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N-terminal specificity of PEGylation of human bone morphogenetic protein-2 at acidic pH

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ABSTRACT

Site-specific PEGylation offers the possibility to modify a therapeutic protein without interfering with its biological activity. Previously, a preferential N-terminal PEGylation has been reported for several proteins when the reaction was performed at acidic pH. In the present study it was explored if acidic pH favors N-terminal PEGylation of bone morphogenetic protein-2 (BMP-2). PEGylation by poly(ethylene glycol) aldehyde (PEG-AL) or poly(ethylene glycol) carboxymethyl succinimidyl ester (PEG-NHS) was performed at moderate acidic pH of 4. Comparing with PEG-NHS, PEG-AL converted more BMP-2 mainly to mono- or di-PEGylated derivatives at much less molar excess and shorter duration. Analysis of Tryptic fragments of the PEGylated derivatives indicated a partial N-terminal PEGylation specificity. PEG-AL exhibited higher specificity than PEG-NHS. UV spectrometry proved that PEGylation improved the solubility of BMP-2 in PBS. Surface plasmon resonance showed that PEGylation decreased the binding of BMP-2 proteins to a type II receptor. Remarkably, mono-PEGylated BMP-2 with PEG-AL showed higher cellular bioactivity than unmodified protein. Higher N-terminal PEGylation specificity correlates with higher receptor binding affinity and cellular activity. In summary, PEGylation of BMP-2 by PEG-AL and PEG-NHS at acidic pH exhibits a partial N-terminal specificity which however might be sufficient for an efficient site-specific PEGylation process.

1. Introduction

Bone morphogenetic protein-2 (BMP-2), as a member of the large transforming growth factor-β (TGF-β) superfamily of multifunctional cytokines, can induce ectopic bone and cartilage formation in adult vertebrates (Reddi, 1997, 1998) and plays important roles in early embryonal development in animals (Hogan, 1996). It has received large interest during the past decade due to its therapeutic use in regenerative medicine. BMP-2 is applied together with a carrier during surgery (Seeherman and Wozney, 2005). Its poor solubility under physiological conditions, the presence of a heparin-binding site, and a short circulation half life promote a desirable localized action of BMP-2 for indications as spinal fusion, repair of non-union fractures, and sinus lift augmentation (Robinson et al., 2008; Kwong and Harris, 2008).

Protein PEGylation (attachment with PEG) has been a successful approach to slow down ultrafiltration in the kidney, to alter tissue distribution, and to reduce phagocytosis, proteolysis and immunogenicity (Veronese and Pasut, 2005; Caliceti and Veronese, 2003). BMP-2 and its analogues TGF-β1 and TGF-β2 were PEGylated by poly(ethylene glycol) carboxymethyl succinimidyl ester (PEG-NHS) under slightly alkaline conditions to immobilize protein in tissue engineering scaffolds in order to improve local bone regeneration (Bentz et al., 1998), lengthen local fibroblastic response (Liu et al., 2007) or increase muscle matrix production (Brebda et al., 2001). This work mostly focused on the immobilization and did not analyze the attachment site of PEG and the subsequent effect on the bioactivity of proteins.

PEGylation protocols frequently employ amine reactive PEGs. This however, can lead to differing attachments and loss of biological activity, since lysine residues are abundant in proteins and are often part of functionally important sites. Thus, site-specific PEGylation is an attractive possibility offering the advantages to retain bioactivity of protein drugs and to generate chemically identical entities with predictable pharmaceutical behavior. Different routes were employed for this purpose. Mutagenesis technique has been the only general route currently employed to delete unde-

Abbreviations: BMP, bone morphogenetic protein; PEGylation, conjugation with poly(ethylene glycol); TGF, transforming growth factor; PEG-NHS, poly(ethylene glycol) carboxymethyl succinimidyl ester; G-CSF, granulocyte-colony stimulating factor; GLP-1, glucagon-like peptide-1; EGF, epidermal growth factor; PEG-AL, PEG aldehyde; DMSO, dimethylsulfoxide; $NaBH_3CN$, sodium cyanoborohydride; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; HCl, hydrogen chloride; NaCl, sodium chloride; PBS, phosphate buffered saline; ALP, alkaline phosphatase; BMP-2-monoPEG-AL, mono-PEGylated BMP-2 by PEG-AL; BMP-2-diPEG-AL, di-PEGylated BMP-2 by PEG-AL; BMP-2-monoPEG-NHS, mono-PEGylated BMP-2 by PEG-NHS; BMP-2-diPEG-NHS, di-PEGylated BMP-2 by PEG-NHS.

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sired potential attachment site or generate single attachment site, and subsequently realize site-specific PEGylation (Rosendahl et al., 2009; Zappe et al., 2008; Veronese et al., 2007). In our previous work, based on the fact that BMP-2 does not contain free cysteine in its homodimer (Scheufler et al., 1999), BMP-2 cysteine analogues were generated by site-directed mutagenesis at amino acid positions Ala 2, Asn 56, and Glu 96 (Hu et al., 2010). PEGylation at engineered Cys 56 and 96 located near the receptor binding epitopes exhibited significant reduction in BMP-2 activity. PEGylation at Cys 2 in the N-terminal segment far away from binding epitopes showed a bioactivity comparable to BMP-2 wild type. However, cysteine specific PEGylation is demanding both with respect to work and time. Often PEGylation products are obtained in low yields, since mutants have to be generated and SH chemistry requires special precautions. Therefore, it would be desirable to develop easier methods to achieve site-specific PEGylation with retention of the bioactivity of proteins.

Kinstler et al. (2002) developed an easy and specific PEGylation method for the N-terminal amino group by using PEG aldehyde or PEG-NHS at moderate acidic pH. The selective reaction of the N-terminal α-amine in the presence of lysine ε-amines was attributed to their different pK_a values (7.6–8.0 for the former and 10.0–10.2 for the latter). Granulocyte-colony stimulating factor (G-CSF) (Kinstler et al., 1997), glucagon-like peptide-1 (GLP-1) (Lee et al., 2005), epidermal growth factor (EGF) (Lee et al., 2003), and endostatin (Nie et al., 2006) were reported to be PEGylated specifically at the N-terminus employing this approach.

The present experiments explore the specificity of PEGylation of BMP-2 at acidic pH. Mature BMP-2 exists as a disulfide-bonded homodimer consisting of nine lysine residues in each monomer. Four lysine residues occur in a flexible N-terminal segment and five in the cysteine knot domain, which is crucial for receptor binding (Scheufler et al., 1999). Six of the lysines are recovered in a large Tryptic fragment of BMP-2. Now, PEG-AL and PEG-NHS were reacted with wild type BMP-2 in a simple one-step reaction. The two PEGs were compared with respect to reactivity, yield and site-specificity. The solubility, the receptor binding activity, and cellular activity of PEGylated proteins were investigated.

2. Materials and methods

2.1. Materials

Recombinant human BMP-2 was expressed in *Escherichia coli* and purified as described previously (Ruppert et al., 1996; Groppe et al., 1998).

Poly(ethylene glycol) aldehyde (PEG-AL, Mn = 5008 Da, SUNBRIGHT ME-050AL), and poly(ethylene glycol) carboxymethyl succinimidyl ester (PEG-NHS, Mn = 5383 Da, SUNBRIGHT ME-050AS) were purchased from NOF, Inc. (Kyoto, Japan). Functional PEGs were dissolved in analytical grade dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO, U.S.A.) and stored at −20 °C. Sodium cyanoborohydride ($NaBH_3CN$) was purchased from Sigma (St. Louis, MO, U.S.A.). Buffer chemicals and SDS-PAGE chemicals were purchased from Carl Roth (Karlsruhe, Germany). Phenylmethylsulfonyl fluoride (PMSF) and Trypsin (excision grade) were purchased from Merck (Darmstadt, Germany).

2.2. PEGylation at analytical scale

BMP-2 (0.1 mg, 1 mg/mL) and different amounts of PEG-AL or PEG-NHS were incubated in pH 4.0 acetic acid buffer (50 mM sodium acetate-acetic acid, pH 4.0) and 10% (v/v) DMSO. 40 mM $NaBH_3CN$ was added during reaction with PEG-AL. The PEGylation mixture was shaken at 700 rpm at 4 °C for up to 10 h for PEG-AL and 48 h for PEG-NHS.

Samples were taken at different time intervals and diluted 4 folds with non-reducing loading buffer for SDS-PAGE.

2.3. PEGylation at preparative scale

2.3.1. With PEG-AL

BMP-2 (5 mg, 1 mg/mL) and PEG-AL (2 mg/mL, molar ratio of PEG to protein of 10:1) reacted in pH 4.0 acetic acid buffer plus 40 mM $NaBH_3CN$ and 10% (v/v) DMSO. The reaction was shaken at 700 rpm at 4 °C for 10 h.

2.3.2. With PEG-NHS

BMP-2 (2 mg, 1 mg/mL) and PEG-NHS (50 mg/mL, molar ratio of PEG to protein of 250:1) reacted in pH 4.0 acetic acid buffer plus 10% (v/v) DMSO. The mixture was shaken at 700 rpm at 4 °C for 48 h.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a 12% polyacrylamide as described previously (Laemmli, 1970). 2–5 folds dilution by non-reducing sample buffer was used for sample preparation. The gels were stained with Coomassie Blue.

2.5. Evaluation of stained gels with Image J

Image J was downloaded from the website of the National Institutes of Health (http://rsbweb.nih.gov/ij/). The intensity distribution of the stained protein bands after SDS-PAGE was determined with Image J at 2-digit accuracy.

2.6. Reversed phase HPLC (RP-HPLC)

RP-HPLC was performed on a liquid chromatography system equipped with a GRACE VYDAC 214TP54 C4 column (250 mm × 4.6 mm), a Merck Hitachi L-6200A intelligent pump, a Merck Hitachi L-4000 UV detector and a Gilson FC203B Fraction Collector. The wavelength in the detector was set at 280 nm.

The PEGylation mixture (2–5 mL) was concentrated 4–10 folds with a centrifugal filter device Centricon® (MwCO 5000) to 0.5 mL. Then buffer was changed to 0.1% trifluoroacetic acid (TFA) by 3 times dilution and concentration to remove most of DMSO. The solution was then loaded on the 0.1% TFA equilibrated RP-HPLC column. For elution, 0.1% TFA (eluent A) and acetonitrile (eluent B) were used. Gradient starts from 0% to 25% eluent B in 10 min, 25–80% B in 55 min, and then 80–100% B in 5 min.

2.7. Trypsin digestion

0.1 mg PEGylated BMP-2 dissolved in 70 μL 1 mM HCl was mixed with 10 μL 0.1 mg/mL Trypsin solution in 1 mM HCl. A 20 μL solution of 2.5 M Tris and 250 mM NaCl, pH 8.5 was then added, and the whole mixtures were kept at 37 °C for 4 h. 1 mM PMSF was added to stop the reaction before sample preparations for SDS-PAGE.

2.8. Solubility analysis in phosphate buffered saline (PBS)

The UV spectra of PEGylated BMP-2 in PBS (150 mM NaCl, 10 mM sodium phosphate salt, pH 7.4) were recorded with an UV–Visible Spectrophotometer (CARY 50 BIO, Varian). Unmodified BMP-2 was used as the control. Stock aqueous solutions containing 4 μM of free or PEGylated BMP-2 were diluted with the same volume of double concentrated PBS, mixed thoroughly and kept at room temperature for 2 h. The spectra of the solution were recorded between 250 nm and 320 nm.