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Use of a non-covalent cell-penetrating peptide strategy to enhance the nasal delivery of interferon beta and its PEGylated form

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ABSTRACT

The conjugation of therapeutic proteins to polyethylene glycol (PEG) is known as PEGylation. It improves their retention in the body and reduces the frequency of injections. Development of noninvasive delivery systems for biopharmaceuticals can improve the patients' quality of life. The present study aimed to evaluate the cell-penetrating peptides (CPPs), which act as bioenhancers, for the nasal delivery of protein drug interferon beta (IFN-β) and its PEGylated form (PEG-IFN-β). The ability of CPPs to enhance the nasal mucosal absorption of unmodified IFN-β was assessed in rats. It was shown that only D-amino acid forms of amphipathic CPPs, penetratin and PenetraMax significantly enhanced the nasal absorption of IFN-β. Especially, D-penetratin (up to 2 mM) enhanced the absorption of INF-β in a dose-dependent manner. The maximum absolute bioavailability reached 8.26% following *in situ* nasal coadministration of IFN-β with D-penetratin (2 mM). Furthermore, it was found that the coadministration of D-penetratin also facilitated the nasal absorption of PEG-IFN-β, which remained in the circulation for more than 6 h. Moreover, the toxicity assessments showed no damage to the epithelial membranes after nasal administration of CPPs including penetratin and PenetraMax. Altogether, this study provides the first evidence that the noncovalent coadministration of PEGylated proteins with CPPs could be a potent strategy for the noninvasive and sustained nasal delivery of therapeutic proteins.

1. Introduction

Several drug delivery systems functionalized with the moieties that provide stealth effects and passive or active targeting functions have been developed in last few decades (Bertrand et al., 2014; Mitragotri et al., 2014; Torchilin, 2014). Among these, PEGylation is one of the most effective and established strategies for improving the therapeutic efficacy of injected drugs, especially protein-based biopharmaceuticals and lipidic or polymeric carriers (Kolate et al., 2014). It provides drug delivery systems with minimal immunogenicity and improves the pharmacokinetic behavior of drugs. For example, it reduces the proteolytic disruption of conjugated drugs owing to steric hindrance and inhibits renal clearance of drugs owing to the increase in molecular weight. PEGylation can reduce the frequency of drug injection by prolonging the drug retention in body. This improves the patients' quality of life (QOL). Several PEGylated protein- or nucleic acid-based drugs have been approved worldwide, such as interferon (IFN) (PegIntron® and Pegasys®), granulocyte colony-stimulating factor (G-CSF) (Neulasta®), erythropoietin (Mircera®), monoclonal antibody to tumor necrosis factor (TNF) (Cimzia®), and vascular endothelial growth factor (VEGF) aptamer (Macugen®) (Calabresi et al., 2014). Despite the pharmacological activity of therapeutic proteins may be often reduced by structural modification with PEG chains (for instance, the relative activities of PegIntron and Pegasys to free interferon α are 28 and 7%, respectively), the improved pharmacokinetics with prolonged half life are significantly valuable for total effectiveness and daily use of injectable drugs. However, all of these approved PEGylated biopharmaceuticals are injectable formulations. The invasive administration is a burden on patients since it is associated with frequent hospital visits, increased medical costs and risks, and difficulty in and discomfort upon self-injection. This has drawn interest to investigate alternative noninvasive methods for administration of biopharmaceuticals to improve the patients' QOL. However, to date, there

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has been limited success in noninvasive oral and nasal administration. The poor permeability of PEGylated biopharmaceuticals through the epithelial membranes is an essential hurdle to overcome for achieving their noninvasive formulations. Therefore, the strategies to effectively deliver the PEGylated drugs into the intestinal and nasal mucosa have to be established for boosting their eventual absorption into systemic circulation.

The cell-penetrating peptides (CPPs) are promising tools for delivering the bioactive macromolecules such as proteins and nucleic acids as well as particulate carrier systems to the several types of cells (Bechara and Sagan, 2013; Copolovici et al., 2014). Some examples of the CPPs include cationic peptides, such as human immunodeficiency virus (HIV)-1 Tat protein-derived peptide and artificially synthesized oligoarginines; and the amphipathic peptides, such as *Drosophila* Antennapedia homeoprotein-derived peptide. Macromolecular drugs, such as peptides, proteins, and nucleic acids, covalently conjugated with CPPs could be efficiently delivered into the cells. Several mechanisms involving the endocytosis and energy-independent routes contribute to the internalization of CPPs and their cargos in cells (Duchardt et al., 2007; Kamei et al., 2013b; Tunnemann et al., 2006). Thus, the CPPs have been thought to have the potential facilitating the permeation of PEGylated biopharmaceuticals through the intestinal and nasal membranes. Recently, we showed that CPPs including oligoarginines (R8) and penetratin dramatically increased the intestinal and nasal mucosal-absorption of therapeutic peptides, insulin, glucagon-like peptide-1 (GLP-1), and exendin-4 (Kamei et al., 2008; Khafagy el et al., 2009b), via noncovalent intermolecular interactions between CPP and drug (Kamei et al., 2009; Khafagy el et al., 2010). Moreover, it was shown that the CPPs could accelerate the intestinal absorption of protein-sized macromolecules (e.g. dextran: molecular weight = 70 kDa) and enhance the epithelial uptake of polystyrene nanoparticles with a diameter of 200 nm (Kamei et al., 2016). Based on this, it was hypothesized that the use of noncovalent CPP strategy could enhance the absorption of PEGylated protein drugs across the nasal mucosal barrier. This enhanced nasal absorption could lead to the development of an ideal formulation, which can be conveniently administered and has sustained therapeutic efficacy.

The present study aimed to evaluate the utility of CPPs to enhance the nasal absorption of PEGylated proteins. Firstly, the effect of three CPPs, penetratin (Derossi et al., 1996), PenetraMax (Kamei et al., 2013a; Khafagy el et al., 2013), and human immunodeficiency virus (HIV)-1 Tat protein-derived peptide (Vives et al., 1977) (shown in Table 1), on the nasal absorption of IFN-β was evaluated in rats. Subsequently, the nasal absorption of the PEG-IFN-β was examined in the presence of CPPs. Furthermore, the safety of CPPs was evaluated by monitoring the epithelial membrane (for any disruption) and by microscopic observations of histopathological changes in nasal mucosa after the treatment with CPPs.

Table 1
Amino acid sequences of CPPs used in this study.

CPPs	Amino acid sequences	Molecular weight
L-penetratin	RQIKIWFQNRRMKWKK	2246.72
D-penetratin	rqikiwfqnrrmkwkk	2246.72
L-PenetraMax	KWFKIQMQIRRWKNKR	2246.72
D-PenetraMax	kwfkiqmqirrwknkr	2246.72
L-Tat	GRKKRRQRRRPPQ	1719.03
D-Tat	grkkrrqrrrppq	1719.03

F: phenylalanine, G: glycine, I: isoleucine, K: lysine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: arginine, W: tryptophan.
Uppercase and lowercase letters indicate the L-and D-forms of amino acids, respectively.

2. Materials and methods

2.1. Materials

Recombinant human interferon β (IFN-β; MW: approximately 22 kDa; 3.0×10^6 IU/0.01 mg/vial) and PEG-IFN-β (MW: approximately 60 kDa; 5.83×10^7 IU/0.516 mg/mL) were obtained from Toray Industries, Inc. (Kanagawa, Japan). The anti-virus activities of IFN-β and PEG-IFN-β per mole are almost equal (6.6×10^9 IU/μmol (IFN-β) vs. 6.8×10^9 IU/μmol (PEG-IFN-β). The CPPs listed in Table 1 were synthesized by Sigma Genosys, Life Science Division of Sigma-Aldrich Japan Co. (Hokkaido, Japan). Sodium caprate (C10) was purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). Purified goat-IgG isotype control antibody was purchased from Southern Biotechnology Associates Inc. (Birmingham, AL, USA). All other chemicals were of analytical grade and were commercially available.

2.2. Preparation of IFN-β or PEG-IFN-β and penetratin solutions

One vial of IFN-β (3.0×10^6 IU = 0.01 mg) was diluted with 0.15 mL of phosphate-buffered saline (PBS, pH 7.4), which contained 0.001% methylcellulose (MC) to prevent the adsorption of IFN-β to the tube surface, to adjust the concentration of IFN-β at 2.0×10^7 IU/mL. PEG-IFN-β solution (0.516 mg/mL = 5.83×10^7 IU/mL) was diluted to 0.4 mg/mL (4.52×10^7 IU/mL). Specific amount of CPPs (penetratin, PenetraMax or Tat peptide) was dissolved in PBS (pH 7.4). IFN-β (2.0×10^7 IU/mL) or PEG-IFN-β (0.4 mg/mL) was added to equal volumes of CPP solutions, mixed gently, and adjusted to the following final concentrations: IFN-β (1.0×10^7 IU/mL) or PEG-IFN-β (0.2 mg/mL) and CPPs (0.5, 1, 2 or 5 mM). All IFN-β or PEG-IFN-β/CPP solutions obtained after mixing were clear.

2.3. In vivo nasal absorption study

2.3.1. Animals

In vivo experiments were performed at Kobe Gakuin University in compliance with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals. Male Sprague-Dawley rats weighing 180–220 g were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were housed in rooms with controlled temperature (23 ± 1 °C) and relative humidity (55 ± 5%) and provided free access to water and food during acclimatization. Animals were fasted for 12 h before the experiments; however, they were allowed to drink water *ad libitum*.

2.3.2. Administration of drugs to the open nasal cavity

The rats were anesthetized using an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg, Somnopentyl®, Kyoritsu Seiyaku Corp., Tokyo, Japan), and restrained in a supine position on a thermostatically controlled board at 37 °C. Additionally, mice were intraperitoneally administered 12.5 mg/kg sodium pentrobarbital every 1 h to maintain the anesthesia. Four aliquots (5 μL each) of IFN-β/CPP solutions (total 20 μL) were administered alternately into the right and left nostrils every 20 s by using a micropipette. The dose of IFN-β was 1.0×10^6 IU/kg and the concentration of CPPs was 2 mM. The effect of C10 (10 mg/mL), which is a commonly used absorption enhancing agent approved for use in clinical formulations (Maher et al., 2009), on the intestinal absorption of IFN-β was assessed and compared with that of CPPs. Blood samples (0.25 mL) were taken from the jugular vein before dosing and at 0.25, 0.5, 1, 2, 4, 6, 10, and 24 h after dosing. A tuberculin syringe (1 mL, Terumo Corp., Japan) was heparinized by aspirating heparin to coat the syringe wall. The remaining heparin was expelled by depressing the plunger to the needle hub. The plasma was separated by centrifugation at 13,400*g*