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Target specific hyaluronic acid–interferon alpha conjugate for the treatment of hepatitis C virus infection

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ABSTRACT

Interferon alpha (IFNα) conjugated with polyethylene glycol (PEG) has been widely used for the treatment of hepatitis C virus (HCV) infection as a once-a-week injection formulation. However, the PEGylated IFNα has a low efficacy of ca. 39% and a side effect after repeated injections possibly due to the non-specific delivery with PEGylation. In this work, target specific long-acting hyaluronic acid–interferon alpha (HA–IFNα) conjugate was successfully developed for the treatment of HCV infection. HA–IFNα conjugate was synthesized by coupling reaction between aldehyde modified HA and the N-terminal group of IFNα. The IFNα content could be controlled in the range of 2–9 molecules per single HA chain with a bioconjugation efficiency higher than 95%. According to *in vitro* anti-proliferation assay using Daudi cells, HA–IFNα conjugate showed a comparable biological activity to PEG-Intron. *In vivo* real-time bioimaging confirmed the target specific delivery of near-infrared fluorescence (NIRF) dye labeled HA–IFNα conjugate to the liver in mice. In addition, pharmacokinetic analysis revealed the enhanced residence time longer than 4 days. After tail-vein injection, HA–IFNα conjugate induced ca. 60% higher expression of 2′,5′-oligoadenylate synthetase 1 (OAS 1) for innate immune responses to viral infection in the murine liver tissues than IFNα and PEG-Intron.

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1. Introduction

Currently, 170 million people are estimated to be infected with hepatitis C virus (HCV), one of the main causes of liver cirrhosis and hepatocellular carcinoma. HCV infection is progressed to the chronic disease in approximately 85% patients [1]. Interferon alpha (IFNα) with 165 amino acids has been widely used for the treatment of chronic HCV infection by three times a week injection and melanoma by daily injection [2,3]. A variety of drug delivery systems have been developed to improve the patient compliance without frequent injection of IFNα such as a long-acting conjugate with polyethylene glycol (PEG) [4–6], a genetic fusion with albumin [7], and biodegradable microparticles for the sustained release [8–10]. Among them, PEGylated IFNα has been successfully commercialized under the trade-names of PEGASYS (peginterferon alpha-2a) [11,12] and PEG-Intron (peginterferon alpha-2b) [13,14] as a once-a-week injection formulation. According to the phase III clinical test results, however, the patient rate with sustained virologic response was only 39% after treatment with PEGASYS. A combination therapy using PEGASYS and Ribavirine showed a success rate of ca. 57% in patients with HCV genotype 1 [15–17]. The low efficacy of PEGylated IFNα might be attributed to the fact that PEGylation is intended for by-passing the liver and enabling long circulation in the body. In other words, the low binding affinity to the liver might be advantageous for long circulation, but result in a low therapeutic efficacy for the treatment of liver diseases. Accordingly, we decided to develop a target specific delivery system of IFNα using hyaluronic acid (HA) for the treatment of chronic HCV infection.

HA is a biodegradable, non-immunogenic, non-toxic, and natural polysaccharide in the body [18–20]. There are various kinds of HA receptors in the body with their unique biological functions [21–24]. HA receptors have been used as target sites for HA based

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drug delivery systems [25,26]. For example, HA receptor for endocytosis (HARE) in the liver sinusoidal epithelial cells (LSEC) was the target site for gene delivery using HA-poly-L-lysine (PLL) conjugate [25]. Moreover, cluster determinant 44 (CD44) expression was reported to increase significantly in the cases of alcoholic liver diseases and dimethylnitrosamine-induced liver cirrhosis [27]. In our previous work, real-time bioimaging of HA derivatives was carried out using quantum dots (QDots) [20,28]. HA derivatives with an HA modification less than 25 mol% were mainly accumulated in the liver, whereas highly modified HA derivatives were evenly distributed in the body [20]. In addition, HA–QDot conjugates were more efficiently delivered to hepatic stellate cells (HSCs) and hepatoma cells than normal hepatocyte. The clearance of HA–QDot conjugates was relatively slow remaining in the cirrhotic liver longer than 8 days after tail-vein injection [28]. From the results, we could confirm the feasibility of HA derivatives as target specific drug delivery carriers for the treatment of chronic liver diseases, such as HCV infection, liver cirrhosis, and liver cancer.

In this work, on the basis of previous real-time bioimaging of HA derivatives using QDots, HA–IFNα conjugate was developed for target specific systemic treatment of liver diseases, especially HCV infection. Aldehyde modified HA (HA-ALD) was synthesized and conjugated with N-terminal primary amine group of IFNα. The resulting HA–IFNα conjugate was characterized by ^{1}H NMR, gel permeation chromatography (GPC), and circular dichroism (CD). After confirmation of *in vitro* anti-proliferation activity and serum stability in Daudi cells, target specific systemic delivery of HA–IFNα conjugate was carried out with real-time bioimaging and pharmacokinetic analysis. *In vivo* antiviral activity of HA–IFNα conjugate was assessed by measuring the expression levels of 2′,5′-oligoadenylate synthetase 1 (OAS 1) which is induced by IFNα and participates in innate immune responses to viral infection in the liver. The target specific HA–IFNα conjugate was discussed for further clinical applications as a once-a-week injection dosage form for the treatment of HCV infection.

2. Materials and methods

2.1. Materials

Sodium hyaluronate, the sodium salt of hyaluronic acid (HA), with a molecular weight of 100 kDa was obtained from Shiseido (Tokyo, Japan). Human interferon alpha-2b (IFNα-2b) and PEG-Intron were kindly provided by Shinpoong Pharmaceutical Co. (Seoul, Korea). Human serum, sodium periodate, sodium cyanoborohydride, ethyl carbazate, and tert-butyl carbazate were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) tablet was purchased from Invitrogen (Carlsbad, CA) and the Bradford protein assay kit from Thermo scientific (Rockford, IL). Cell Titer 96 AQueous One Solution Reagent was purchased from Promega (Madison, WI). Near-infrared fluorescence (NIRF) HiLyte Fluor™ 647 protein labeling kit was purchased from AnaSpec (Fremont, CA). IFNα ELISA kit was purchased from PBL InterferonSource (Piscataway, NJ). Rabbit anti-OAS1 polyclonal antibody was purchased from Abgen (San Diego, CA) and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG were purchased from Kirkegaard and Perry Laboratories (Gaitherburg, MD). The human Daudi cell line was purchased from Korean Cell Line Bank. All reagents were used without further purification.

2.2. Preparation of aldehyde modified HA

Aldehyde modified HA (HA–ALD) was prepared as described elsewhere [29]. Briefly, 1 g of HA with a MW of 100 kDa was dissolved in 100 mL of water. Sodium periodate (2 molar ratio of HA repeating unit) was added to the HA solution. After reaction in a dark place for 2 h, 6 h, and 12 h, excess amount of ethylene glycol (1 g) was added to each solution for the reaction termination. Finally, the resulting solution was dialyzed against a large excess amount of water using a prewashed dialysis membrane tube (MWCO of 10 kDa) and lyophilized for 3 days. In order to analyze the degree of aldehyde modification, the HA–ALD was dissolved in sodium acetate buffer (5 mg/mL) at pH 5.2, which was reacted with tert-butyl carbazate in the presence of sodium cyanoborohydride both at 5 molar ratio of HA repeating unit for 24 h. Then, the reaction solution was poured into the prewashed dialysis membrane tube (MWCO of 10 kDa) and dialyzed against a large excess amount of water. The solution was lyophilized for 3 days, which was characterized by ^{1}H NMR (DPX500, Bruker, Germany).

2.3. Synthesis of HA–interferon alpha conjugate

HA–ALD with an aldehyde content of 10 mol% was dissolved in sodium acetate buffer (pH 5.5) at a concentration of 5 mg/mL. The number of IFNα molecules per single HA chain in the feed was varied from 2, 4, 6, and 9. The conjugation reaction was performed at room temperature for 24 h with mild stirring. For the reduction of hydrazone linkage, 5 molar excess of sodium cyanoborohydride to aldehyde group was added into the reaction solution. The unreacted aldehyde groups in HA–IFNα conjugates were blocked with 5 molar excess of ethyl carbazate in the presence of sodium cyanoborohydride at room temperature for another 24 h. Then, HA–IFNα conjugate was purified by dialysis against a large excess amount of PBS for 2 days. For the following experiments, three kinds of HA–IFNα conjugates were synthesized to contain 6 IFNα molecules in a single HA chain using HA–ALD with 10, 25, and 45 mol% aldehyde contents, and represented as HA–IFNα (10%/6), HA–IFNα (25%/6), and HA–IFNα (45%/6), respectively.

2.4. Characterization of HA–interferon alpha conjugate

The successful synthesis of HA–IFNα conjugates and blocking of the remaining aldehyde groups with ethyl carbazate were confirmed by ^{1}H NMR analysis (DPX500, Bruker, Germany). GPC analysis was carried out to check the retention time before and after conjugation of IFNα to HA. The number of IFNα molecules in HA–IFNα conjugates was calculated from the GPC peak area at 280 nm. The standard curve of IFNα was prepared by several dilutions of the protein stock solution at a concentration of 1 mg/mL. The GPC analysis was performed using the following systems: Waters 717 plus autosampler, Waters 1525 binary HPLC pump, Waters 2487 dual λ absorbance detector, Ultrahydrogel™ 500 connected with Ultrahydrogel™ 250 column. The eluent was phosphate buffered saline at pH 7.4 and the flow rate was 0.3 mL/min. The detection wavelengths were 210 nm for HA and 280 nm for IFNα. The secondary structure of HA–IFNα conjugate was analyzed by CD. The CD spectra for IFNα and HA–IFNα 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 2 mm was used and the data were acquired at 0.2 mm intervals with a response time of 1 s. Each spectrum was subtracted by the spectrum of PBS and the residual ellipticity was calculated as an average of three scans.

2.5. In vitro biological activity of HA–interferon alpha conjugate

The human Daudi cell line was maintained in RPMI 1640 media supplemented with 10 vol% fetal bovine serum (FBS) and 10 IU/mL of antibiotics (penicillin). The cells were resuspended at a concentration of 4×10^5 cells/mL in assay media and 50 μL of the cell suspension containing 2×10^4 cells was seeded to the flat bottom of 96 well tissue culture plate. Serial dilutions of the protein samples were prepared in assay media and 50 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 4 days. Then, 20 μL of Cell Titer 96 AQueous One Solution Reagent was added to each well and the plates were incubated at 37 °C in the tissue culture incubator for 2 h. The absorbance was measured at 490 nm using a microplate reader (EMax, Molecular Devices, CA).

2.6. In vitro serum stability test of HA–interferon alpha conjugate

The serum stability of IFNα and HA–IFNα (10%/6) conjugate was assessed by anti-proliferation assay using Daudi cells after incubation in human serum at a concentration of 0.5 mg/mL at 37 °C for up to 7 days. At the predetermined time intervals, each sample was immediately diluted with PBS by 10,000 times and stored at −80 °C before the biological activity tests.

2.7. In vivo bioimaging of NIRF dye labeled HA–interferon alpha conjugate

To investigate the systemic body distribution, HA–IFNα conjugates were labeled with NIRF dye following the protocol of AnaTag™ HiLyte Fluor™ 647 protein labeling kit. The HiLyte Fluor™ 647 dye solution (7.5 mM) in DMSO was added to the protein solutions of IFNα and HA–IFNα conjugate (10 molar ratio to protein molecule). The conjugation reaction was performed at room temperature for 2 h with mild stirring. The NIRF dye labeled IFNα and HA–IFNα conjugates were purified using desalting columns. The degree of substitution was calculated by measuring the absorbance at 280 nm and 652 nm. Then, NIRF dye labeled IFNα and HA–IFNα conjugates were administered to female Balb/c mice at an age of 5 weeks via tail vein injections. The dose was 5 nmol IFNα with 2 nmol dye. After 30 min and 1 h post-injection, mice were anesthetized via intraperitoneal injection of a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg). The fluorescence of injected mice was captured with a luminescent image analyzer (Maestro 2.8, CRI, MA). All

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