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In Vitro Characteristics of Heparin/Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Nanocomplexes

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In an attempt to improve the stability of tumor necrosis factor-related apoptosis inducing ligand (TRAIL), we prepared the nano-sized complexes by mixing the positively charged TRAIL with heparin, a highly sulfated polysaccharide. The increase in the feed ratio (FR) of heparin to TRAIL reduced the particle size and decreased the zeta-potential, indicating that the TRAIL surface was covered by heparin via the ionic interactions. TRAIL in the complexes was demonstrated to effectively induce apoptosis of the cancer cells. The results indicated that heparin is the useful, anionic polysaccharide as the carrier of TRAIL to form stable nano-sized complexes.

Keywords: heparin; nano-complex; protein stability; TRAIL

1. INTRODUCTION

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is the emerging protein drug for cancer therapy because it induces cell death by apoptosis in a number of human cancer cells [1–3]. TRAIL

[*]These authors contributed equally to this work.

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has specific, binding affinity to the death receptors that are over-expressed on the cancer cells, thus exhibiting anti-cancer activity without significant cytotoxicity to the normal cells [4–6]. Clinical applications of TRAIL, however, have been limited by its instability in biological fluids. For example, when TRAIL is dispersed in an aqueous solution *in vitro*, its biological activity rapidly decreases by forming large aggregates via hydrophobic interactions. The *in vivo* half-life of TRAIL is reported to be less than 30 min., which may require frequent administration for preserving the therapeutic level in blood [7,8]. Therefore, it is of high interest to improve stability of TRAIL for its clinical trials.

Heparin, a highly sulfated polysaccharide composed of glucuronic acid and glucosamine derivatives, has been used as an anti-coagulant agent in the clinic to prevent deep vein thrombosis and pulmonary embolism. Owing to its potent anti-coagulant activity, heparin has also been considered as the coating material to generate anti-coagulant surface on various medical devices such as artificial blood vessels, stents, and catheters [9–11]. In recent years, it has been reported that heparin can affect tumor metastasis and angiogenesis, implying its potential as an anti-cancer agent [12–14].

In this study, nano-sized complexes were prepared by combining TRAIL with heparin as the potential therapeutics for cancer. By varying the feed ratio (FR) of heparin to TRAIL, the particle size and zeta potential was investigated. Also, the effect of the complex formation on protein stability was determined of TRAIL.

2. EXPERIMENTAL

2.1. Materials

TRAIL was prepared as described previously [15]. Heparin with a molecular weight of 12 kDa was supplied from GlaxoSmithKline (Brentford, Middlesex, UK). Human TRAIL ELISA kit was purchased from BioSource International, Inc. (Camarillo, CA, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals used in this study were analytical grades and were used without further purification.

2.2. Preparation and Characterization of heparin/TRAIL Complexes

Heparin/TRAIL formulations were prepared by simply mixing heparin and TRAIL in the buffer, as shown in Figure 1. In brief,

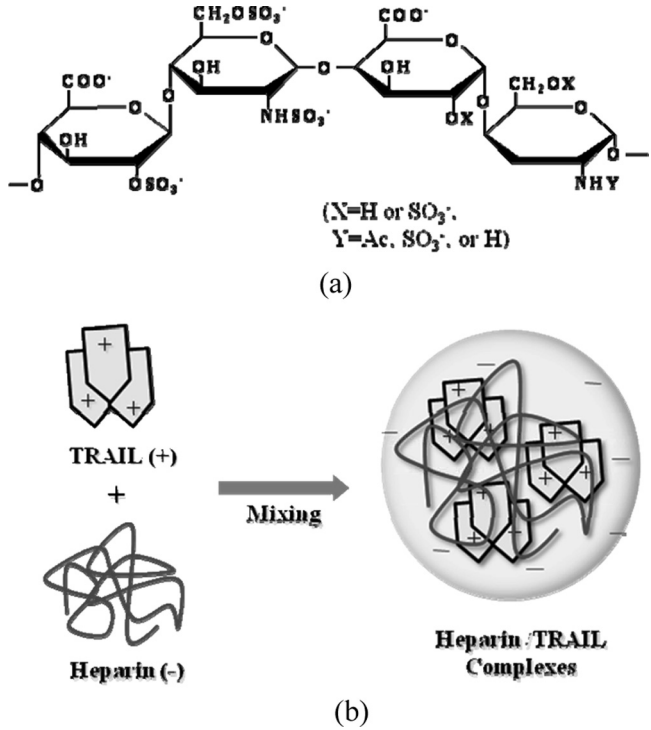

FIGURE 1 Ionic complex formation between heparin and TRAIL: (a) chemical structure of heparin; (b) schematic illustration of nanocomplex formation in the presence of heparin and TRAIL.

TRAIL and Heparin were separately dissolved in an imidazole buffer (20 mM imidazole, 100 mM NaCl, pH 5.0). The complexes were obtained by addition of a predetermined amount of TRAIL to the heparin solution, through which the FR of heparin to TRAIL was adjusted in the range of 0.2 to 5.

The mean diameter of heparin/TRAIL complexes was measured using a dynamic light scattering (DLS, Brookhaven Instrument Co., NY, USA) at a wavelength of 633 nm with a 90° detection angle. For the stability test, the nanocomplex solution (100 μg/ml of TRAIL in an imidazole buffer, pH 5.0) was stored at 37°C in an incubator, and the particle size of the complex was measured at a defined time interval over 6 days. The zeta potentials of heparin/TRAIL complexes were evaluated using a photon correlation spectroscopy (Malvern Zetasizer, Malvern Instruments, UK). The data reported were the mean value of at least three separate samples.

2.3. *In Vitro* Bioactivity of Heparin/TRAIL Complexes

Bioactivity of TRAIL in the complexes was estimated by measuring its cytotoxicity to human cervical cancer cells (HeLa) and human colorectal tumor cells (HCT-116) with the MTT assay. The cells were cultured at 37°C in a humidified atmosphere of 5% CO in the culture flask containing Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), 50 μg/ml streptomycin, and 50 U/ml penicillin. Cells were then seeded on 96-well plates at a density of 1×10^4 cells/well. After a 24 h incubation, the medium of each well was replaced with the fresh medium (1% FBS) containing various concentrations of heparin/TRAIL nanocomplexes. After 24 h, the medium was aspirated, and the cells were washed twice with a phosphate-buffered saline (PBS, pH 7.4). 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.

To determine the apoptotic activity of TRAIL on the cancer cells, the cell death was further investigated using Annexin-V-FLUOS staining kit (Roche, Penzberg, Germany), according to the manufacturer's instruction. Briefly, 1×10^6 HeLa cells were placed into a 6-well plate and incubated for 24 hr to insure the cell adhesion. Then, the cells were treated with TRAIL (100 ng/ml) for 0, 3, 6, 12, and 24 hr. After the treatment, the cells were washed with PBS (pH 7.4) and stained with 100 μl of Annexin-V-FLUOS labeling solution for 15 min. Finally, the apoptotic and necrotic cells were analyzed by the fluorescence microscopy.

3. RESULTS AND DISCUSSION

3.1. Particle Size and Zeta-Potential of Complexes

Heparin is negatively charged polysaccharide due to the presence of carboxylates and sulfates at the sugar units. This may allow forming ionic complexes with oppositely charged biomolecules including TRAIL in an aqueous condition, as shown in Figure 1. In this study, we have attempted to prepare nano-sized ionic complexes by varying the FR of heparin to TRAIL. Figure 2 shows the effect of the FR on the particle size the complexes. As the FR increased from 0.2 to 5, the particle size decreased from 300 to 50 nm. The large particle size at the low FR might be due to formation of large aggregates, in which heparin may play a role as the physical crosslinker between TRAILs.

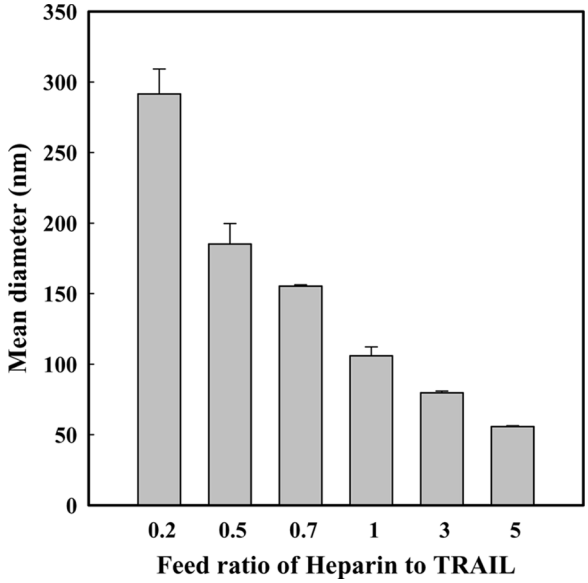

FIGURE 2 Particle sizes of complexes at different feed ratios of heparin to TRAIL. The error bar is for standard deviation ($n = 3$).

One the other hand, the large amount of heparin in the complex may induce to form separate and compact nanoparticles because heparin can effectively cover the surface of the TRAIL. This is supported by the results of the zeta-potential that gradually decreases as the FR increases (Fig. 3).

3.2. Stability of TRAIL in Nanocomplexes

Native TRAIL is a highly unstable protein in an aqueous solution. In fact, when native TRAIL is dispersed in the buffer, it is precipitated within a day by forming large aggregates. The stability of nanocomplexes was observed by measuring the particle sizes of the complexes with three different FRs at 37°C in an incubator as a function of time (Fig. 4). For the complex with FR of 0.2, the particle size rapidly increased from 291 nm to 833 nm for the whole period of time, applied in this study. Also, standard deviation of its size became larger after 2 days. On the contrary, for the other complexes with higher FRs, their particles sizes slightly increased for 6 days without significant changes in standard deviations. These results indicate that the complexes, prepared at higher FRs, possess high stability in an aqueous

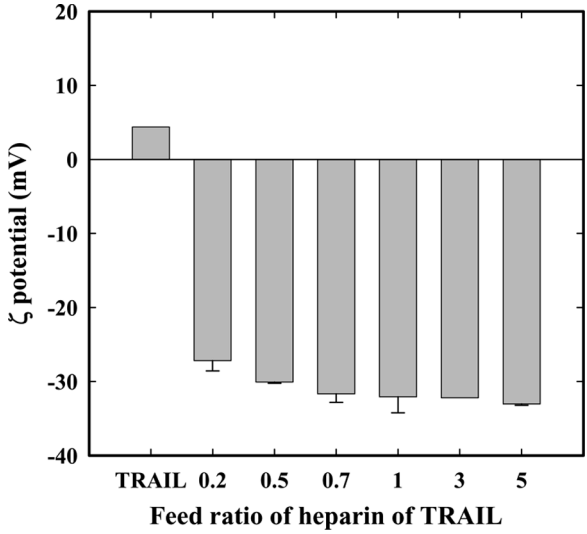

FIGURE 3 Zeta potentials of complexes at different feed ratios of heparin to TRAIL. The error bar is for standard deviation ($n = 3$).

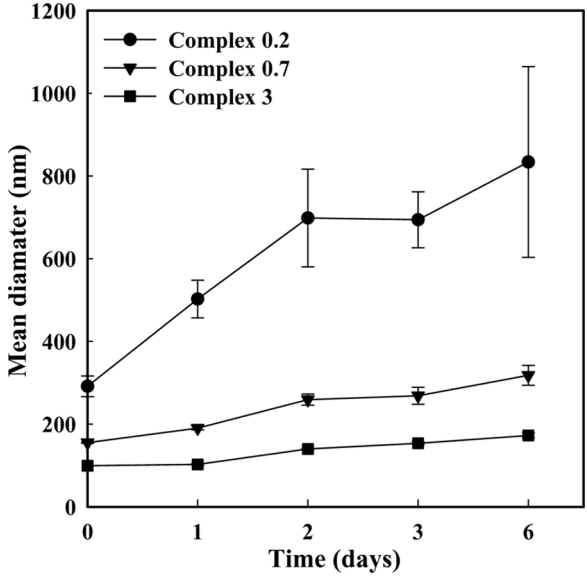

FIGURE 4 Particle sizes of complexes at 37°C as a function of time. Three complexes with different feed ratios (0.2, 0.7, and 3) were tested for 6 days. The error bar is for standard deviation ($n = 3$).

environment. Therefore, the next experiment for *in vitro* cellular study was carried out using the complex with FR of 3 (complex 3) as the representative example of the complexes with high stability.

In an attempt to observe the effect of complex formation on the TRAIL stability, the biological activity of TRAIL in the complex 3 was compared to that of native TRAIL. The biological activity was evaluated by the MTT assay which provided information on the cytotoxicity of TRAIL to cancer cells including HCT and HeLa cells. As expected, heparin did not show any cytotoxicity to the cancer cells because it is highly biocompatible polysaccharides found in the body. Both of HCT and HeLa cells, however, were effectively killed by the tiny amount of TRAIL: e.g., IC_{50} values for HCT and HeLa cells were 1.8 and 34.0 ng/ml, respectively. It is worthy of note that the complex 3 showed comparable bioactivity to native TRAIL, indicating that the complex formation of TRAIL did not affect its bioactivity. The mechanism of cell death was evaluated using the Annexin-V-FLUOS staining method (Fig. 6). The cells killed by apoptosis and necrosis emitted the green and red colors, respectively. As shown in Figure 6, intensity of the green color gradually increased in the presence of TRAIL and complex 3 as a function of time. Therefore, it is evident that both of TRAIL and complex 3 can effectively kill the cancer cells via the apoptotic pathway.

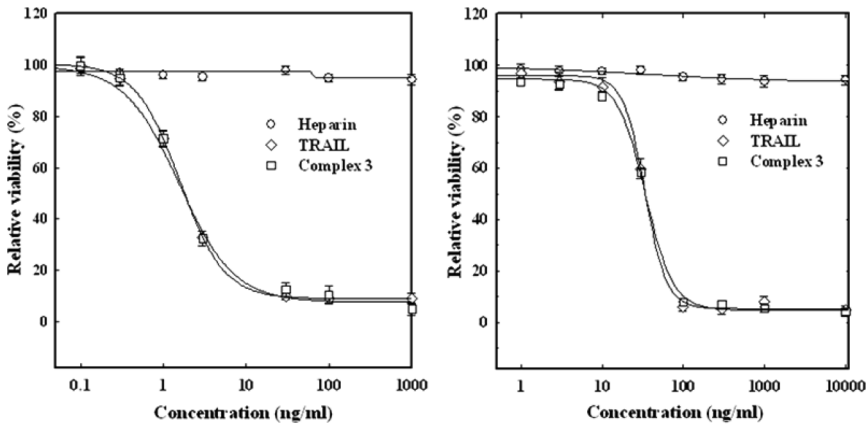

FIGURE 5 *In vitro* cytotoxicity of heparin, native TRAIL, and nanocomplex. The cell viability was obtained from the MTT assay. The complex with FR of 3 (complex 3) was chosen as the representative example of the complexes with high stability. The results represent the mean ± SD ($n = 3$).

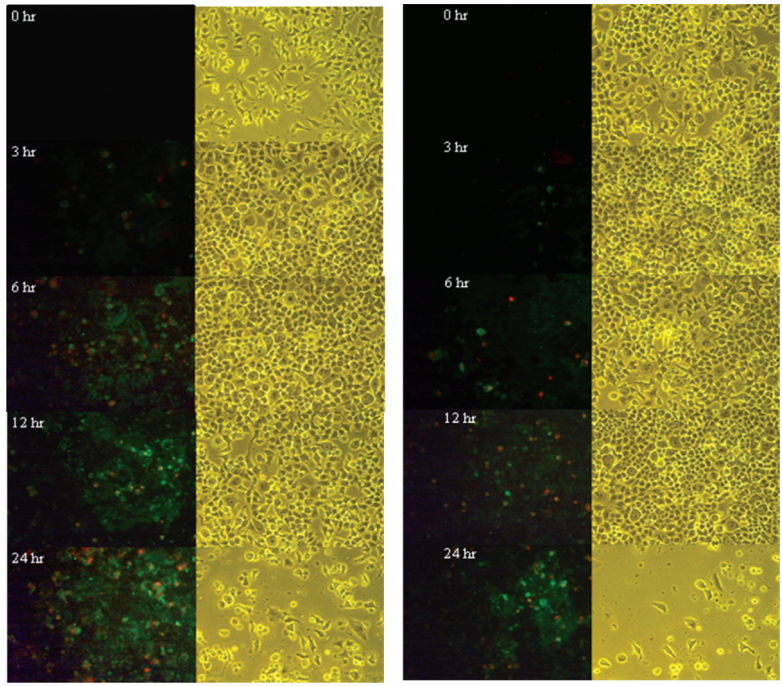

FIGURE 6 Apoptotic activity of TRAIL and nanocomplex as a function of time. The right panel of each sample was obtained using optical microscopy which shows the cell morphology. The left panel of each sample was produced by fluorescence microscopy, exhibiting the cell death by apoptosis (green: apoptotic cells, yellow and red: late apoptotic or necrotic cells).

4. DISCUSSION

Proteins have gained much recognition as the potent therapeutics for many diseases. Nevertheless, clinical applications of the proteins have been limited by their short half-lives and susceptibility to proteolytic enzymes in a biological environment. In this regard, TRAIL is a good model protein because it is highly unstable but has the potential as the drug for diseases such as cancer and arthritis. Our approach to stabilize TRAIL was on the basis of ionic complex formation with an oppositely charged polysaccharide, heparin. The surface coating of TRAIL with heparin was expected to protect TRAIL from enzymatic degradation. In recent years, it has been demonstrated that heparin can inhibit tumor growth and angiogenesis because it binds to angiogenic growth factors and cytokines [12–14]. Therefore, the TRAIL/heparin

complex may have a potential to show the synergistic effect on treatment of cancer.

To prolong therapeutic activity and improve the protein stability, many approaches have been made for the past few decades. For prolonged therapy, biodegradable microspheres have often been investigated [16–18]. Although this approach allows delivering the proteins in a sustained manner, significant amounts of proteins inside microspheres are known to be denatured because the acidic compounds are generated by degradation of microspheres. Also, proteins may lose their bioactivity by the harsh conditions of preparing microspheres such as high temperature, physical stress, and organic solvents. For improving the protein stability, many researchers have attempted to conjugate poly(ethylene glycol) (PEG) to the functional groups of proteins [19,20]. Because PEG can effectively shield the protein surface and thus can minimize degradation by proteolytic enzymes, many of PEGylated proteins have shown improved stability and prolonged activity in the body. Unfortunately, PEGylation often reduces the biological activity of protein drugs because PEG can affect binding affinity of active domains in proteins. In this study, we have prepared protein complexes with an oppositely charged polysaccharide by simply mixing them in an aqueous environment. This approach does not involve harsh environments because the complex is prepared using a biocompatible polysaccharide in an aqueous medium. As shown in Figure 5, complex formation of TRAIL did not affect its biological activity. These results may suggest that the complex system, developed in this study, may be useful for improving protein stability.

5. CONCLUSION

TRAIL was successfully formulated to improve its stability in an aqueous condition by forming the complex with heparin. Depending on the FR of heparin to TRAIL, various nano-sized complexes were prepared and they showed comparable bioactivity to native TRAIL. The particle size of the complex was not significantly changed for at least 6 days. The nanocomplex system might have a potential as a stable therapeutic drugs for cancer therapy.

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