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Characterization of a monoPEG20000-Endostar

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ABSTRACT

In this study, we investigated the PEG attachment site of mono-PEGylated Endostar, a modified recombinant human endostatin approved in China for lung cancer. N-terminal site-directed mono-PEGylation of Endostar was accomplished using mPEG-propionaldehyde derivatives (Mw = 20 kDa) under slightly acidic pH conditions (pH 5.5). One-step cation exchange chromatography was used to purify the mono-PEGylated Endostar. Following tryptic digestion, the peptide fragment containing PEG was separated by SDS-PAGE. Barium iodide staining and Western blotting were used to detect the PEG moiety and the N-terminus of Endostar, respectively. The peptide fragment stained by barium iodide showed a positive response to anti-(His) 6 mAb, demonstrating that PEG was located at the N-terminus of Endostar. LC/MS was applied to verify the occurrence of mono-PEGylation at the N-terminus of Endostar.

1. Introduction

Endostatin, the 20 kDa C-terminal fragment of collagen XVIII, inhibits endothelial proliferation and potently suppresses angiogenesis and tumor growth [1]. Many animal and human tumors in mice have been inhibited by the injection of endostatin protein, such as Lewis lung carcinoma, neuroblastoma, and lung metastasis of mouse lung adenocarcinoma [2–4]. As an effective anticancer agent, endostatin was quickly evaluated in clinical trials. However, it was difficult to obtain sufficient quantities of endostatin for clinical evaluation.

Endostar, a novel recombinant human endostatin, is superior to other recombinant endostatins. At the N-terminus of the protein, there is an additional nine-amino acid sequence, MGGSHHHHH, including a six-histidine tag. These nine amino acids not only simplified the purification and increased the yield but also stabilized the protein [5]. Endostar was approved in China for the treatment of non-small-cell lung cancer in 2005.

As a polypeptide drug, Endostar inevitably has several limitations during clinical application, such as susceptibility to degradation by proteolytic enzymes and hence a short half-life *in vivo*. Many therapeutic proteins such as enzymes and cytokines have been significantly improved by PEGylation. It is known that covalent modification with poly(ethylene glycol) (PEG) masks the surface of the protein and increases the molecular size of the polypeptide, thus decreasing immunogenicity and kidney filtration, and increasing solubility and *in vivo* residence time [6]. PEGylation also protects the protein from proteolytic enzymes, thus increasing its stability and prolonging its half-life *in vivo* [7].

Amino groups are the first target of PEGylation. Amino modification, so far, represents the most common strategy and often the first approach in many new PEG-protein drugs. However, it is unavoidable that high numbers of isomers will emerge. These isomers often have different activities and it is usually difficult to purify the mixtures [6]. Therefore, a site-directed modification is necessary, not only for purification but also for the preservation of bioactivity. Recently, conjugation of PEG to thiol or amide groups has been attempted by using several specific chemical or enzymatic methods [8,9]. These methods, however, may affect the structure of the protein. Kinstler et al. devised a method based on the low pK_a of the N-terminal α-amino groups compared with that of the ε-amino group in lysines [10]. When the PEG-aldehyde was conjugated at low pH conditions, PEGylation at the N-terminus site occurred preferentially. This strategy has been successfully used in the PEGylation of G-CSF that was approved by the FDA in 2002 [11].

Nie et al. PEGylated human recombinant endostatin using PEG-aldehyde and proved N-terminal PEGylation was important for the stability of endostatin [12]. However, the PEG attachment site was not investigated. It is known that determination of the PEGylation site is one of the most important requirements for fully understanding PEGylated molecules. In previous studies, mass spectrometry (MS) was indispensable for the localization of the PEGylation site [13,14]. In this study, Endostar was PEGylated in a site-directed manner using PEG-propionaldehyde derivatives. Western blotting was introduced to locate the modification site. In addition, the result was confirmed by the application of peptide mapping.

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2. Experimental

2.1. Materials

Endostar, expressed in *E. coli*, was provided by Simcere Pharmaceutical R&D Co., Ltd. mPEG-propionaldehyde (methoxy-poly(ethylene glycol)-propionaldehyde) (Mw = 20,000) was purchased from Jemken Technology (Beijing, China). CM Sepharose FF was obtained from GE Healthcare (Piscataway, NJ, USA). The sequencing-grade endoproteinase, trypsin was from Roche. Anti-(His) 6 mAb was from GenScript Corporation (Piscataway, NJ, USA). Goat anti-mouse IgG/horseradish peroxidase conjugate and the DAB Kit were purchased from ZhongShan Goldenbridge Biotechnology Co., Ltd. (Beijing, China). 5 μm particle size, 30 nm pore size C_4 reversed phase column (4.6 mm × 250 mm) was obtained from YMC (Kyoto, Japan). The protein & peptide C18 column (4.6 mm × 250 mm) was supplied by Vydac (Hesperia, CA, USA).

2.2. PEGylation of Endostar with mPEG-propionaldehyde derivatives

Endostar and mPEG-propionaldehyde derivatives (Mw = 20 kDa) were dissolved in sodium acetate buffer (20 mM, pH 5.5) in the presence of sodium cyanoborohydride (20 mM) as a reducing agent. The reaction was conducted for 12 h with stirring at 4 °C. Reaction mixture was analyzed by SDS-PAGE. To examine the PEG, the gel was stained based on the formation of a barium iodide complex with PEG [15]. After electrophoresis, the gel was placed in 5% barium chloride solution and then 0.1 M iodine solution was added. The gel was then re-stained with Coomassie brilliant blue to detect the protein.

2.3. Separation and purification of mono-PEGylated Endostar

Endostar conjugates were purified by CM Sepharose FF cation exchange chromatography. The resin was packed using a 1.6 cm internal diameter column with a bed height of 5 cm. The column was equilibrated with 20 mM sodium acetate, pH 5.5 (buffer E) at a speed of 100 cm/h. The reaction mixture was diluted with buffer E and then loaded onto the column. Following loading, the column was washed with 2 column volumes of buffer E. The protein was eluted using a linear gradient to 1 M NaCl in buffer E. Fractions were collected and analyzed by SDS-PAGE and RP-HPLC.

2.4. Reversed phase HPLC (RP-HPLC)

PEGylated Endostar was analyzed on an YMC-Pack C_4 column (4.6 mm × 250 mm). The column was equilibrated in 60% (v/v) buffer A (HPLC grade H_2O containing 0.1% TFA, v/v) and 40% (v/v) buffer B (CH_3CN containing 0.1% TFA) with a flow rate of 1.0 mL/min. The elution consisted of a linear gradient program from 40% to 45% buffer B over 40 min, maintained at 45% buffer B for 5 min, then returned to 40% buffer B over 5 min, and maintained at 40% buffer B for 15 min. Eluates were monitored at the wavelength of 280 nm.

2.5. MALDI-TOF

MALDI-TOF-MS was carried out using the 4700 Proteomics Analyze (Applied Biosystems, CA, USA). A 1% (w/v) solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) in 50% methanol (v/v) was used as a matrix solution. Two microliters of sample containing 20 μg of protein was added. The spectra were obtained in the linear mode with an accelerating voltage of 20,000 V. A Nd-YAG laser was set to deliver 355 nm wavelength pulses onto the sample.

2.6. Tryptic digestion

PEGylated Endostar was adjusted to 1 mg/ml. Following dialysis (twice) in a 1% NH_4HCO_3 buffer, trypsin was added with a PEGylated Endostar/trypsin weight ratio of 50:1. Endostar was treated in the same way as control. The proteolytic digestion was carried out at 37 °C. After 24 h, the reaction was terminated by the addition of 50% (v/v) acetic acid.

2.7. Western blotting

After digestion, the mixture was analyzed by 12% SDS-PAGE. The mono-PEGylated Endostar without digestion, unreacted PEG and unmodified Endostar were used as controls. The gel was stained by both barium iodide and Coomassie brilliant blue. Four duplicate samples on the same gel were used for Western blotting. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane. The anti-(His) 6 mAb (1:5000) was used as primary antibody to detect the N-terminus of PEGylated Endostar. Goat anti-mouse IgG/horseradish peroxidase conjugate was used as secondary antibody (1:5000). Immunoreactivity was detected by the DAB Kit.

2.8. LC–MS analysis

Chromatographic analysis was performed using an Agilent 1200 HPLC system with a reversed phase chromatographic Vydac 218TP54 C18 column, 4.6 mm × 250 mm (5 μm). Mobile phase was comprised of 0.05% TFA in 100% water (A) and in 100% acetonitrile (B). Gradient applied was 0–70% B over 70 min. Approximately 100 μg of trypsin-treated peptide fragments were loaded onto the column. UV detection was monitored at 214 nm. The Agilent 1200 LC instrument was connected to a micrOTOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The ESI-TOF mass spectra were obtained under the following conditions: ionization was performed in the positive mode; nitrogen was used both as a nebulizer gas (pressure 0.8 bar) and a drying gas (flow rate 8 L/min); drying temperature was 200 °C; applied capillary voltage was set at 4500 V, capillary exit was 100.0 V and skimmer 1 was set to 40.0 V; transfer time of the ions from hexapole to orthogonal acceleration was 80 μs and hexapole radio frequency was 400.0 Vpp. TOF MS was operated with micrOTOF control version 2.2 from Bruker Daltonics.

3. Results and discussion

3.1. PEGylation of Endostar

Endostar consists of 192 amino acids and two disulfide bonds. Besides the α-amino group at the N-terminus, there are additional five potential PEGylation sites: Lys84, Lys104, Lys115, Lys126 and Lys192 (Fig. 1). The conjugation reaction was conducted under slightly acidic conditions (pH 5.5) for 12 h. Under these conditions, PEG-propionaldehyde derivatives preferentially reacted with the N-terminal α-amino group of Endostar to produce an alkylated linkage between PEG and Endostar due to the different pK_a values between the ε-amino group and the α-amino group.

However, ε-amino group of Lys was still slightly reactive to PEGylation. After forming a mono-PEGylated protein, a diPEGylated molecule and species with a higher degree of PEGylation subsequently appeared. As indicated in Fig. 2, several bands with molecular sizes larger than mono-PEGylated Endostar were observed on the gel, which likely represented oligo-PEGylated Endostar. The mono-PEGylated Endostar was separated by cation exchange chromatography. The purity of the mono-PEGylated Endostar was measured by SDS-PAGE (Fig. 2) and