camarillo, CA, USA). All other chemicals used in this study were analytical grades and were used without further purification.

2.2. Preparation and characterization of HA/TRAIL formulations

HA/TRAIL formulation was prepared by making a complex of TRAIL with HA. TRAIL and HA was dissolved in 20 mM sodium acetate buffer containing 100 mM NaCl, pH 5.0. The formulation was prepared by the addition of a predetermined amount of TRAIL to the HA solution, through which the feed ratio (FR) of HA to TRAIL were adjusted in the range of 0.1–10.

The mean diameter of each HA/TRAIL complex was measured using a dynamic light scattering (DLS, Brookhaven Instrument Co., New York, USA) at a wavelength of 633 nm with a 90° detection angle. The DLS measurement was performed at 4 °C, and a final concentration of TRAIL was 100 μg/ml.

The zeta-potentials of HA/TRAIL complexes were evaluated using a photon correlation spectroscopy (Malvern Zetasizer, Malvern Instruments, UK). The measurement was performed at 4 °C, and the data reported were the mean value of at least three separate samples for each complex.

For the structural evaluation of the HA/TRAIL complexes, circular dichroism (CD) measurements were carried out using a Jasco J-720 Spectropolarimeter (Tokyo, Japan), and three to five scans for far-UV region (200–250 nm) were averaged to obtain the final spectra. Data (ellipticity in mdeg) were transformed to mean residue ellipticity (θ_m) using the expression, $\theta_m = (\theta \times M)/(C \times l)$, where θ is the observed ellipticity (mdeg), M is the mean residue molecular weight (g/mol), C is the protein concentration (g/ml), and l is the optical path length (cm).

2.3. Biological activity and stability

The biological activity of TRAIL formulated in HA/TRAIL nanocomplexes was evaluated by determination of cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. HeLa cells (ATCC, Manassas, VA) were cultured at 37 °C in a humidified atmosphere of 5% CO in culture flask containing Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 μg/ml streptomycin, and 50 U/ml penicillin. Cells were then seeded on 96-well flat-bottomed tissue-culture plates at a density of 1×10^4 cells per well and incubated for 24 h. The medium of each well was replaced with fresh medium (1% FBS) containing various concentrations of HA/TRAIL nanocomplexes. After 24 h, the medium was aspirated, and the cells were washed twice with phosphate-buffered saline (PBS). Next, 100 μl of fresh culture medium was added to each well, followed by addition of 20 μl MTT solution (2.5 mg/ml in PBS). The cells were then incubated for 4 h at 37 °C, after which the medium was carefully aspirated. After 200 μl of DMSO was added to each well, the absorbance at 570 nm was measured using a FL600 microplate reader (Bio-Tek Inc., Winooski, VT). The data were expressed as the percent of viable cells compared to the control group.

HA/TRAIL nanocomplexes with different fractions of HA were prepared at a TRAIL concentration of 100 ng/ml. To measure stability of HA/TRAIL nanocomplexes, samples were stored at 4 °C and biological activity of TRAIL was measured by using the MTT assay as described above once a day for a week.

2.4. In vivo pharmacokinetic study

The pharmacokinetics of HA/TRAIL formulations were evaluated using male Sprague–Dawley rats (Jail Co., Suwon, Korea). Prior to experimentation, the rats (5–6 weeks of age, 200–220 g) were anesthetized by i.p. injection of ketamine and xylazine (90/10 mg/kg) and cannulated with polyethylene tubing in the jugular veins. After overnight acclimation, native TRAIL and HA/TRAIL formulations were administered subcutaneously at a dose of 50 μg TRAIL/rat. After administration, blood samples (200 μl) were obtained from the jugular vein at scheduled time and heparinized, followed by plasma recovery through centrifugation at 10,000 × *g*. The plasma samples were then frozen immediately. The plasma concentrations of TRAIL were analyzed by using a human TRAIL ELISA Kit.

3. Results and discussion

3.1. Particle size and zeta-potential of nanocomplexes

HA is a natural linear polysaccharide, composed of *N*-acetyl-D-glucosamine and D-glucuronic acid, as shown in Fig. 1a. Since HA has the carboxylic acid in the repeating unit that exists as the ionized form in the biological fluids, it can form an ionic complex with positively charged biomolecules including TRAIL. Fig. 1b shows the schematic diagram of HA/TRAIL nanocomplex formation, which was significantly dependent on the feed ratio (FR) between HA and TRAIL. Fig. 2 shows the effect of the FR on the particle size and zeta-potential of the complexes. In general, the increase in the FR reduced the particle size, indicating the formation of compact ionic complexes (Fig. 2a). For example, at the FRs of 0.1 and 0.2,

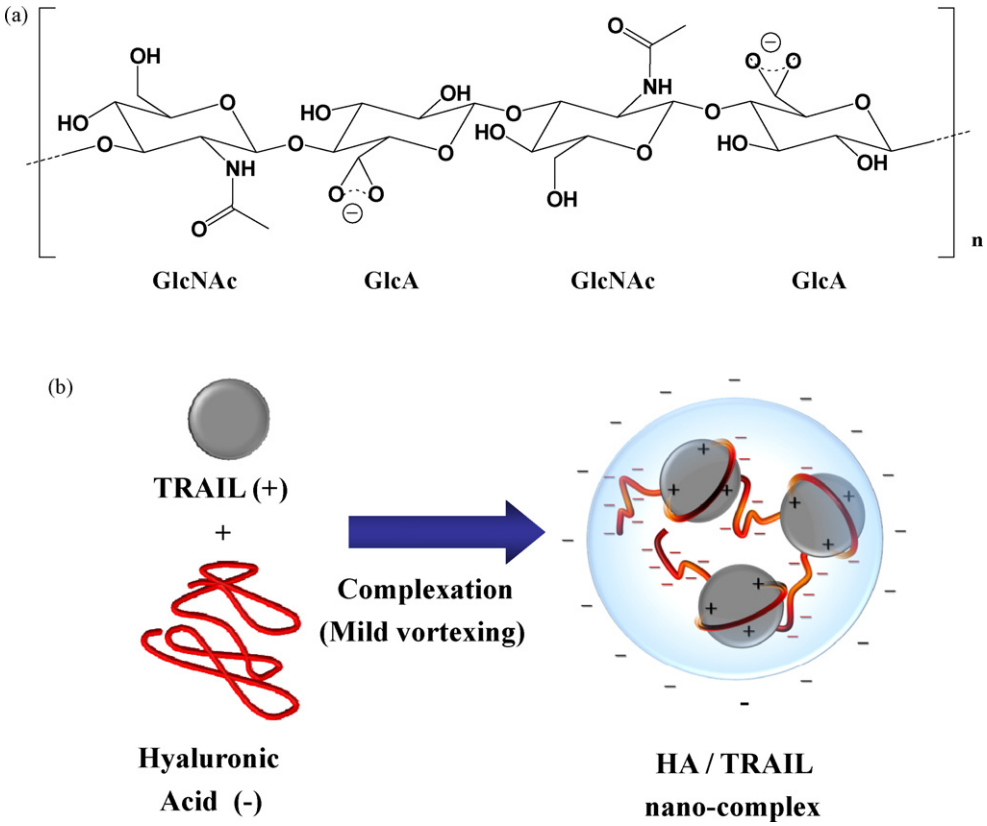

Fig. 1. Ionic complex formation of hyaluronic acid (HA) and TRAIL: (a) the chemical structure of HA and (b) schematic diagram of nanocomplex formation in the presence of HA and TRAIL.

the particle sizes were 1079 ± 149 and 558 ± 83 nm, respectively. The smallest particle based on the mean value was prepared at the FR of 3 (182 ± 25 nm in diameter). Formation of the large particle at the low FR might be due to loose aggregation between HA and TRAIL, in which the high molecular weight of HA can be role as the bridge between TRAILs. On the other hand, the TRAIL surface would be sufficiently covered by HA at the high FR, thus forming separate and compact nanoparticles. This was supported by the results of the zeta-potential that gradually decreased as the FR increased. In particular, the zeta-potential was saturated to −43 mV at the FR higher than 3, indicating that the TRAIL surface was fully covered by HA.

3.2. Biological activity and stability of TRAIL in nanocomplexes

In order to observe the effect of complex formation on the TRAIL stability, its structural information was estimated using the CD (Fig. 3). The CD spectrum of native TRAIL was slightly different from that of TRAIL in the complex. This might imply that the ionic complex formation with HA can change the structure of TRAIL, which may lead to loss of its biological activity. Therefore, in the next experiments, the biological activity of TRAIL in the complex was compared to that of the native TRAIL using the cytotoxicity test because TRAIL is known to induce apoptosis of cancer cells (Walczak et al., 1999; Fesik, 2005; Bremer et al., 2006). Fig. 4a shows

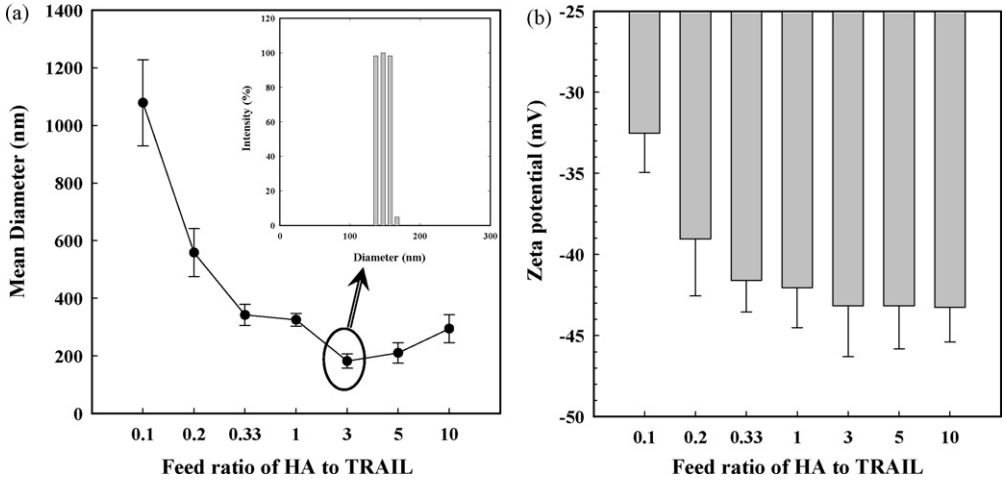

Fig. 2. Effect of the feed ratio on (a) particles size and (b) zeta-potential. The results represent the means ± S.D. ($n = 3$).

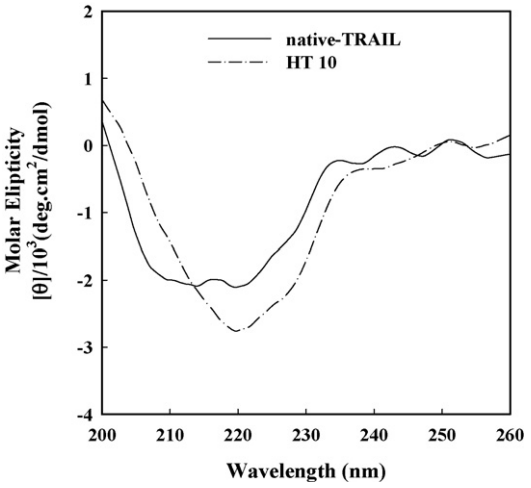

Fig. 3. CD spectra of TRAIL and TRAIL/HA nanocomplex. TRAIL and the complex with FR of 10 (HT10) were dissolved in 20 mM sodium acetate buffer containing 100 mM NaCl (pH 5.0). The concentration of TRAIL was fixed at 50 μg/ml.

the cell viability results for HA (1 μg/ml), native TRAIL (100 ng/ml), and complexes (100 ng/ml of TRAIL). As expected, HA alone did not show any effect on the cell viability because of its good biocompatibility. Native TRAIL, however, could effectively kill the HeLa cells. The death mechanism of the cells was demonstrated to be an apoptosis (data not shown). Interestingly, the cell viability of HA/TRAIL formulations with the FR higher than 3 was comparable to that of native TRAIL, although the structure of TRAIL was slightly affect by the complex formation as demonstrated in the CD spectra. As a matter of fact, such nanoparticular formulations with the high FR (>3) showed similar cell viability at the whole range of TRAIL concentrations to native TRAIL (IC_{50} = 40 ng/ml) as shown in Fig. 4b. This may suggest that the biological activity of TRAIL is not deteriorated by the complex formation with HA. In other words, although the complex formation can slightly change the structure of TRAIL, the active site of TRAIL is still capable of binding to the death receptor on the cancer cells. Although the cell viability was exhibited to be higher for complexes with the low FR (<3) than native TRAIL at the concentration of 100 ng/ml, this might not be due to loss of bioactivity of TRAIL: i.e., the aggregation of TRAIL may induce uneven local concentration of TRAIL. This assumption was supported by the same bioactivity of the complexes with the low FR as native TRAIL at the high concentrations (>1000 ng/ml) of TRAIL.

To date, the most successful method to improve stability of the protein drugs is considered to be chemical attachment of poly(ethylene glycol) (PEG) to the functional groups of proteins (Duncan, 2003, 2006). The PEGylated protein drugs have shown prolonged activity in the body, compared to native proteins, because PEG can effectively shield the surface of globular proteins and thus can minimize degradation by proteolytic enzymes. Several PEGylated proteins are already on the market, and they account for sales more than $3 billion (Wagner et al., 2006). PEGylation, however, reduces substantially the biological activity of protein drugs. For example, the prolonged in vivo half-life of PEGylated IFN-α species has compensated for the decreased bioactivity (Veronese and Pasut, 2005; Lee et al., 2007a). Further, recent studies have demonstrated that the repeated dose of PEGylated therapeutics can generate antibodies against PEG and thus result in rapid clearance from the blood, accumulation in the liver, and acute hypersensitivity (Kelly et al., 2001; Ganson et al., 2006; Ishida et al., 2006; Judge et al., 2006). On the other hand, the nanosized ionic complexes, prepared in this study, showed the comparable bioactivity to native protein. Also, it is expected that HA-based protein complex dose not induce immunogenic responses by the presence of HA because it is highly biocompatible polysaccharide found in our body. In this regard, ionic complexation of protein drugs might be the promising approach to stability and the protein drugs.

The biological activity of HA/TRAIL formulations was further investigated after being stored at 4 °C to determine if the complex formation with HA can improve the stability of TRAIL under long-term storage (Fig. 5). For native TRAIL at concentration of 100 ng/ml, cell viability increased to 67.4 ± 2.9% only after 1 day, and more than 90% of cells were survived after a 3-day storage due to the loss of biological activity. Indeed, a significant amount of TRAIL was precipitated in the buffer after 1 day, probably owing to aggregation by the hydrophobic interaction. In contrast, all the nanocomplexes with the FR higher than 3 showed better biological activities than native TRAIL during the whole time of period, applied in this study. Of the different formulations, the nanocomplexes with the FR of 10 exhibited the lowest cell viability for the whole time period of time. Therefore, it could be suggested that the increase in the HA ratio improve the stability of TRAIL by shielding its surface of protein, which may minimize protein aggregation.

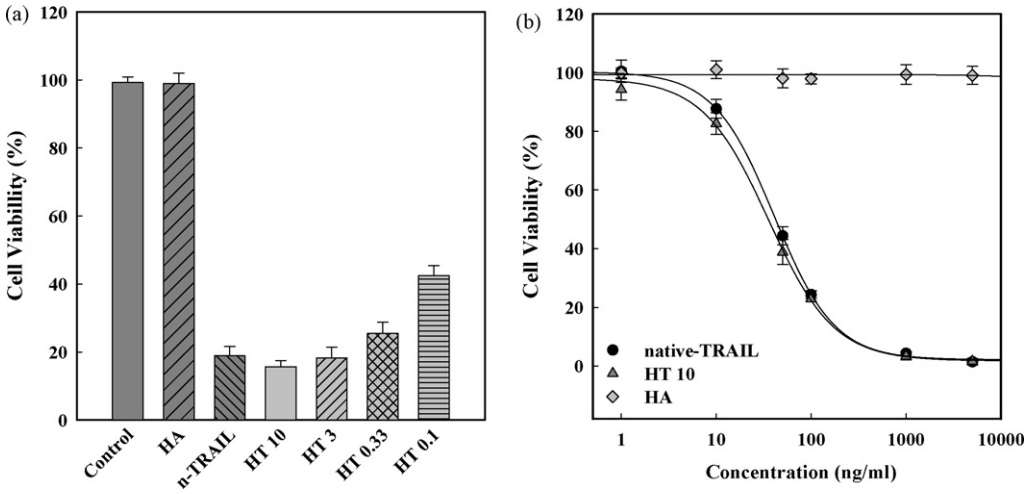

Fig. 4. In vitro biological activity: (a) the cytotoxicity of HA, native TRAIL (100 ng/ml), and nanocomplexes (100 ng/ml of TRAIL) to HeLa cells and (b) the cytotoxicity of the native TRAIL and HT10 as a function of TRAIL concentration. The number behind HT indicates the FR of the HA/TRAIL complex. The results represent the means ± S.D. (n = 3).

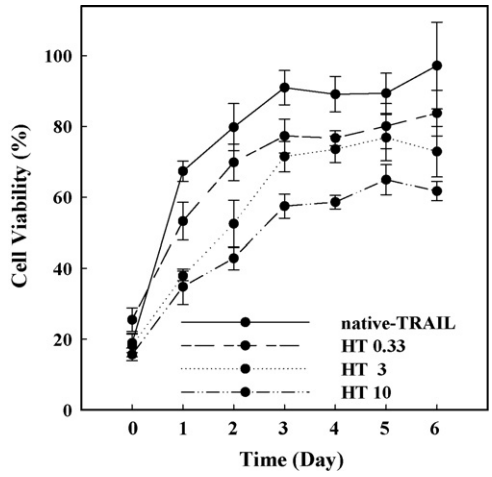

Fig. 5. The stability of TRAIL in nanocomplexes. The cytotoxicity of the samples was evaluated as a function of storage time. The final concentration of TRAIL was 100 ng/ml. The number behind HT indicates the FR of the HA/TRAIL complex. The results represent the means ± S.D. ($n = 3$).

3.3. Pharmacokinetics of HA/TRAIL formulations

The pharmacokinetics of HA/TRAIL formulations was evaluated after being subcutaneously administered into the SD rat. The HA/TRAIL nanocomplex with FR of 10 (HT10) in the acetate buffer was selected for the animal test because bioactivity and stability of TRAIL in HT10 were comparable or better than in nanocomplexes with other FRs. Also, in order to induce more sustained release of TRAIL in tissues as induced by high viscosity of HA, the HT10 dispersed in 1% HA solution was evaluated. The pharmacokinetic profiles of native TRAIL, HT10, and HT10 in 1% HA are shown in Fig. 6, and the pharmacokinetic parameters from non-compartmental analysis are listed in Table 1. After subcutaneous administration, native TRAIL was rapidly absorbed into systemic circulation with maximum peak concentrations occurring around 30 min (T_{max}: 0.5 ± 0.14 h, C_{max}: 14.05 ± 0.97 ng/ml). Then, the plasma TRAIL concentrations rapidly reduced and reached the basal level (<1 ng/ml) at 3 h after injection with slight prolongation of the basal plasma levels up to 12 h. The fast elimination of the

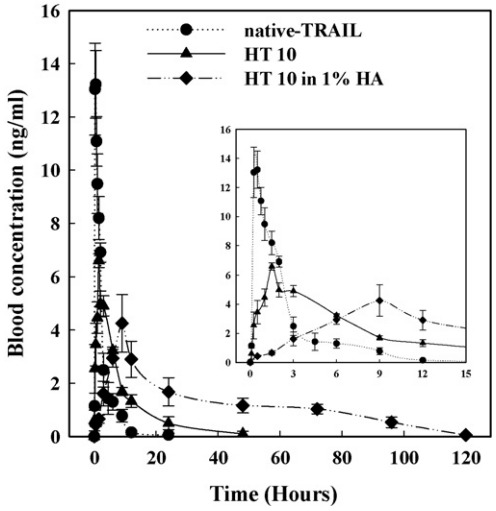

Fig. 6. Plasma concentration of TRAIL as a function of time. Native TRAIL complex, with the FR of 10 (HT10), and HT10 in 1% HA were administered subcutaneously at a dose of 50 μg TRAIL/rat. The results represent the mean ± S.D. ($n = 3$).

Table 1
Pharmacokinetic parameters obtained after subcutaneous administration of native TRAIL and HA/TRAIL formulations

Parameters (units)	Native-TRAIL[a]	HT10[b]	HT10 in 1% HA
C_{max} (ng/ml)	14.05 (±0.97)	6.61 (±0.25)	4.87 (±0.79)
T_{max} (h)	0.5 (±0.14)	1.5 (±0.00)	7.2 (±1.2)
AUC (h ng/ml)	33.76 (±4.51)	55.76 (±3.76)	144.20 (±22.76)

The results represent the mean ± S.E. of three animals per group at least.
[a] Leucine Zipper human TRAIL.
[b] HA/TRAIL nanocomplex with FR of 10.

TRAIL from the systemic circulation might be originated from the fast renal clearance mechanism (10). On the other hand, quite different pharmacokinetic profiles were obtained from HT10 and HT10 in 1% HA formulations. The HT10 group showed delayed absorption phenomena with T_{max} value of 1.5 h, followed by slightly retarded plasma TRAIL disappearance. The dramatic change of pharmacokinetic profile was obtained from HT10 in 1% HA injections. Owing to the limited diffusion of ion-complex nanoparticle from subcutaneous injection site to the bloodstream and high viscosity of HA medium, the plasma TRAIL concentration was continuously increased and reached maximum point at 7.2 ± 1.2 h (T_{max}) after injection. Similar to the delayed absorption phase, the elimination phase also significantly delayed and the substantial TRAIL circulation was detected at 5 days after injection. The sustained TRAIL release also affected the bioavailability of the TRAIL. The values of areas under the concentration–time curve for HT10 and HT10 in 1% HA were approximately 1.7- and 4.3-fold higher than native TRAIL, respectively, representing the enhanced drug availability. In this respect, HA/TRAIL formulations might have a potential as a long-lasting TRAIL delivery system.

4. Conclusion

The injectable TRAIL formulation was prepared with the aim of prolonged therapeutic effect in vivo. TRAIL was simply formulated as the nano-sized ionic complex with a negatively charged polysaccharide, HA. The resulting complex retained biological activity of TRAIL and showed improved stability in vitro. Furthermore, the pharmacokinetic study substantiated the in vivo long-lasting characteristics of HA/TRAIL formulations. Overall, this HA/TRAIL formulation could be evaluated as stable TRAIL-based therapeutics for clinical application.

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