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A dimeric form of a small-sized protein binder exhibits enhanced anti-tumor activity through prolonged blood circulation

Tae Yoon Kim[a,1], Hyo-Deok Seo[a,1], Joong-jae Lee[b], Jung Ae Kang[c], Woo Sik Kim[c], Hye-Min Kim[c], Ha-Yeon Song[c], Ji Min Park[c], Dong-Eun Lee[c,*], Hak-Sung Kim[a,*]

[a] Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Republic of Korea
[b] Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea
[c] Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute (KAERI), Jeongeup, Jeonbuk 580-185, Republic of Korea

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ABSTRACT

Small-sized non-antibody scaffolds have attracted considerable interest as alternatives to immunoglobulin antibodies. However, their short half-life is considered a drawback in the development of therapeutic agents. Here we demonstrate that a homo-dimeric form of a repebody enhances the anti-tumor activity than a monomeric form through prolonged blood circulation. Spytag and spycatcher were genetically fused to the C-terminus of a respective human IL-6-specific repebody, and the resulting two repebody constructs were mixed at an equimolar ratio to produce a homo-dimeric form through interaction between spytag and spycatcher. The homo-dimeric repebody was detected as a single band in the SDS-PAGE analysis with an expected molecular size (78 kDa), showing high stability and homogeneity. The dimeric repebody was shown to simultaneously accommodate two hIL-6 molecules, and its binding affinity for hIL-6 was estimated to be comparable to a monomeric repebody. The serum concentration of the dimeric repebody was observed to be about 5.5 times higher than a monomeric repebody, consequently leading to considerably higher tumor suppression effect in human tumor xenograft mice. The present approach can be effectively used for prolonging the blood half-life of small-sized protein binders, resulting in enhanced therapeutic efficacy.

1. Introduction

Over the past decade, non-antibody protein scaffolds with a small size (< ~30 kDa) have drawn considerable attention as alternatives to immunoglobulin antibodies, and diverse scaffolds have been developed, showing the possibility of their wide applications in many areas [1–8]. Small-sized protein scaffolds have been shown to possess distinct advantages compared to antibodies, including a low manufacturing cost, easy design and engineering capability, and high tissue penetration [9]. Nonetheless, the short half-life of small-sized scaffolds in blood owing to fast renal clearance is considered a limitation to their development as therapeutic agents [9–11]. Since pharmacokinetics and blood half-life of the therapeutic agents are critical for determining the therapeutic efficacy, tremendous efforts have been made to improve the pharmacokinetic property of small-sized protein scaffolds [12,13]. Indeed, modifications of the small-sized protein scaffolds, including the fusion with Fc domain of antibody [14–17], PEGylation [18–22], and fusion with the albumin or albumin-binding domain [23–27], have been attempted to achieve prolonged circulation of such protein scaffolds in blood. However, they have some drawbacks such as difficulty with bacterial expression and requirement of multiple purification steps or refolding process. In addition, they often generate highly heterogeneous and unstable constructs. It is thus of great significance to develop an alternative method avoiding shortcomings of conventional approaches for prolonging the blood circulation of small-sized protein scaffolds.

We previously developed a non-antibody protein scaffold, called “repebody”, which is composed of leucine-rich repeat (LRR) modules [28]. A repebody with a molecular mass of 31 kDa has unique structural and biophysical features: easy design and engineering, simple to manufacture and mass production using *E. coli*, high stability against heat, pH, and proteolytic digestion, high tissue penetration. In addition, a modular architecture enables the easy development of a target-specific repebody with high affinity through a phage display and modular engineering approach. In practice, target-specific repebodies were shown to effectively block the cell signaling processes, exhibiting strong anti-tumor activities in xenograft mice [29–31]. Despite high therapeutic

* Corresponding authors.
E-mail addresses: delee@kaeri.re.kr (D.-E. Lee), hskim76@kaist.ac.kr (H.-S. Kim).
[1] These authors contributed equally to this work.

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potential, however, the repebodies exhibited a short half-life mainly owing to the rapid renal clearance similar to other non-antibody protein scaffolds [32,33].

Herein, we present the development of a homo-dimeric repebody specific for human interleukin 6 (hIL-6) as an approach to achieve an efficient cancer therapy through a prolonged half-life. Spytag and spycatcher were genetically fused to the C-terminus of a respective anti-hIL-6 repebody using a flexible G_4S linker, and the resulting two repebody constructs were simply mixed at an equimolar ratio to produce a homodimeric repebody. Spytag and spycatcher are known to associate with each other through a covalent bond [34]. The homo-dimeric repebody has an expected molecular size of 78 kDa, exhibiting high stability and homogeneity. The homo-dimeric repebody was observed to accommodate two hIL-6 molecules with a comparable binding affinity to the monomeric one. We demonstrated enhanced antitumor activity of the homo-dimeric repebody in xenograft mice through a prolonged blood circulation compared to a monomeric one. Details are reported herein.

2. Materials and methods

2.1. Materials

All the gene constructs, including engineered repebodies and human interleukin 6 (hIL-6), were cloned into a pET21a expression vector (Novagen, Madison, WI, USA) using *Nde*I, *Xho*I restriction enzymes and T4 DNA Ligase (Takara Bio, Shiga, Japan). LB broth media and antibiotics were purchased from Duchefa (Haarlem, The Netherlands). Origami B (DE3) competent cells for the repebody expression were from Novagen (Madison, WI, USA). IPTG was purchased from LPS Solution (Seoul, Korea). cOmplete™, EDTA-free Protease Inhibitor Cocktail Tablet (Roche Diagnostics GmbH, Mannheim, Germany,) was used to prevent protein degradation by the proteases. The Ni-NTA agarose resin applied was from Qiagen (Germantown, MD, USA). HiTrap Q HP, Hiload Superdex 75 and Hiload Superdex 200 columns were purchased from GE Healthcare (Uppsala, Sweden). All other reagents were of analytical grade.

2.2. Construction of a homo-dimeric from of a repebody

To construct a homo-dimeric form of a repebody, spytag and spycatcher were genetically fused to the C-terminal end of a respective hIL-6-specific repebody through a flexible G_4S linker. The six-histidine tag was introduced at the C-terminal end of the spytag and spycatcher for purification using the Ni-NTA agarose resin. The resulting vector constructs were transformed into Origami B (DE3) competent cells. A single colony expressing a respective repebody construct was used for seeding in an LB medium containing ampicillin (100 μg/mL), kanamycin (50 μg/mL), and tetracycline (5 μg/mL) and grown overnight in a shaking incubator at 37 °C. The cells were inoculated into 1 L of an LB medium with the same concentration of antibiotics at 37 °C until OD_{600} reached 0.5. The cells were induced with 0.5 M of isopropyl-b-D-thiogalactopyranoside (IPTG), followed by further incubation for 20 h at 18 °C. Cells expressing a respective repebody construct were harvested through centrifugation at 8000 rpm for 20 min. The cell pellets were re-suspended using a lysis buffer (20 mM Tris, 150 mM NaCl. 10 mM Imidazole, pH 8.0), followed by ultra-sonication and centrifugation at 16,000 rpm at 4 °C for 1 h in the presence of protease inhibitors. Supernatants were filtered through a 0.22 μm syringe filter (EMD Millipore, Bedford, MA, USA) and subjected to purification using a Ni-NTA agarose resin column. The columns were washed thoroughly using a washing buffer (20 mM Tris, 150 mM NaCl, 20 mM Imidazole, pH 8.0), and the proteins were eluted using an elution buffer (20 mM Tris, 150 mM NaCl, 250 mM Imidazole, pH 8.0). Purified two repebody constructs were mixed at an equimolar ratio and incubated overnight at 4 °C for the spontaneous association. The resulting homo-dimeric form of a repebody was further purified using a HiTrap Q HP anion exchange column (0.1 M MOPS buffer, pH 6.5) and size exclusion chromatography (Superdex 200) using PBS (pH 7.4) to remove the residual spytag and spycatcher. A native hIL-6-specific repebody and hIL-6 were purified as described in our previous work [29]. The protein concentration was determined using UV–Vis spectrophotometry at 280 nm. The SDS-PAGE gel images were analyzed using a Gel Doc EZ Imager (Bio-Rad, Hercules, CA, USA).

2.3. Circular dichroism (CD) analysis

The molar ellipticity of a dimeric repebody was measured from 190 nm to 280 nm at 25 °C by circular dichroism (Jasco J-815, Easton, MD, USA). The melting temperature of the dimeric repebody was determined by measuring the molar ellipticity at 222 nm with a gradual increase of temperature at a rate of 1 °C/min, ranging from 25 to 95 °C. The pH stability of the dimeric repebody was also determined based on the melting temperature at each pH value. The protein was diluted with each buffer at a final concentration of 2 mg/mL and incubated for 24 h at 4 °C. The molar ellipticity was measured at 222 nm within a temperature range of 25 to 95 °C. The buffers used for the pH stability test are as follows: 0.3 M NaCl - 5 mM glycine buffer (pH 3.0), 0.1 M citric acid - 0.2 M Na_2HPO_4 buffer (pH 4.0–7.0), phosphate buffered saline (pH 7.4), 0.2 M Na_2HPO_4–0.2 M NaH_2PO_4 buffer (pH 8.0–9.0), 0.2 M sodium carbonate - 0.2 M sodium bicarbonate buffer (pH 10.0–11.0), and 0.2 M KCl - 0.2 M NaOH buffer (pH 12.0).

2.4. Enzyme linked immunosorbent assay (ELISA)

The binding capacity of a homo-dimeric repebody for hIL-6 was determined using ELISA. Briefly, 10 μg/mL of either hIL-6 or human EGFR ectodomain was coated onto a 96-well Immuno Plate (SPL Life Sciences, Seoul, Korea) at 4 °C overnight, followed by a blocking using 1% BSA in PBST (PBS pH 7.4, 0.05% Tween 20) for 1 h at room temperature. Human EGFR ectodomain was used as an off-target. A spytag-fused anti-hIL-6 repebody, spycatcher-fused anti-hIL-6 repebody, and anti-hIL-6 dimeric repebody were diluted to 10 μg/mL with a blocking buffer, and 100 μL of the respective proteins were added to each well. The wells were washed five times using PBST (PBS pH 7.4, 0.05% Tween 20), and biotinylated hIL-6 or biotinylated EGFR ectodomain was added to each well and incubated for 1 h at room temperature. Human IL-6 and human EGFR ectodomain were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Waltham, MA, USA). HRP-conjugated streptavidin (BioLegend, San Diego, CA, USA) was added to each well, followed by the addition of 3,3′,5,5′-Tetramethylbenzidien (TMB). After incubation for 1 min at room temperature, 1N H_2SO_4 was added, and the signals were measured at 450 nm using an Infinite M200 microplate reader (Tecan GmbH, Crailsheim, Germany). The ELISA experiments were conducted in triplicate, and the wells were washed with PBST (PBS pH 7.4, 0.05% Tween 20) five times between each step.

2.5. Isothermal titration calorimetry (ITC)

The binding affinity of a dimeric repebody was determined using MicroCal iTC200 (Malvern Instruments, Worcestershire, UK) at 25 °C. Briefly, 0.2 mM of a dimeric repebody was titrated by adding 0.02 mM of hIL-6. The titration curve was fitted into a one-site binding model, and the binding affinity was calculated using the Origin program (OrginLab, Northampton, MA, USA).

2.6. Radio isotope labeling

2.6.1. Radiolabeling of a repebody with $[^{99m}Tc(OH_2)_3(CO)_3]^+$

Homo-dimeric and monomeric repebodies were labeled with $[^{99m}Tc(OH_2)_3(CO)_3]^+$ as follows. To synthesize ^{99m}Tc-tricarbonyl, a 1 mL