Tuning of Poly-*S*-Nitrosated Human Serum Albumin as Superior Antitumor Nanomedicine

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ABSTRACT: Macromolecules have been developed as carriers of low-molecular-weight drugs in drug delivery systems (DDS) to improve their pharmacokinetic profile or to promote their uptake in tumor tissue via enhanced permeability and retention (EPR) effects. We have previously demonstrated that poly-nitric oxide (NO) conjugated human serum albumin (Poly-SNO-HSA) has the potential to be a DDS carrier capable of accumulating NO in tumors. However, the stability of Poly-SNO-HSA in the circulation has to be improved, and its optimal molecular size for using the EPR effects has to be evaluated. In the present study, we performed two tuning methods for refining Poly-SNO-HSA, namely, pegylation and dimerization. We observed that pegylation enhanced the stability of Poly-SNO-HSA both *in vitro* and *in vivo*, and that dimerization of Poly-SNO-HSA enhanced the antitumor activity via more efficient delivery of NO in Colon 26 tumor-bearing mice. Intriguingly, dimerization resulted in a 10 times higher antitumor activity. These data suggest that pegylation and dimerization of Poly-SNO-HSA are very important tuners to optimize NO stability and accumulation, and thereby effect, in tumors. Thus, polyethylene glycol-Poly-SNO-HSA dimer seems to be a very appealing and safe NO carrier and thereby a strong candidate as an antitumor drug in future development of cancer therapeutics. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:2184–2188, 2014

Keywords: human serum albumin; nitric oxide; drug delivery system; antitumor activity; pegylation; dimerization; cancer; controlled release/delivery; macromolecular drug delivery; nanoparticles

INTRODUCTION

Cancer treatment remains one of the most important clinical challenges. One problem in treating various cancers is the development of multidrug resistance (MDR) by the cancer cells. Various approaches have been tested to overcome MDR such as the use of agents that inhibit P-glycoprotein directly or indirectly through altering the cell membrane. Most of the approaches have shown some success in small animal models but their clinical application has been limited. Because nitric oxide (NO) seems to be able to overcome MDR, and has other promising properties and effects, it has the potential of becoming a useful anticancer therapeutic.[1,2]

Nitric oxide is a free-radical gas involved in diverse biological processes, such as neurotransmission, blood pressure control, inhibition of platelet aggregation, and innate immunity.[3] However, under certain circumstances, NO can be cytotoxic. For example, high concentrations of NO can inhibit tumor cell growth and induce apoptosis.[4,5] Recent studies have revealed that NO is associated not only with apoptosis of cancer cells, but also with inhibition of cancer progression and metastasis, as well as cancer angiogenesis and microenvironment. It also functions as a modulator for chemo/radio/immunotherapy.[6] Despite highly useful properties, the *in vivo* half-life of NO is so short that NO itself cannot be used as a therapeutic agent. Such a short half-life can be overcome using continuous release of NO from a reservoir, such as the *S*-nitrosated form of human serum albumin (HSA).[7–9]

Our previous studies with cell cultures demonstrated that the NO influx from poly-nitric oxide conjugated human serum albumin (Poly-SNO-HSA) via cell surface protein disulfide isomerase is very fast and pronounced and leads to cell death caused by apoptosis. However, the apparent *in vivo* half-life of *S*-nitroso moieties in Poly-SNO-HSA has been estimated to be 18.9 min in tumor-bearing rats, indicating that it is not always a long-acting medicine.[10] Therefore, the release rate of NO from Poly-SNO-HSA needs to be well controlled and prolonged to develop more effective NO delivery systems. Shishido and de Oliveira[11] have shown that the thermal stability of *S*-nitrosothiols is increased in polyethylene glycol (PEG) solution because the cage effect of PEG increased the stability of S–NO bonding. Perhaps, this effect can be applied to construct more useful Poly-SNO-HSA preparations.

In addition to *in vivo* half-life, the problem of drug targeting should be addressed. In this respect, the enhanced permeability

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and retention (EPR) effect is relevant. This effect is now known to play a major role in the tumor-selective delivery of macromolecular or polymeric drugs, or of so-called nanomedicines including antibodies.[12–14] It is well known that long-circulating liposomes with an average diameter of 100–200 nm accumulate efficiently in tumor tissues.[15] Matsumura and Maeda[16] found that high-molecular-weight (40 kDa or higher), long-circulating macromolecules, as well as various long-circulating nanoparticulate pharmaceutical carriers are capable of spontaneously accumulating in various pathological sites, such as solid tumors and infracted areas. However, recently, Kataoka's group demonstrated that only 30-nm micelles could penetrate poorly permeable pancreatic tumors to achieve an antitumor effect.[17] These data suggest that macromolecular therapeutics with a size of 30 nm could efficiently deliver to even poorly permeable tumors. For testing this possibility, we have designed a HSA dimer, in which the C-terminus of one HSA molecule is linked to the N-terminus of another HSA molecule by the amino acid linker $(GGGGS)_2$, that can be successfully produced by the yeast *Pichia pastoris*. We have previously reported that HSA dimer has a longer circulation time than the monomeric form of HSA, HSA monomer, in rats and mice.[18] Moreover, HSA dimer could have an enhanced accumulation in solid and poorly permeable tumors via the EPR mechanism because of its molecular size of approximately 30 nm (130 kDa). Therefore, it is possible that HSA dimer could be of great clinical use as a new DDS material with a superior plasma retention property (e.g., prolonged plasma half-life) as well as with increased tumor-specific accumulation.

In this study, we performed two tuning methods of pegylation and dimerization of Poly-SNO-HSA. We examined the effect of pegylation on the stability of *S*-nitrosated sites of Poly-SNO-HSA *in vitro* and *in vivo*, and the effect of dimerization of Poly-SNO-HSA on its antitumor activity in Colon 26 (C26) tumor-bearing mice.

MATERIALS AND METHODS

Materials

Human serum albumin and HSA dimer were synthesized using the yeast *Pichia pastoris* (*P. pastoris*) (strain GS115) as previously described,[18] and the proteins were defatted by means of charcoal treatment.[19] Sephadex G-25 desalting column (ϕ 1.6 × 2.5 cm) was obtained from GE Healthcare (Kyoto, Japan). 1,4-dithiothreitol was obtained from Sigma–Aldrich (St. Louis, Missouri). Isopentyl nitrite (IAN) was bought from Wako Chemicals (Osaka, Japan). Diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid were purchased from Nacalai Tesque (Kyoto, Japan). $^{111}InCl_3$ was a gift from Nihon Medi-Physics Company, Ltd. (Hyogo, Japan).

Cells and Animals

C26 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (Sanko Junyaku Co, Ltd.,Tokyo, Japan), 100 units/mL penicillin, and 100 μg/mL streptomycin, incubated in a humidified (37°C, 5% CO_2 and 95% air) incubator, grown in 75-cm^2 flasks (Falcon BD) Tokyo, Japan, and passaged when 75% confluency was reached. Male BALB/cAnNCrlCrlj mice (5 weeks old, 17–22 g) were purchased from Charles River Laboratories (Ibaraki, Japan). A C26 solid tumor model was established by subcutaneously implanting 2×10^6 C26 cells into the back of the mice. Other animals, that is, male ddY mice and male Donryu rats, were from Kyudo Inc. (Kumamoto, Japan).

Pegylation and *S*-Nitrosation

To perform the pegylation of HSA, HSA–polyethylene glycol conjugate-5000 (PEG–HSA) was synthesized by reacting activated PEG with HSA in 50 mM borate buffer (pH 9.2) for 24 h at 4°C in the dark to react with available amino acids.[20] The reaction mixture was then washed and concentrated by ultrafiltration against distilled water. The average number of PEG moieties is 4.6 mol PEG/mol HSA, and the molecular weight of the product is estimated to be 90 kDa by using SDS-PAGE (data not shown).

Poly-nitric oxide conjugated human serum albumins were prepared according to a previous report.[4] In brief, terminal sulfhydryl groups were added to HSA, PEG–HSA, and HSA dimer by incubating 0.15 mM HSA, 0.15 mM PEG–HSA or 0.075 mM HSA dimer with 3 mM Traut's Reagent (2-imminothiolane) in 100 mM potassium phosphate buffer containing 0.5 mM DTPA (pH 7.8) for 1 h at 25°C. The resultant modified HSA, PEG–HSA, and HSA dimer was then *S*-nitrosated by 3 h incubation with 15 mM IAN at 25°C. The resulting Poly-SNO-HSA, PEG-Poly-SNO-HSA, or Poly-SNO-HSA dimer was concentrated, exchanged with saline using a PelliconXL filtration device (Millipore Corporation, Billerica, Massachusetts), and the final concentration was adjusted to 2 mM Poly-SNO-HSA, 2 mM PEG-Poly-SNO-HSA, or 1 mM Poly-SNO-HSA dimer. The samples were stored at 80°C until use . The number of NO moieties bound to Poly-SNO-HSA and PEG-Poly-SNO-HSA is 6.7 mol NO/mol protein, whereas the number bound to Poly-SNO-HSA dimer is 13.5 mol NO/mol protein.

Pharmacokinetic Experiments

Poly-nitric oxide conjugated human serum albumin was labeled with ^{111}In by using DTPA anhydride as a bifunctional chelating agent.[21] Labeled Poly-SNO-HSA was injected via the tail vein into male ddY mice (weighing 25–27 g) at a dose of 5 μmol NO/kg. At appropriate times after injection, blood was collected from the vena cava with the mouse under ether anesthesia. Heparin sulfate was used as an anticoagulant, and plasma was obtained from the blood by centrifugation. The radioactivity in each sample was counted using a well-type NaI scintillation counter ARC-2000 (Aloka, Tokyo, Japan). ^{111}In radioactivity concentrations in plasma were normalized as a percentage of the dose injected per milliliter and were analyzed by means of the nonlinear least-squares program MULTI.[22] We also measured the amount of *S*-nitrosated moieties in the plasma samples. That was performed by means of the Saville assay.[23]

Effect of Pegylation on the Stability of S-Nitrosated Sites of Poly-SNO-HSA

For *in vitro* study, Poly-SNO-HSA or PEG-Poly-SNO-HSA at a NO concentration of 100 μM was incubated in mouse blood or plasma at 37°C for different times in the dark. For *in vivo* study, Poly-SNO-HSA or PEG-Poly-SNO-HSA at doses of 5 μmol NO/kg were injected as a bolus through the tail vein of 5-week-old male Donryu rats (200–250 g), and NO concentrations in plasma were determined at 1, 15, 30, 60, 180, and 360 min after injection. The amounts of the *S*-nitrosated moieties of Poly-SNO-HSA and PEG-Poly-SNO-HSA were quantified using a 96-well plate. First, 20 μL aliquots of Poly-SNO-HSA or