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Genetically engineered and self-assembled oncolytic protein nanoparticles for targeted cancer therapy

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ABSTRACT

The integration of a targeted delivery with a tumour-selective agent has been considered an ideal platform for achieving high therapeutic efficacy and negligible side effects in cancer therapy. Here, we present engineered protein nanoparticles comprising a tumour-selective oncolytic protein and a targeting moiety as a new format for the targeted cancer therapy. Apoptin from chicken anaemia virus (CAV) was used as a tumour-selective apoptotic protein. An EGFR-specific repebody, which is composed of LRR (Leucine-rich repeat) modules, was employed to play a dual role as a tumour-targeting moiety and a fusion partner for producing apoptin nanoparticles in *E. coli*, respectively. The repebody was genetically fused to apoptin, and the resulting fusion protein was shown to self-assemble into supramolecular repebody-apoptin nanoparticles with high homogeneity and stability as a soluble form when expressed in *E. coli*. The repebody-apoptin nanoparticles showed a remarkable anti-tumour activity with negligible side effects in xenograft mice through a cooperative action of the two protein components with distinct functional roles. The repebody-apoptin nanoparticles can be developed as a systemic injectable and tumour-selective therapeutic protein for targeted cancer treatment.

1. Introduction

The development of cancer medicines with profound therapeutic efficacy, but negligible toxicity, is an ultimate goal in the field of clinical oncology [1,2]. Key issues for achieving such ideal platforms, particularly for cancer prevention and treatment, include selective cytotoxicity and the preferential delivery of anti-cancer agents toward tumour cells [3]. Over the decades, an increasing number of chemotherapeutics have been developed and have shown partial or significant tumour regression in patients [4,5]. However, the systemic delivery of small-chemical drugs still suffers from poor selectivity, low bioavailability, and severe side effects [6,7]. As a promising strategy to address such issues, considerable attention has focused on protein-based biologics including drug conjugates, and many types of protein therapeutics are clinically used for treating various cancers [8–10]. Despite notable outcomes, however, low-dose exposure of protein therapeutics on normal cells with a low expression level of a surface antigen can induce unwanted on-target toxicity, resulting in a narrow therapeutic index [11,12]. Molecularly targeted therapeutics has recently emerged as a new paradigm in cancer treatment owing to its highly efficient and tumour-selective cytotoxicity, which depends on the intracellular oncogenic activity of tumour cells.

Pro-apoptotic proteins with tumour-selective properties, including oncolytic virus-derived proteins and other human tumour suppressors, have been of great interest owing to their ability to selectively kill cancer cells [13]. Among them, apoptin, which is a 13.6 kDa protein from the chicken anaemia virus (CAV), has attracted considerable attention as a promising anti-cancer therapeutic because it triggers tumour-selective cell death, while leaving normal cells unaffected [14,15]. Interestingly, the oncolytic

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activity of apoptin proceeds independently of tumour suppressor p53, and its biological function is stimulated by various intracellular oncoproteins [16–18]. To clinically use apoptin as an anticancer agent, however, an intracellular delivery system is a prerequisite. The virus-mediated delivery of the apoptin gene has been a primary choice [14,16], but has certain drawbacks, including low transduction efficiency, uncontrolled gene expression, and immunotoxicity, limiting the systemic delivery and therapeutic benefit of apoptin [16,19,20]. The direct delivery of active apoptin protein is thus considered a preferred approach to the efficacious and manageable treatment of cancers. Several direct delivery systems have been attempted in a non-targeted manner using protein transduction domains and a degradable cationic polymer [21–23], and most of them have relied on epithelial and intra-tumoural injection in mouse models to facilitate the delivery of apoptin to tumour sites. Considering that such administrations are difficult to implement practically in cancer therapy, a targeted and systemic delivery of apoptin protein is crucial for translation into the clinical domain.

Here, we present engineered protein nanoparticles comprising apoptin and a targeting moiety as a new format for targeted cancer therapy. Anti-EGFR repebody, which is composed of leucine-rich repeat (LRR) modules, was employed to play a dual role as a tumour-targeting moiety and a fusion partner for bacterial production of a supramolecular apoptin complex, respectively. Genetically engineered apoptin with the repebody was shown to self-assemble into the repebody-apoptin nanoparticles with high homogeneity and stability as a soluble form when expressed in *E. coli*. We demonstrate the utility and potential of the repebody-apoptin nanoparticles for targeted cancer therapy by showing a remarkable tumour regression in xenograft mice through a cooperative action of the two protein components with distinct functional roles. Details are reported herein.

2. Materials and methods

2.1. Protein expression and purification

All gene constructs were cloned into a pET21a expression vector (Novagen, USA) using *Nde*I and *Xho*I restriction enzyme sites. For protein purification, a poly-histidine tag was introduced at the N-terminal end of the repebody-apoptin fusion protein (Rb-Apo) and maltose binding protein-fused apoptin (MBP-Apo), and at the C-terminal end of the free repebody (rEgH9), respectively. The resulting constructs of the Rb-Apo nanoparticles (Rb-Apo-NPs) and repebody (rEgH9) were transformed into BL21 (DE3) and Origami B (DE3), respectively. A single colony expressing each construct was inoculated into 10 ml of an LB medium with 100 μg/ml ampicillin and grown overnight at 37 °C. In the case of repebody (rEgH9), kanamycin (50 μg/ml) and tetracycline (10 μg/ml) were additionally supplemented. The overnight culture was diluted to 1:100 for inoculation in an LB medium supplemented with the above-mentioned concentration of antibiotics. The cells were grown at 37 °C, and isopropyl-b-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM for Rb-Apo and MBP-Apo or 0.5 mM for the free repebody when the culture reached an absorbance (600 nm) value of 0.5. Following incubation at 18 °C overnight, the cells were harvested by centrifugation at 6000 rpm for 15 min, and the pellet was resuspended using a lysis buffer (20 mM Tris, 150 mM NaCl, 10 mM Imidazole, and pH 8.0). The resuspended cells were disrupted using ultra-sonication, and subjected to centrifugation at 13,000 rpm for 60 min. Supernatant was filtered through a 0.22-μm filter membrane, and the filtered solution was loaded into a Ni-NTA chromatography column (Qiagen, USA). Following three washings with a washing buffer (20 mM Tris, 500 mM NaCl (for the repebody, 150 mM NaCl was used), 20 mM Imidazole, and pH 8.0), proteins were eluted using an elution buffer (20 mM Tris, 150 mM NaCl, 250 mM Imidazole, and pH 8.0), and the buffer was changed to a Tris or PBS buffer (pH 7.4). Purified proteins were stored at 4 °C for the repebody or at 25 °C for the Rb-Apo and MBP-Apo for further experiments. The concentration of each apoptin fusion protein was determined using a UV–visible spectrometer (GE healthcare, USA) based on UV absorption at 280 nm, and the molar concentration of apoptin particles used in the present study was expressed as relative to the molar concentration of the monomeric fusion proteins.

2.2. Atomic force microscopy

20 μl of proteins (1 mg/ml) were dissolved in PBS (pH 7.4) with 10 mM $MgCl_2$, and deposited onto freshly cleaved mica (Ted Pella Corp., USA). Following dropping and incubation for 5 min, the mica surface was rinsed with DDW (double distilled water) three times and carefully dried using nitrogen gas. The resulting mica substrate was additionally dried in a vacuum desiccator overnight, and subjected to scanning in non-contact mode using a Park NX-10 ADM with an NC-NCH tip (Park System Corp., Korea).

2.3. Dynamic light scattering

1 mg/ml of protein was dissolved in PBS (pH 7.4), followed by filtration using a 0.22-μm filter membrane. To assess the stability of Rb-Apo-NPs under physiological conditions, Rb-Apo-NPs (final 1 mg/ml) were diluted in the Tris buffer (20 mM Tris, 150 mM NaCl and pH 8.0), and incubated with 10% mouse serum or 10 NIH units/ml of thrombin (Sigma, USA) at 37 °C. A total of 150 μl of the sample was transferred into a micro cuvette (ZEN0040; Malvern, UK), and the hydrodynamic size was measured using a Zetasizer instrument (Malvern, UK) at 25 °C. All measurements were conducted in triplicate.

2.4. Circular dichroism analysis

Circular dichroism spectra of the repebody (rEgH9) and the Rb-Apo-NPs were measured from 190 to 260 nm at 25 °C using a J-815 CD spectropolarimeter (Jasco, Japan). The path length of the quartz cuvettes used for the repebody (rEgH9) and the Rb-Apo-NPs is 0.2 and 1.0 mm, respectively. All samples were diluted in PBS (pH 7.4) at a concentration of 1 mg/ml. To determine the melting temperatures, a thermal denaturation analysis was conducted by measuring the molar ellipticity at 222 nm with a gradual increase in temperature from 25 to 90 °C. The resulting data were fitted using the SigmaPlot program (Systat Software, USA) for a melting temperature calculation.

2.5. Enzyme-linked immunosorbent assay

For identification of the target specificity of the Rb-Apo-NPs, 96-well Maxisorp plates (SPL, Korea) were coated with BSA and EGFR family proteins at 4 °C overnight and blocked with 2% BSA (20 mg/ml) in PBST (PBS supplemented with 0.1% Tween 20) at room temperature for 1 h. The coating concentration of all proteins was 10 μg/ml. After incubation with the Rb-Apo-NPs (5 nM) for 1 h, a rabbit anti-repebody antibody (10.1 mg/ml; Abclon, Korea) was added into each well at a dilution of 1:5000 for 1 h. Next, the binding signals were developed using a subsequent incubation of a 1:3000 dilution of HRP-conjugated goat anti-rabbit antibody (Bio-Rad, USA) and tetramethylbenzidine (TMB) solution (Sigma, USA). After the addition of a 1 N sulfuric acid solution, the absorbance at 450 nm was measured using a microplate reader (Tecan, Swiss).