Combination of hybrid peptide with biodegradable gelatin hydrogel for controlled release and enhancement of anti-tumor activity *in vivo*

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ABSTRACT

We previously reported that the EGFR2R-lytic hybrid peptide has cytotoxic and anti-tumor activities both *in vitro* and *in vivo*. In this study, to improve the peptide pharmacokinetics and its anti-tumor activity after intravenous injection, we prepared biodegradable gelatin hydrogel nanoparticles as the delivery system of peptide. The complex is formed through the electrostatic interaction between the cationic peptide and anionic gelatin. *In vitro* release studies confirmed that the peptide was released from the complex in phosphate-buffered saline (PBS) solution containing fetal bovine serum at 37 °C within 48 h, whereas little release was observed in PBS solution. *In vivo* release studies indicated that the anti-tumor activity of the complex was more effective than that of peptide treatment alone, and high tumor accumulation of the peptide was observed in the mice treated with the complex. Furthermore, the plasma area under the concentration curve (AUC) and half-life ($T_{1/2}$) values of the complex were higher than those of the peptide treatment alone, respectively. These results demonstrate that the rate of peptide release was controlled by the gelatin, and that the complex had a longer circulation time and enhanced its anti-tumor activity *in vivo*.

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1. Introduction

We previously designed and synthesized a novel peptide that is chemically conjugated between targeted-binding and cell-killing peptide components and has selective cytotoxic activity to discriminate between normal cells and cancer cells. *In vivo* analysis revealed that these hybrid peptides had significant anti-tumor activity in xenograft models [1–5]. The EGFR-lytic peptide is a hybrid peptide synthesized by the chemical conjugation of the targeted-binding peptide to epidermal growth factor receptor (EGFR) and the cell-killing lytic peptide [1]. Furthermore, to improve the selective anti-tumor activity of the EGFR-lytic peptide, we modified the EGFR-binding peptide by introducing a mutation of a single amino acid, in which second histidine (H) of EGFR-lytic peptide was replaced to arginine (R), to form a new peptide named "EGFR2R-lytic peptide". This modified peptide has a higher cytotoxic activity and anti-tumor activity than the original EGFR-lytic peptide [2].

However, this hybrid peptide, like other peptide drugs, has several problems associated with its short half-life after intravenous (i.v.) administration. Therefore, frequent administration at an excessively high dose is required to obtain their therapeutic effects *in vivo* [1–5]. The short plasma half-life is usually due to fast renal clearance, which is connected to the hydrophilic properties of most of these agents as well as their typical small size (molecular size under 5 kDa), or due to enzymatic degradation by enzymes in the blood, liver, and kidney. Strategies to prolong plasma half-life may improve the therapeutic effectiveness of peptide-based therapies. Moreover, prolongation of plasma half-life is often a prerequisite for the clinical use of drug candidates. Therefore, improvements in the peptide concentration in the bloodstream after i.v. administration and enhancement of their therapeutic efficacy are necessary for pre-clinical trials.

Recently, drug delivery system (DDS) has been extensively explored to achieve a therapeutic effect with a controlled release profile for an extended time periods. Various polymers have been used in drug delivery research as they can enhance the delivery of drugs to the target site and thus improve the therapeutic efficacy, while minimizing side effects [6–9]. High molecular weight polymers and nano-sized particles accumulate in solid tumors at much higher concentrations than in normal tissues or organs due to the enhanced permeability and retention effect [10]. The exploitation of such mechanisms for tumor targeting has been successfully proven with various anticancer drugs, such as cisplatin [8,9] and doxorubicin [11–13].

Gelatin is a non-toxic, natural, biodegradable polymer consisting of denatured protein which is obtained by acid and alkaline processing of collagen. It has been widely used in food, pharmaceutical, and medical applications because of its biodegradability [14–17] and biocompatibility

[18,19]. These characteristics have contributed to gelatin's proven record of safety and efficacy in drug formulations and as a DDS. We previously reported that gelatin carrier-mediated pharmaceutical drug delivery can enable drug release from the gelatin complex and enhance their biological activities, such as growth factor delivery for bone infection and repair [20–22] and cancer chemotherapy [23–25]. Gelatin is composed of 18 different types of amino acids linked together to form a random-coiled structure with free amino and carboxyl groups. Based on the electrostatic interactions, it is conceivable that the increased number of free carboxyl groups in alkalized gelatin interacts with lysine-rich cationic peptides. These findings prompted us to investigate the effect of combination with anionic gelatin for controlled release of cationic peptide and its influence on the anti-tumor activity and peptide stability after i.v. administration.

Here, we prepared a peptide–gelatin ion complex, and then compared the anti-tumor activity and pharmacokinetic behaviors of the complex with the unmodified peptide in mice.

2. Materials and methods

2.1. Materials

The EGFR2R-lytic hybrid peptide and cysteine- or FITC-C-terminal conjugated peptides (EGFR2R-lytic-C or EGFR2R-lytic-FITC) used in this study were synthesized by the American Peptide Company (Sunnyvale, CA, USA) and Sigma (St. Louis, MO, USA), respectively. All peptides were synthesized by solid-phase chemistry, purified to homogeneity (>90% purity) by reversed-phase high-pressure liquid chromatography (HPLC), and assessed by mass spectrometry. The sequence of the EGFR2R-lytic peptide is as follows: YRWYGYTPQNVIGGGK**LLKLLKKLLKLLKKK** (bold letters indicate D-amino acids) [2]. A gelatin sample with an isoelectric point of 5.0 (molecular weight, 60 kDa), isolated by an alkaline process from the bovine bone, was kindly supplied from Nitta Gelatin (Osaka, Japan). ATTO 740 dye was purchased from ATTO-TEC (Am Eichenhang, Germany). Other agents were mostly from Nacalai Tesque (Kyoto, Japan). All agents were of reagent grade quality.

2.2. Animals

Female 7-week old BALB/c nu/nu mice or BALB/c mice (body weight, 17–20 g) were purchased from SLC Japan (Shizuoka, Japan) and maintained under specific pathogen-free conditions. All animal experiments were approved by the Animal Research Committee of Kyoto University and carried out in accordance with its guidelines.

2.3. Cell culture

The human pancreatic cancer cell line BxPC-3 was purchased from the European Collection of Cell Culture Collection (ECACC, Salisbury, UK), and the luciferase-expressing pancreatic cancer cell line BxPC-3-luc was obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan). The human embryonic kidney HEK293 cell line was purchased from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). Cells were cultured in RPMI-1640 (BxPC-3 and BxPC-3-luc) or MEM (HEK293) containing with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.4. Preparation and characterization of the peptide–gelatin complex

The peptide–gelatin ion complex is formed through electrostatic interaction between negatively charged gelatin and positively charged peptide. Gelatin (8 mg) was dissolved in 1 ml of phosphate-buffered saline (PBS; pH 7.4) overnight at 37 °C with stirring to obtain a stable gelatin solution. The peptide powder was dissolved in 1 ml of water and

buffered to pH 7.4. Both solutions were mixed at various molar ratios, and the reaction mixture was incubated for 3 h with stirring at room temperature to form a uniformly sized gelatin-based nanoparticles. Unreacted peptide was removed by ultrafiltration with Amicon Ultra-0.5 centrifugal filter devices (Millipore, Billerica, MA, USA) with a 30 kDa cutoff [26]. The concentration of peptide in the complex was determined with a NanoDrop Spectrophotometer (Thermo Fisher Scientific Rochester, NY, USA). The rate of complex formation was calculated with the following equation according to the manufacturer's protocol: $[(V_f \times C_f / V_o \times C_o)_{peptide\ solution} - (V_f \times C_f / V_o \times C_o)_{peptide–gelatin\ mixed\ solution}] \times 100$ (V_f, volume of filtrate solution; C_f, filtrate peptide concentration; V_o, volume of original starting peptide or mixed solution; C_o, original starting peptide concentration).

The mean particle size, size distribution, and zeta potential values of the complex were obtained with a Malvern Zetasizer Nano ZS (Westborough, MA, USA). The samples were diluted to a concentration of 1 mg/ml immediately prior to measurement. The complex formulation process was confirmed by using size-exclusion column chromatography (Shimadzu, Kyoto, Japan). A TSK-gel G3000SW$_{XL}$ 300 × 7.8 mm column (TOSOH, Tokyo, Japan) was used and the column eluent was detected at 280 nm with an SPD-6A UV detector (Shimadzu, Kyoto, Japan). The PBS (pH 7.4) mobile phase was delivered at a flow rate of 1 ml/min.

2.5. Evaluation of peptide release from the peptide–gelatin complex

In vitro release studies were performed with the FITC-labeled peptide. The FITC-labeled peptide–gelatin complex (20 μl) was incubated in PBS solution containing different concentrations of FBS (5%, 10%, 20%, 50% and 100%) or PBS alone (negative control) at 37 °C with continuous stirring. After different incubation periods, the released peptide samples were recovered in an Amicon Ultra centrifuge tube with a centrifugal filter device (cutoff 30 kDa). The samples were then analyzed with a Fluoroskan Ascent fluorescence microplate reader (Thermo Scientific, Waltham, MA, USA).

2.6. Cell viability assay

Cell viability was determined by a WST-8 assay as described previously [1–5]. Briefly, a total of 3×10^3 cells per well were seeded into 96-well microplates and incubated for 24 h in medium containing 10% heat-inactivated FBS. The cells were then incubated with increasing concentrations of peptide or complex in 100 μl of medium for 24 h or 120 h at 37 °C. Cell viability was then measured with WST-8 solution (cell count reagent SF, Nacalai Tesque, Kyoto, Japan).

2.7. Evaluation of the in vivo anti-tumor activity of the peptide–gelatin complex

Evaluation of anti-tumor activity with a xenografted mice model was carried out as described previously [1–5]. Briefly, 5×10^6 cells of BxPC-3 were resuspended in 150 μl of PBS, and subcutaneously transplanted into the right flank region of nude mice (day 0). On day 5, the mice were randomized into three groups: saline (control), peptide (1 mg/kg) and equivalent peptide-loading complex (1 mg/kg). To make sure that the same molar amount of peptide was injected, the doses of the peptide content were determined by a NanoDrop Spectrophotometer (Shimadzu, Kyoto, Japan). The mice were i.v. injected with the corresponding saline or peptide solution (50 μl/mice) three times a week for a total of nine doses. Tumors were measured with a caliper, and the tumor volume was calculated as follows: width^2 × length × 0.5. At the end of the treatment, the mice were killed and the main organs were removed. Histological examination was then performed by light microscopy after hematoxylin and eosin (H&E) staining.