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Research Paper

Enhancement of anti-tumor activity of hybrid peptide in conjugation with carboxymethyl dextran via disulfide linkers

Arong Gaowa [a], Tomohisa Horibe [a], Masayuki Kohno [a], Yasuhiko Tabata [b], Hiroshi Harada [c], Masahiro Hiraoka [c], Koji Kawakami [a,*]

[a] Department of Pharmacoepidemiology, Graduate School of Medicine and Public Health, Kyoto University, Kyoto, Japan
[b] Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan
[c] Department of Radiation Oncology and Image-applied Therapy, Graduate School of Medicine, Kyoto University, Kyoto, Japan

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ABSTRACT

To improve the anti-tumor activity of EGFR2R-lytic hybrid peptide, we prepared peptide-modified dextran conjugates with the disulfide bonds between thiolated carboxymethyl dextran (CMD-Cys) and cysteine-conjugated peptide (EGFR2R-lytic-Cys). *In vitro* release studies showed that the peptide was released from the CMD-s-s-peptide conjugate in a concentration-dependent manner in the presence of glutathione (GSH, 2 μM–2 mM). The CMD-s-s-peptide conjugate exhibited a similar cytotoxic activity with free peptide alone against human pancreatic cancer BxPC-3 cells *in vitro*. Furthermore, it was shown that the CMD-s-s-peptide conjugates were highly accumulated in tumor tissue in a mouse xenograft model using BxPC-3 cells, and the anti-tumor activity of the conjugate was more effective than that of the free peptide. In addition, the plasma concentrations of peptide were moderately increased and the elimination half-life of the peptide was prolonged after intravenous injection of CMD-s-s-peptide conjugates. These results demonstrated that the conjugate based on thiolated CMD polymer would be potentially useful carriers for the sustained release of the hybrid peptide *in vivo*.

1. Introduction

We previously reported that the EGFR2R-lytic hybrid peptide has cytotoxic and anti-tumor activities against EGFR over-expressing cancers both *in vitro* and *in vivo* [1,2]. To improve the pharmacokinetics of this hybrid peptide and its anti-tumor activity after intravenous (i.v.) injection, we subsequently prepared gelatin hydrogel nanoparticles based on the ionic interactions between anionic gelatin and the cationic peptide, and demonstrated that gelatin hydrogel nanoparticles exhibited a longer circulation time in the blood and higher anti-tumor activity than the free peptide [3]. However, gelatin hydrogel as a carrier system has a low capacity for the encapsulation of biological drugs, because the viscosity of gelatin solutions increases with increasing gelatin concentration and decreasing temperature [4]. Hence, the synthesis of injectable gelatin-based nanoparticles with high drug loading capacity is limited.

Stimuli-responsive drug release mechanisms developed over the past few decades present further promising strategies to improve the pharmacokinetics and biodistribution of drugs and significantly enhance their therapeutic efficacies. The design of the stimuli-responsive drug delivery system has generally been developed based on environmental properties, such as temperature [5,6], pH [7,8], and light [9,10], or on stimulation of biological agents such as enzymes [11,12] and glutathione (GSH) [13]. It is well known that the concentration of GSH is substantially higher in the intracellular environment than in the extracellular space [14], and higher in tumors than in normal tissues; thus, the differences of GSH concentration are important target for the delivery of anti-cancer drugs [15–18]. Various carriers such as gold [13] and gelatin nanoparticles [19,20] are used for GSH-responsive targeted delivery systems with cleavable disulfide spacers under reduced conditions. Glucose polymers in the form of dextrans have been used for more than 50 years as plasma volume expanders because of their relatively low immunogenicity [21]. Carboxymethyl dextran (CMD) is a dextran derivative and is frequently used as a macromolecular carrier for the delivery of drugs because of its low glomerular filtration rate and lower hepatic uptake [22–25]. Furthermore, it has been previously reported that thiolated CMD is a potential candidate for GSH-responsive drug release and could enhance the therapeutic efficacies of drugs [26,27]. Hence, we chose thiolated CMD for the preparation of GSH-responsive

* Corresponding author at: Department of Pharmacoepidemiology, Graduate School of Medicine and Public Health, Kyoto University, Yoshida Konoecho, Sakyoku, Kyoto 606-8501, Japan. Tel.: +81 75 753 4459; fax: +81 75 753 4469.
E-mail address: kawakami.koji.4e@kyoto-u.ac.jp (K. Kawakami).

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nanoconjugate as potential carriers for the delivery of the hybrid peptide in this study.

Here, we investigated the capacity of disulfide-based drug carriers for controlled release of EGFR2R-lytic hybrid peptide and its effect on the anti-tumor activity of the hybrid peptide *in vivo*. The amount of peptide released from conjugates during incubation with a phosphate buffered saline (PBS) solution containing GSH at 37 °C was measured *in vitro*. We then examined the cytotoxic activity of the peptide-loaded CMD conjugates against EGFR-expressing human pancreatic cancer cells, BxPC-3, *in vitro*. Finally, we investigated the effect of *in vivo* sustained release of peptide from conjugates on peptide pharmacokinetics, tumor accumulation, and the anti-tumor activity *in vivo*.

2. Materials and methods

2.1. Materials

Carboxymethyl dextran sodium salt (CMD, Mw: 15–20 kDa), L-cysteine, hydrogen peroxide, and glutathione reduced ethyl ester (GSH-OEt) were purchased from SIGMA (St. Louis, MO, USA). Ellman's reagent, 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from Thermo Fisher Scientific Inc., (Rockford, IL, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was obtained from Tokyo Chemical Industry Co., (Tokyo, Japan). Cysteine-conjugated hybrid peptide, EGFR2R-lytic-Cys, was synthesized by SIGMA, as previously reported [3]. ATTO 740-NHS fluorescence dye was obtained from ATTO-TEC (Am Eichenhang, Germany). Other agents were mostly from Nacalai Tesque (Kyoto, Japan). All agents were of reagent-grade quality.

The BxPC-3 (human pancreatic cancer) cell line was obtained from the European Collection of Cell Culture Collection (ECACC, Salisbury, UK), and the luciferase-expressing cell line BxPC-3-luc was obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan).

2.2. Synthesis of thiolated carboxymethyl dextran (CMD-Cys) polymer

The coupling reaction of L-cysteine to CMD was prepared as described previously, with some modifications [26,27]. The sulfhydryl group-containing compound, cysteine was attached to the carboxyl groups of the CMD via the formation of amide bonds following the synthetic scheme shown in Fig. 1. One gram of CMD was dissolved in 100 mL of demineralized water. The carboxylic acid moieties of the polymer were activated by the addition of EDAC to the final concentrations listed in Table 1. The reaction was allowed to proceed for 35 min. L-Cysteine was added in a weight ratio of 1:1 (polymer: cysteine) and the pH was adjusted to 4.0 with 1 M HCl. The reaction mixture was incubated and stirred for 3.5 h at room temperature. For purification of the CMD-Cys conjugate, dialysis was performed using Spectra/Por® 3 membrane (MWCO: 3.5 kDa; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) at (low acidic) pH ~ 3, as described previously [26,27]. Any unreacted ingredients were removed by dialysis repeated twice against 5 mM HCl, followed by 5 mM HCl containing 1% NaCl twice, and finally by 1 mM HCl solution. After dialysis, the purified polymers were lyophilized by freeze-drier for four days at −80 °C under reduced pressure and stored at 4 °C until use.

The amounts of free thiol groups immobilized to the CMD were quantified by Ellman's reagent and spectrophotometrical analysis as previously described [26–28].

2.3. Synthesis of CMD-s-s-peptide conjugates

The covalent attachment of peptide to CMD-Cys polymer was achieved by the formation of disulfide bonds between the thiol groups of the cysteine-conjugated peptide EGFR2R-lytic-Cys [(YRWYGYTPQNVIGGGKLLLK**LL**K**K**LLK**L**LKKK-Cys (bold letters indicate D-amino acids)] [3] and the CMD-Cys polymer. CMD-Cys polymer (10 mg) was dissolved in 1 mL of 0.1 M PBS solution (pH 3.5). Cysteine-conjugated peptide EGFR2R-lytic-Cys was dissolved in PBS solution (pH 7.4). Both solutions were mixed in a ratio of 1:2 (peptide:polymer) and hydrogen peroxide was added to the mixture drop by drop to a final concentration of 0.06% (*v*/*v*). The

Table 1
Synthesis of carboxymethyl dextran-cysteine (CMD-Cys) conjugates.

CMD/H_2O (g/mL)	EDAC (final conc., mM)	L-Cysteine (g)	SH-groups (mM)[a]
0.2/20	5	0.2	0.19
0.2/20	50	0.2	0.85
0.2/20	250	0.2	0.58
0.2/20	500	0.2	0.61

[a] The total amounts of thiol groups attached to the polymers quantified by Ellman's reagent.

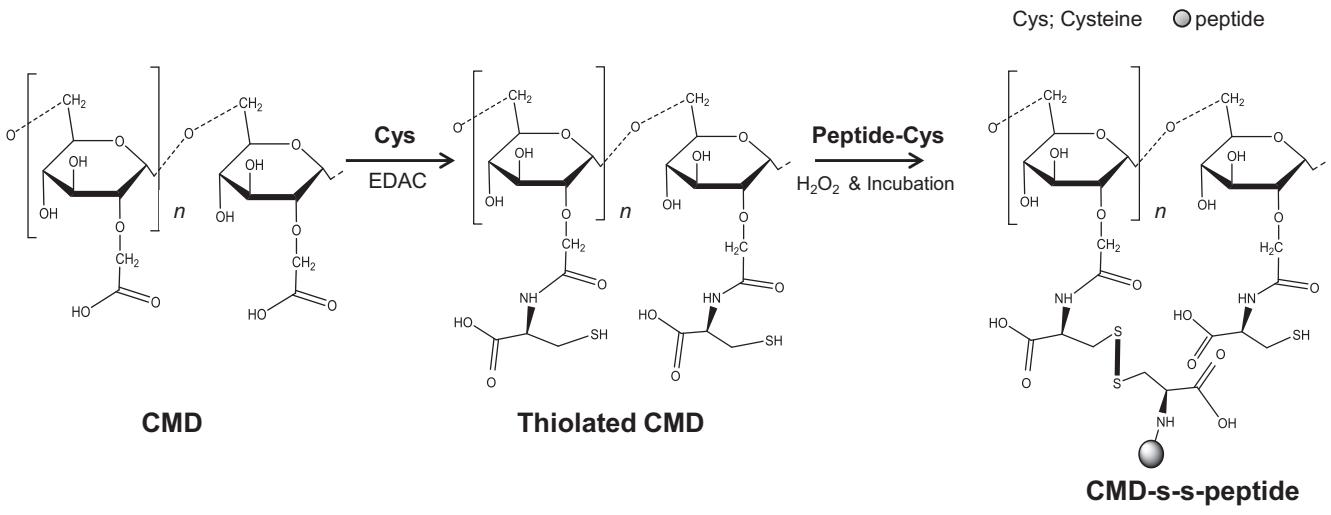

Fig. 1. Schematic diagrams of the synthetic process of CMD-s-s-peptide conjugate.