Original Article

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N-terminal PEGylation of human serum albumin and investigation of its pharmacokinetics and pulmonary microvascular retention

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Summary **Human serum albumin (HSA) is used as an important plasma volume expander in clinical practice. In the present study, HSA was N-terminally PEGylated and a PEGylated HSA (PEG-HSA) carrying one chain of PEG (20 kDa) per HSA molecule was obtained. The purity, secondary structure and hydrodynamic radius of the modified protein were characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis, circular dichroism measurements, and dynamic light scattering, respectively. The pharmacokinetics in normal mice and vascular permeability of the PEG-HSA in a lipopolysaccharide-induced acute lung injury mice model were evaluated. The results showed that the biological half-life of the modified HSA was approximately 2.2 times of that of native HSA, and PEG-HSA had a lower vascular permeability which suggested that PEGylation of HSA could reduce extravasation into interstitial space. It can be inferred that due to the prolonged half-life time and enhanced vascular retention, the molecularly homogeneous PEG-HSA may be a superior candidate as a plasma volume expander in treating capillary permeability increase related illness.**

Keywords: Human serum albumin, N-terminal PEGylation, vascular permeability, pharmacokinetics

1. Introduction

Human serum albumin (HSA) is the major protein component of human plasma with a plasma content of 42 ± 3.5 g/L. It consists of a single non-glycosylated polypeptide chain of 585 amino acids forming a heart-shaped molecule with molecular weight of 66.5 kDa (*1*). HSA is responsible for 80% of the colloid osmotic pressure of plasma (25-33 mmHg). Because of this, its main clinical use is in maintaining colloid osmotic pressure and increasing circulating plasma volume. HSA is widely used to treat hypovolemia caused by traumatic shock, massive hemorrhage, burns, cirrhosis with ascites and hypoalbuminemia, with a usual dosage of 10 g/dose or more (*2*).

In spite of the many theoretical benefits of albumin infusion in critically ill patients, the clinical use of albumin is still controversial. It is reported that the loss rate of albumin to tissue spaces rose by more than 300% in patients with septic shock and the average increase occurring within 7 h of cardiac surgery was 100% (*3,4*). The transcapillary escape rate in cachectic cancer patients was twice that of healthy individuals (*5*). Increased vascular permeability is an important cause of the hypoalbuminemia commonly seen in acute and chronic disease (*6*). Under these conditions, correction of low plasma volume therefore becomes essential. However, the plasma expanding effect

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of HSA is transient due to a continuous leakage of macromolecules into the interstitial space (*7*), such poor intravascular retention does not only lead to interstitial edema but also demands frequent HSA infusion to maintain the desired blood concentration (*8*).

In addition, commercial production of HSA is primarily based on fractionation of plasma obtained from blood donors, which is limited in supply but has high clinical demand globally. Many efforts have been made to develop a substitute for plasma-derived HSA (pdHSA) by means of gene manipulation techniques (*2,9*). However, due to the high dosage of HSA in clinical applications, there is still an unsettled problem concerning the establishment of techniques for high purity, low cost, industrial, large-scale production of recombinant HSA (rHSA). Therefore, at present, plasma is still the most important source of HSA as a plasma volume expander.

In the present study, we hypothesized whether an albumin alternative agent with properties of good intravascular retention and a long life time could be developed to solve the problems of interstitial edema and frequent infusion. Increasing the molecular weight of HSA may be a very good potential approach to make a modified HSA as a superior volume expander. We conjugated HSA with polyethylene glycol (PEG) in a site-specific manner by taking advantage of the differences between the p*Ka* values of the α-amino group of the N-terminal amino acid residue and the ε-amino groups of the Lys residues in the protein backbone. This N-terminal PEGylation could generate a chemically well-defined and molecularly homogeneous modified HSA product. The secondary structure and hydrodynamic radius was characterized. In addition, a pharmacokinetics study of native HSA and PEGylated HSA (PEG-HSA) was conducted using an iodine-125 (^{125}I) isotope tracing technique in mice, and vascular permeability was evaluated in a murine model of lipopolysaccharide (LPS)-induced lung injury.

2. Materials and Methods

2.1. *Materials*

Propionaldehyde-derivatized 20 kDa linear monofunctional PEG (mPEG) was purchased from Beijing Kaizheng Biotech Development Co., Ltd., Beijing, China. HSA was kindly provided by Shandong Taibang Biologic Products Co., Ltd., Taian, China. ^{125}I was purchased from the China Institute of Atomic Energy, Beijing, China. DEAE Sepharose Fast Flow was purchased from GE Healthcare Bio-Sciences, Piscataway, NJ, USA. LPS from *Escherichia coli* 055:B5 was a product of Sigma-Aldrich, St Louis, MO, USA. Other reagents and chemicals were of commercially available analytical grade. Circular dichroism (CD) spectra were measured using a Chirascan spectropolarimeter, Applied Photophysics, Surrey, UK. Dynamic light scattering (DLS) measurements were performed on a DAWN® HELEOS™ multi-angle laser light scattering photometer, Wyatt Technology, Santa Barbara, CA, USA. Histology examination was conducted with an inverted fluorescence microscope (Olympus, Tokyo, Japan).

2.2. *Animals*

Four to five week-old male Kunming mice weighing 18-20 g used in the experiments were supplied by the experimental animal center of Shandong University. The animals were housed in animal facilities accredited by the Shandong Council on Animal Care and treated in accordance with approved protocols. Animals were maintained in a specific pathogen-free environment that was temperature-controlled (23 ± 2°C) and humidity-controlled (60 ± 10%), under a 12:12 h light/dark cycle.

2.3. *Preparation of 20 kDa mPEG-propionaldehyde-modified HSA*

The reaction was carried out with stirring at 4°C for 36 h using 25 mg freeze-dried HSA at a 5 mg/mL protein concentration in 10 mM sodium phosphate buffer, pH 6.0, containing 20 mM sodium cyanoborohydride. The PEG-propionalde derivative (Mw = 20 kDa, 23 mg) was added to give a 3:1 molar ratio of PEG/protein.

The reaction mixture was stopped by diluting the mixture with 10 volumes of buffer A (10 mM phosphate buffer, pH 6.5) and applied to a DEAE Sepharose FF column (2.6 cm × 20 cm) which was initially equilibrated with buffer A. The column was washed with buffer A to remove unconjugated PEG and then, bound proteins were eluted with an ascending linear gradient up to 40% buffer B (10 mM phosphate buffer, pH 6.5 containing 1 M NaCl) over 5 column volumes. The elution was monitored at 280 nm. The fraction containing mono-PEGylated HSA was collected and desalted by ultrafiltration employing a membrane with a 30 kDa cut-off. Protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Solarbio, Beijing, China) according to the manufacturer's instructions.

2.4. *Purity identification of PEGylated HSA using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

Reaction mixtures and fractions from the DEAE column were analyzed by SDS-PAGE. Samples in loading buffer were incubated at 100°C for 5 min, and loaded on a 10% polyacrylamide Tris-glycine gel according to Laemmli (*10*). Electrophoresis was programmed using a two-step mode applying constant voltages of 80 V in the stacking gel and 130 V in the separation gel. After approximately

2 h, gels were stained with Coomassie Brilliant Blue R-250 overnight and destained afterwards. Gel images were scanned immediately after the destaining steps on a gel imaging system.

2.5. *Secondary structure analysis by far-UV CD measurement*

CD analysis was used to examine the secondary structural conformation of HSA and PEG-HSA. CD measurements were taken on an Applied Photophysics Chirascan spectropolarimeter using a 1 mm circular quartz cell at room temperature. The spectra were recorded in the far UV (190~260 nm) using 5 μM HSA solution or an equimolar quantity of PEGylated HSA in 50 mM phosphate buffer, pH 7.4 (*8*). The bandwidth was 1 nm and time-per-point was 1 sec.

2.6. *Hydrodynamic radius determination by DLS*

DLS was used to characterize the hydrodynamic radius (R_h) of HSA and PEG-HSA molecules. A linearly polarized gallium arsenide (GaAs) laser operating at 658 nm was used to illuminate the sample which was maintained at 25°C. Observations were made at a scattering angle of 99°. Scattering intensity data were processed using the instrumental software to obtain the R_h and size distribution of each sample. All calculations were based on the Stokes-Einstein equation: $R_h = k_BT/6\pi\eta_0D$ where R_h is the hydrodynamic radius, k_B is Boltzmann's constant, T is the absolute temperature, η_0 is the solvent viscosity, and D is the translational diffusion coefficient (*11*). The protein samples were prepared by dissolution of an appropriated amount of freeze-dried powder in saline and filtered through a 0.22 μm filter (Millipore, Billerica, MA, USA).

2.7. *Pharmacokinetic study*

HSA and PEG-HSA were iodinated with $Na^{125}I$ using iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluri) methods as described previously (*12*). ^{125}I-labeled HSA and PEG-HSA solutions were administered as a single *i.v.* bolus injection in saline *via* the tail vein of Kunming mice. Blood samples (100 μL) were collected at 5, 20, and 45 min and at 1, 4, 8, 12, 24, 36, 48, 60, 72, 84, and 96 h post injection and plasma was harvested by centrifugation. ^{125}I-HSA or ^{125}I-PEG-HSA associated radioactivity in the plasma samples was determined after precipitation with 10% trichloroacetic acid (TCA) (*13*). The resultant TCA precipitates were counted for radioactivity using a Packard Cobra II Auto-Gamma Counter to determine the ^{125}I radioactivity associated with HSA. Pharmacokinetic parameters were evaluated using practical pharmacokinetic program version 97 (supplied by Chinese Pharmacological Society).

2.8. *Pulmonary microvascular permeability in the LPS-induced capillary leak model*

The vascular permeability of HSA and PEG-HSA was evaluated using LPS-induced capillary leak as a model system. Pulmonary capillary leak was induced by intranasal (*i.n.*) administration of 10 μg LPS in 50 μL saline, and 50 μL of saline solution alone was administrated to Kunming mice as a control (*14*). HSA and PEGylated HSA were labeled with fluorescein isothiocyanate (FITC) as described by Monsigny *et al*. (*15*). Mice were randomly allocated to three experimental groups: control group (saline + FITC-HSA), HSA-treated model group (LPS + FITC-HSA), and PEG-HSA-treated model group (LPS + FITC-PEG-HSA). FITC-labeled protein (0.42 mg/kg) were administered intravenously (*i.v.*) *via* a tail vein 20 min after the injection of LPS or saline. At 4 h post LPS administration, mice were anesthetized, and the thorax was opened, left atrium incised, and the lung was perfused *in situ* with isotonic saline *via* the pulmonary artery. Then the flushed pulmonary tissue was removed and fixed in 10% formalin for fluorescence micrograph studies. The fluorescence intensities of mice lung frozen sections were examined with an inverted fluorescence microscope using 40× objectives.

2.9. *Statistical analysis*

All data are expressed as mean ± S.D., except where otherwise noted. The data were statistically analyzed using student's *t*-test. Statistic analysis was performed using *3P97* (Chinese Academy of Sciences Mathematics Research Institute).

3. Results

3.1. *Preparation of 20 kDa mPEG-propionaldehyde-modified HSA*

The PEG-HSA conjugate was prepared by reductive alkylation between propionaldehyde derivatives of a 20 kDa mPEG molecule and the free α-amino group of the N-terminal amino acid residue of HSA. By taking advantage of the differences between the p*Ka* values of the α-amino group of the N-terminal amino acid residue and the ε-amino groups of the Lys residues in the protein backbone (*16*), the mPEG propionaldehyde was expected to be selectively conjugated to the N-terminal α-amino group of HSA and a homogeneous conjugate would be obtained. Figure 1 shows the results of SDS-PAGE analysis and DEAE Sepharose FF column chromatography for the products of the PEGylation reaction. SDS-PAGE analysis of native HSA and conjugation reaction mixture (lanes 2 and 3, respectively, in Figure 1A) showed that, compared with the lane of native HSA (Mw = 66.5 kDa) and the protein molecular

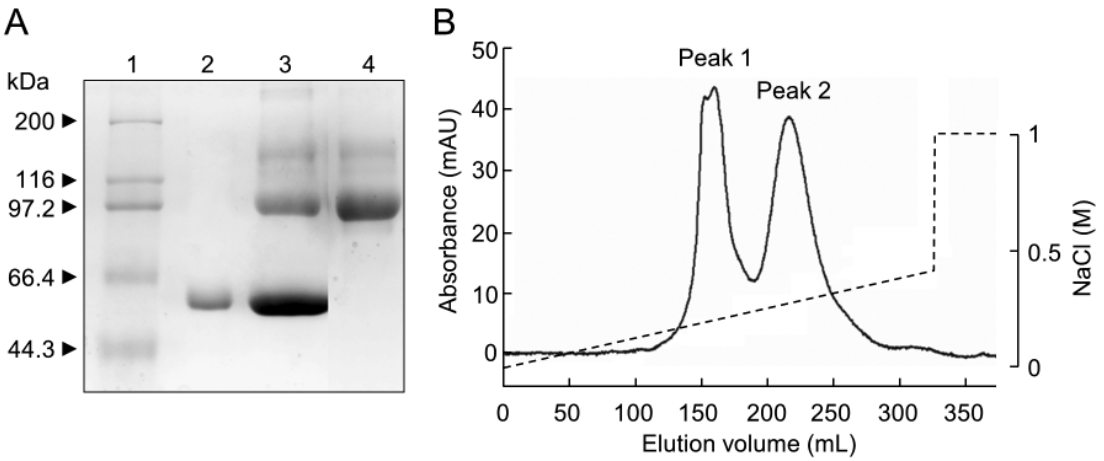

Figure 1. Purification of the PEGylated HSA. (A) SDS-PAGE analysis of column fractions. Gel was stained with Coomassie Brilliant Blue R-250. Lane 1, markers; lane 2, native HSA; lane 3, PEGylation reaction mixture; lane 4, peak 1 eluted from DEAE Sepharose FF column. Five micrograms of protein was applied in each lane. **(B)** Elution profile for DEAE Sepharose FF column chromatography of the HSA PEGylation reaction mixture. Solid line, absorbance at 280 nm; dashed line, NaCl concentration (M).

weight markers, a new protein band with an apparent molecular weight of about 100 kDa was observed in the reaction mixture lane. According to the molecular weight of the new protein, it could be inferred that only one PEG chain was attached onto each HSA molecule, and thus the mono-PEGylated HSA was predominantly produced by using mPEG-propionaldehyde. The modification rate was up to 35% as estimated by optical density scanning of the electrophoretic bands.

The PEGylated HSA was purified from non-PEGylated protein and unreacted PEG by ion exchange chromatography using a DEAE Sepharose FF column. As shown in Figure 1B, two major peaks were obtained with NaCl linear gradient elution. SDS-PAGE of the fractions showed a band with an apparent molecular weight of 90 kDa eluted at 0.18 M NaCl was PEG-HSA (lane 4 in Figure 1A, peak 1 in Figure 1B), whereas the unmodified PEG was eluted at 0.3 M NaCl (peak 2 in Figure 1B). The yield of the purified mono-PEGylated HSA was about 32%.

3.2. *Secondary structure analysis by far-UV CD measurement*

To obtain information of the secondary structure changes of PEG-HSA, CD measurements of HSA and PEG-HSA were performed in the far-UV region (Figure 2). The far-UV CD spectrum of the PEGylated HSA showed the same shape as that of native HSA. The result indicated that the PEG-HSA structure was almost identical to that of native HSA and PEGylation did not cause significant alteration of the secondary structure of HSA.

3.3. *Hydrodynamic radius determination by DLS*

The distribution of the hydrodynamic radius of HSA and PEG-HSA in saline obtained by DLS is shown in Figure 3. The protein concentration determined by BCA assay was 0.01 mg/mL. The hydrodynamic radius (R_h) distribution of PEG-HSA was more heterogeneous than that of native HSA due to polydispersity of the conjugated mPEG molecules. The calculated R_h of PEG-HSA was 50.7 nm, which was increased about 6.5 times as compared to that of the HSA molecule (R_h = 7.8 nm).

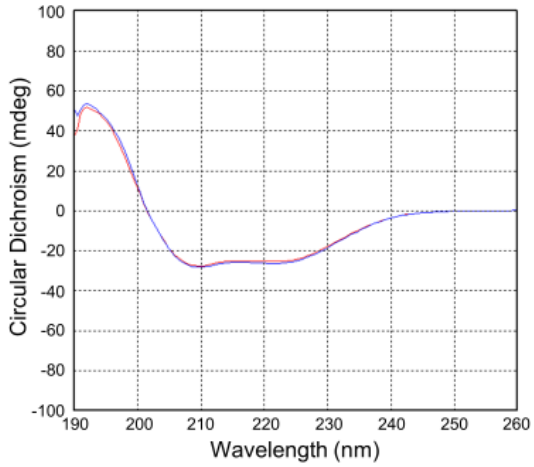

Figure 2. CD profiles of native HSA and PEG-HSA. CD spectra were recorded at room temperature with a 0.1 cm light path cuvette. All samples were dissolved in 50 mM phosphate buffer, pH 7.4. Red line, HSA; blue line, PEG-HSA.

3.4. *Pharmacokinetic study*

PEGylation of therapeutic peptides and proteins is a useful method for prolonging their circulation time in blood (*17*). The pharmacokinetics study of native HSA and PEG-HSA conjugate was conducted by the ^{125}I isotope tracing method. Blood levels of the proteins were measured from 5 min to 96 h post

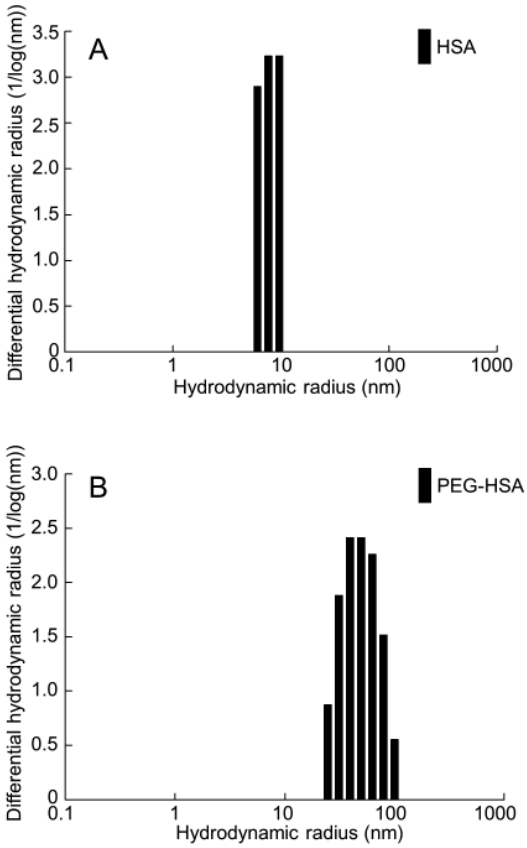

Figure 3. Hydrodynamic radius distribution of HSA and PEG-HSA. Hydrodynamic radius was determined by DLS at a concentration of 0.01 mg/mL of HSA **(A)** or PEG-HSA **(B)** in saline at 25°C.

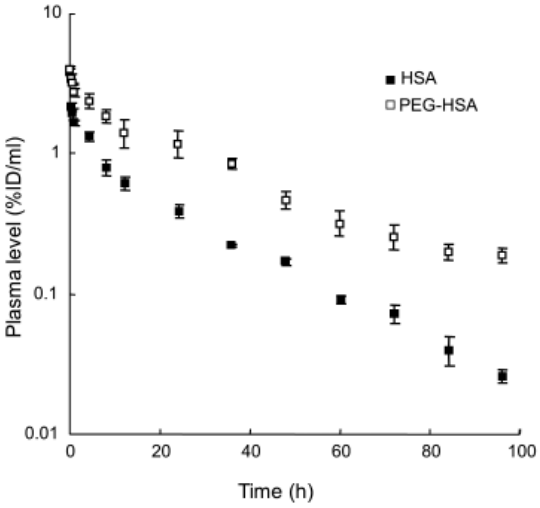

Figure 4. Plasma level of radiolabeled proteins after a single intravenous administration of ^{125}I-HSA or ^{125}I-PEG-HSA to normal mice. Closed symbol, ^{125}I-HSA; open symbol, ^{125}I-PEG-HSA. Data is expressed as a percentage of injected dose per milliliter plasma (%ID/mL) and each point represents the mean ± S.D. (n = 3).

Table 1. Pharmacokinetic parameters estimated by two-compartmental model analysis following a single *i.v.* bolus injection of ^{125}I-HSA or ^{125}I-PEG-HSA to mice.

Paramaters	HSA	PEG-HSA
$AUC_{0-\infty}$ (μg•h/mL)	2.79 ± 0.09	10.95 ± 1.06**
$t_{1/2\alpha}$ (h)	0.17 ± 0.08	0.82 ± 0.65
$t_{1/2\beta}$ (h)	10.3 ± 0.4	22.5 ± 1.7**
CL (mL/h)	0.39 ± 0.03	0.13 ± 0.01**
MRT (h)	22.6 ± 0.8	42.5 ± 3.4*
Vdss (mL)	5.62 ± 0.12	4.08 ± 0.44*

Data are expressed as mean ± S.D. (n = 3).* $p < 0.05$, ** $p < 0.01$, ^{125}I-PEG-HSA *vs.* ^{125}I-HSA.Abbreviations: HSA, human serum albumin; PEG-HSA, PEGylated HSA; AUC, area under the curve; CL, clearance; MRT, mean residence time; Vdss, steady state volume of distribution.

injection. The plasma radiolabeled HSA and PEG-HSA concentration versus time profiles following a single *i.v.* bolus injection in mice is shown in Figure 4, and the pharmacokinetic parameters for these two proteins using a two-compartment model are listed in Table 1. It can be seen in Figure 4, that the plasma concentrations of native HSA and PEG-HSA showed an exponential declining pattern after *i.v.* administration. The elimination of PEG-HSA was slower than that of native HSA. The half-life of the PEG-HSA (22.5 ± 1.7 h) was increased about 2.2 times, as compared to that of native HSA (10.3 ± 0.4 h). On the other hand, the ^{125}I-PEG-HSA showed a two-thirds decrease in clearance (CL), and a marked increase in the area under the plasma concentration-time curve (AUC) was observed. These results suggest that PEG-HSA has longer retention in blood circulation.

3.5. *Pulmonary microvascular permeability in LPS-induced capillary leak model*

Endotoxin, the bacterial LPS released from the bacterial cell wall, is considered to be an important eliciting factor in the development of acute lung injury (*18*). The increment of endothelial permeability is an indicator of lung injury in murine models (*19*). In this study, vascular permeability of native and PEGylated HSA was evaluated using a LPS-induced acute lung injury model. The extravasation of FITC-labeled protein into pulmonary tissue was used as an index of vascular permeability. As shown in Figure 5, under a fluorescent microscope, an increase in fluorescence intensity was observed in the HSA-treated model group compared with the control group, which meant the modeling succeeded. Meanwhile, a lower vascular permeability of FITC-PEG-HSA was observed compared to FITC-HSA. These results suggested that the PEGylation of HSA could reduce extravasation into lung parenchyma.

4. Discussion

HSA has been widely used for the treatment of shocks, burns, trauma, and various critical illnesses that result in albumin loss, but previous studies have produced conflicting results regarding the safety and efficacy of the use of albumin in critical illness with increased capillary permeability (*20,21*). Under such

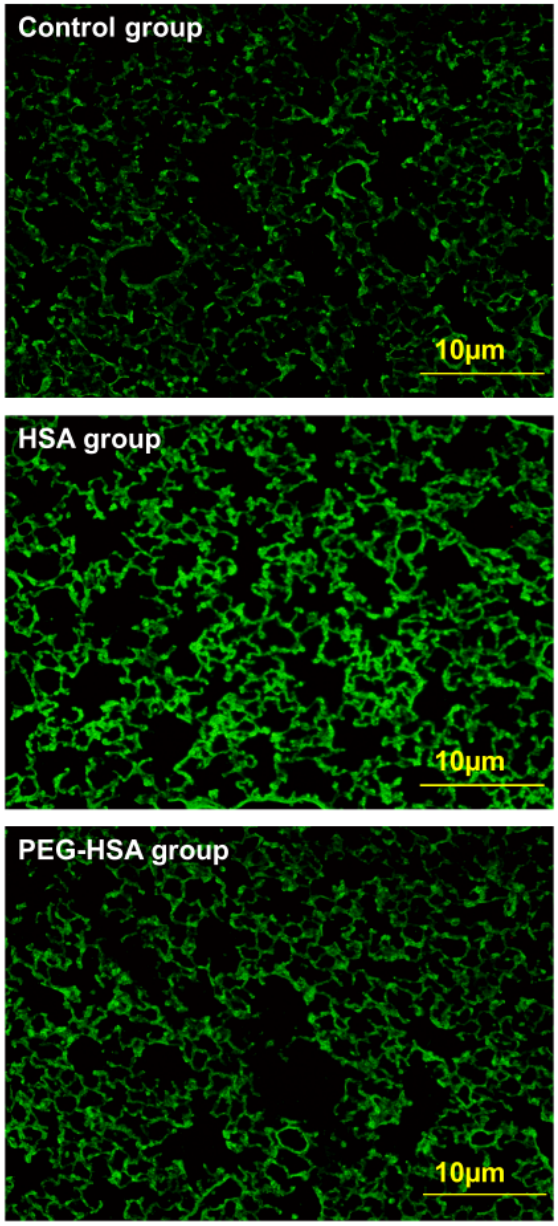

Figure 5. Typical fluorescence micrographs of pulmonary tissues at 4 h after LPS intranasal administration in acute lung injury mice. Vascular permeability of HSA and PEG-HSA was evaluated using a LPS-induced capillary leak model. Top, control group; middle, HSA-treated group; bottom, PEG-HSA-treated group. Bars, 10 μm.

conditions, the HSA infused as a plasma expander may be transported to tissue and bring on further edema. In this study, we modified HSA in a site-specific manner by covalent conjugation of commercial propionaldehyde-derivatized 20 kDa mPEG to the HSA molecule N-terminus. The structurally uniform PEG-HSA was easy to obtain and the preparation method was reproducible. As can be inferred from CD spectra, the structural characteristics of the PEGylated HSA are almost identical to that of native HSA (Figure 2), which indicated that PEGylation did not influence the secondary structure of HSA.

PEG conjugation is regarded as a valuable technique in applied biotechnology that makes protein drugs more water-soluble, non-aggregating, non-immunogenic and more stable to proteolytic digestion (*22*). PEG-propionaldehyde with a large molecular weight of 20 kDa was selected in order to endow the protein with a marked masking effect and a larger hydrodynamic radius. As each ethylene oxide unit of PEG binds 2-3 water molecules by hydrogen bonds, the PEG molecule acts as if it were five to ten times larger than a protein of comparable molecular weight (*23*). In our study, the hydrodynamic radius of PEG-HSA was increased by over 6.5 times, although the molecular weight of PEG-HSA was just 1.3 times that of native HSA (Figure 3, Table 1). The conjugation of HSA with the 20 kDa mPEG endows the HSA molecule with a great hydrated volume.

The pharmacokinetic analysis of PEG-HSA indicated that the PEGylated HSA showed a longer intravascular residence time (Figure 4). It has been reported that the mechanism of HSA breakdown involves endocytotic uptake into vesicles, which could fuse with lysosomes in endothelial cells (*24*). This process involves binding to the scavenger receptors (gp18 and gp30) on the endothelial surface membrane (*25*). Hence, it is likely that the degradation reduction effect of chemical modification of proteins is due to the masking effect of PEG attached to the surface of the HSA molecule, which can prevent recognition of the scavenger receptors, reticuloendothelial system and proteolytic enzymes.

Under normal physiological conditions, the HSA molecule distributes between the intravascular and extravascular compartments. Each day, 120~145 g of HSA is lost into the extravascular space and most of this is recovered back into the circulation by lymphatic drainage (*26*). Critical illness, such as sepsis, shock and major injury, breaks the distribution balance of HSA between the two compartments. The altered distribution is related to an increase in capillary leakage. In this situation, using exogenous albumin to increase the plasma albumin concentration seems beneficial, but it is argued that the infused albumin was inefficient in reducing liquid shift and may contribute to delayed pulmonary edema (*27*). In this study, we expected that increasing the molecular size of albumin by PEG modification could reduce the flow of albumin across capillary membranes under conditions with increased capillary leakage. LPS-induced acute lung injury was used to compare the pulmonary microvascular permeability properties of HSA and PEG-HSA. In this model, inflammatory cascade reactions induce increased microvascular permeability and capillary leakage, especially in pulmonary tissue (*23*). We observed a significant lower vascular permeability of the PEG-HSA at 4 h after injection (Figure 5), which indicated that increasing the molecular size of albumin by PEG modification could reduce transcapillary loss

of albumin molecules. Based on the hydrodynamic radius determination and pulmonary microvascular permeability measurement, it can be inferred that increasing the molecular weight of HSA by PEGylation may contribute to improvement of intravascular retention, and prevent intravascular fluid loss and interstitial liquid accumulation.

The results of the pharmacokinetic study and pulmonary microvascular permeability of N-terminal PEGylated HSA in this study are very similar to those of Cys34-specific PEGylated HSA as we described previously (*29*). The molecular weights of PEG reagents applied to these two conjugations targeting different modification sites were both 20 kDa. Many studies have shown that the *in vivo* behavior and biological properties of drug-PEG conjugates were markedly dependent on the molecular weight of the PEG conjugated (*30,31*). This information provides a reasonable explanation for our results. However, the PEGylation on the two modification sites (N-terminal amino group and sulfydryl group at Cys34) may have different influences on the drug binding functions of HSA, and this work is being carried out in our lab.

In conclusion, the present study offers a simple, reliable method to reduce vascular permeability and improve half-life in circulation of infused HSA, and consequently promotes intravascular retention and decreases administration frequency. Our main task in the future is investigation of pharmacodynamics of PEGylated HSA, optimization of modification strategies and *in vivo* safety evaluation of different modified products.

Acknowledgements

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