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Hyaluronic acid–tumor necrosis factor-related apoptosis-inducing ligand conjugate for targeted treatment of liver fibrosis

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ABSTRACT

Liver fibrosis is a chronic liver disease caused by viral infection and/or metabolic, genetic and cholestatic disorders. The inhibition of hepatic stellate cell (HSC) activation and the selective apoptosis of activated HSCs can be a good strategy to treat liver fibrosis. The activated HSCs are known to be more susceptible to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis than normal HSCs because death receptor 5 is overexpressed on the cell surface. In this work, a target-specific and long-acting hyaluronic acid (HA)–TRAIL conjugate was successfully developed for the treatment of liver fibrosis. The HA–TRAIL conjugate was synthesized by a coupling reaction between aldehyde-modified HA and the N-terminal amine group of TRAIL. The biological activity of the HA–TRAIL conjugate was confirmed by an in vitro anti-proliferation assay and caspase-3 expression in human colon cancer HCT116 cells. In vivo real-time bioimaging exhibited the target-specific delivery of near-infrared fluorescence dye-labeled HA–TRAIL conjugate to the liver in mice. According to pharmacokinetic analysis, the HA–TRAIL conjugate was detected for more than 4 days after single intravenous injection into Sprague–Dawley (SD) rats. Finally, we could confirm the antifibrotic effect of HA–TRAIL conjugate in an *N*-nitrosodimethylamine-induced liver fibrosis model SD rats.

1. Introduction

Liver diseases generally proceed from normal liver to fatty liver, liver fibrosis, liver cirrhosis and liver cancer [1,2]. With the progress of liver fibrosis, scar extracellular matrix is deposited and accumulated in the liver parenchyma, and the hepatic architecture is distorted by the scar tissue formation [3,4]. There is no standard treatment for liver fibrosis; the only way is to protect the liver and retard the fibrosis process therein. Thus, there is a strong need to develop a new treatment method for liver fibrosis and cirrhosis. Recently, activated hepatic stellate cells (HSCs) have been considered as the main target cells for the treatment of liver fibrosis. Type 1 collagen is deposited as the principal matrix protein in liver fibrosis [5–7]. The inhibition of HSC activation and the selective apoptosis of activated HSCs would be a good potential strategy for the treatment of liver fibrosis. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been investigated as an anticancer drug for selectively induced apoptosis of tumor cells [8–12]. In addition, it was reported that TRAIL-related death receptor 5 was overexpressed on the surface of activated HSCs and the activated HSCs were more susceptible to TRAIL-induced apoptosis than normal HSCs [13]. Despite the feasibility of TRAIL as a drug candidate for the treatment of liver fibrosis, there are several factors limiting the further use of TRAIL in clinical applications, including its low stability and short half-life in the body. A variety of drug delivery systems have been developed to improve the therapeutic effect of TRAIL and patient compliance. TRAIL was conjugated to poly(ethylene glycol) [14–16] or transferrin [17], and encapsulated within biodegradable poly(lactic-*co*-glycolic acid) microparticles for sustained long-term release [18,19].

The ubiquitous linear polysaccharide hyaluronic acid (HA) has been extensively investigated for various biomedical applications [20–22]. There are a variety of HA receptors in the body, each with its own biological function. They have been used as target sites for HA-based drug delivery systems [23–25]. In particular, cluster-determinant 44 (CD44) expression has been reported to increase significantly in the cases of alcoholic liver diseases [26]

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and *N*-nitrosodimethylamine (NDMA)-induced liver cirrhosis [27]. We previously carried out real-time bioimaging of HA derivatives using quantum dots (QDots) in normal and cirrhotic liver model mice [20,28]. HA derivatives with an HA modification of less than 25 mol.% were mainly accumulated in the liver. HA–QDot conjugates were delivered more efficiently to HSCs and hepatoma cells than to normal hepatocytes [28]. In addition, the clearance of HA–QDot conjugates was relatively slow, so that it remained in the cirrhotic liver for more than 8 days after intravenous (i.v.) injection. From the results, we could confirm the feasibility of HA derivatives as target-specific drug delivery carriers for the treatment of chronic liver diseases. As one of the model applications, we successfully developed HA–interferon α conjugate for the treatment of hepatitis C virus infection [29].

In this work, HA–TRAIL conjugate was developed for target-specific long-term systemic treatment of liver fibrosis. Aldehyde-modified HA (HA–ALD) was synthesized and conjugated with the N-terminal primary amine group of TRAIL. The resulting HA–TRAIL conjugate was analyzed by gel permeation chromatography (GPC) and circular dichroism (CD). After confirmation of in vitro biological activity in human colon cancer HCT116 cells, real-time bioimaging for the target-specific systemic delivery of HA–TRAIL conjugate was carried out with pharmacokinetic (PK) analysis. The in vivo therapeutic activity of HA–TRAIL conjugate was assessed in NDMA-induced liver fibrosis model Sprague–Dawley (SD) rats. The target-specific HA–TRAIL conjugate was discussed for further clinical applications in a once-a-week injection dosage form for the treatment of liver fibrosis.

2. Materials and methods

2.1. Materials

Sodium hyaluronate, a sodium salt of HA with a molecular weight (MW) of 17 kDa, was obtained from Lifecore (Chaska, MN) and HA with a MW of 100 kDa was obtained from Shiseido (Tokyo, Japan). His-tag cleaved TRAIL and the HCT 116 human colon cancer cell line were kindly provided by Prof. K.C. Lee of SungKyunKwan University (Seoul, Korea). Sodium periodate, sodium cyanoborohydride, ethyl carbazate and tert-butyl carbazate were purchased from Sigma–Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS) tablets were purchased from Invitrogen (Carlsbad, CA) and Bradford protein assay kits were obtained from Thermo scientific (Rockford, IL). A near-infrared fluorescence (NIRF) HiLyte Fluor™ 647 protein labeling kit was purchased from AnaSpec (Fremont, CA). A TRAIL ELISA kit was purchased from R&D system (Minneapolis, MN). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Promega (Madison, WI). All reagents were used without purification.

2.2. Synthesis of HA–TRAIL conjugate

As we reported elsewhere [29], HA–ALD, with an aldehyde content of 20 mol.%, was prepared and dissolved in 20 mM sodium acetate buffer with 100 mM NaCl (pH 5) at a concentration of 5 mg ml^{-1}. The numbers of TRAIL molecules per HA chain (MW = 17 or 100 kDa) in the feed were 1 molecule for $HA_{17\ kDa}$–TRAIL conjugates and 2 molecules for $HA_{100\ kDa}$–TRAIL conjugates. The conjugation reaction was performed at 4 °C for 24 h with mild stirring. For the reduction of hydrazone linkage, 10 M excess of sodium cyanoborohydride to aldehyde group was added into the reaction solution. The unreacted aldehyde groups in the HA–TRAIL conjugates were blocked with a 5 M excess of ethyl carbazate in the presence of sodium cyanoborohydride at 4 °C for another 24 h. HA–TRAIL conjugates were purified by dialysis against a large excess amount of PBS for 1 day. The HA–TRAIL conjugates were then concentrated using a centrifugal concentrator for the following experiments.

2.3. Characterization of HA–TRAIL conjugate

The synthesized HA–TRAIL conjugates were analyzed by GPC to check the retention time before and after conjugation of TRAIL to HA. The GPC analysis was performed using the following systems: Waters 717 plus autosampler, Waters 1525 binary HPLC pump, Waters 2487 dual λ absorbance detector, and Ultrahydrogel™ 1000 connected with Ultrahydrogel™ 500 column. The eluent was phosphate buffered saline at pH 7.4 and the flow rate was 0.4 ml min^{-1}. The detection wavelengths were 210 nm for HA and 280 nm for TRAIL. The secondary structure of the HA–TRAIL conjugate was analyzed by CD. The CD spectra for TRAIL and the HA–TRAIL conjugate in PBS (pH 7.4) were obtained with a UV spectrophotometer (JASCO J-715, Essex, UK) at 25 °C over the range of 200–250 nm under a nitrogen atmosphere. A quartz cuvette with a path length of 1 mm was used and the data were acquired at 0.5 nm intervals, with a response time of 1 s. Each spectrum was subtracted from the spectrum of PBS and the residual ellipticity was calculated as an average of three scans.

2.4. In vitro biological activity of HA–TRAIL conjugate

Human colon cancer cell line of HCT116 was cultured in DMEM medium supplemented with 10 vol.% fetal bovine serum and 10 IU ml^{-1} of antibiotics (penicillin). The cells were resuspended at a concentration of 1×10^5 cells ml^{-1} in assay medium and 100 μl of the cell suspension containing 1×10^4 cells was seeded on the flat bottom of a 96-well tissue culture plate. After 24 h, serial dilutions of the protein samples were prepared in assay medium and 100 μl of the diluted protein samples were added to the test wells in triplicate. The plates were incubated at 37 °C in a humidified 5% CO_2 tissue culture incubator for 1 day. The medium in each well was then replaced by MTT reagent-containing medium and the plates were incubated at 37 °C in the tissue culture incubator for 2 h. The medium of each well was aspirated and dimethylsulfoxide (DMSO) was added to dissolve the formazan crystal. The absorbance was measured at 540 nm using a microplate reader (EMax, Molecular Devices, CA). The caspase-3 expression levels of HCT116 cells treated with TRAIL and HA–TRAIL conjugates were measured by Western blot analysis of the cell lysate.

2.5. In vivo bioimaging of NIRF dye-labeled HA–TRAIL conjugate

HA–TRAIL conjugates were labeled with NIRF dye following the protocol of the AnaTag™ HiLyte Fluor™ 647 protein labeling kit for the bioimaging of their target-specific delivery into liver tissues. The HiLyte Fluor™ 647 dye solution (7.5 mM) in DMSO was added to the protein solutions of TRAIL and HA–TRAIL conjugates at a molar ratio to protein molecule of 5. The conjugation reaction was performed at room temperature for 12 h with mild stirring. The NIRF dye-labeled TRAIL and HA–TRAIL conjugates were purified using desalting columns. The degree of substitution was calculated by measuring the absorbance at 280 and 652 nm. The NIRF dye-labeled TRAIL and HA–TRAIL conjugates were then administered to female BALB/c mice, aged 5 weeks, via tail-vein injection. The dose was 5 nmol of TRAIL with 10 nmol of dye. After 1, 2, 5 and 7 days post-injection, the fluorescence of the injected mice was captured with an IVIS® imaging system (Caliper Life Sciences, Hopkinton, MA). All procedures with animals were performed in accordance with Pohang University of Science and Technology guidelines for animal care and exploitation.

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