Supplementary Materials

Pre-Clinical Studies of a Novel Bispecific Fusion Protein Targeting C3b and VEGF in Neovascular and Nonexudative AMD Models

Yeri Lee[1#], Donggeon Kim[1#], Philip E.D. Chung[1], Minkyeong Lee[1], Nahmju Kim[1], Jihoon Chang[1], Byoung Chul Lee[1*]

[1] KANAPH Therapeutics, Yongsan-gu, Seoul, Republic of Korea.

[#] These authors contributed equally to this work.

Correspondence to:

Byoung-Chul Lee, Ph.D., KANAPH Therapeutics, 3, Itaewon-ro 55ga-gil, Yongsan-gu, Seoul, Republic of Korea, Tel: +82-2-792-8642, Fax: +82-2-792-8638, E-mail: blee@kanaphtx.com

Constructs

The synthesized DNA fragment was amplified through polymerase chain reaction (PCR), and the PCR product was purified by gel. The pTT5 vector was cut with restriction enzymes, and then purified by gel. Each PCR product and the linear vector were ligated using the In-Fusion Kit. The produced vector was transformed in ECOS101 DH5α competent cells, and cultured on a 2xYT agar plate containing 100 μg/ml of ampicillin. All manipulation processes were performed according to standard transformation protocols. Positive recombinants were confirmed through colony PCR, and sequence-verify sequencing was performed on a recombinant plasmid. A single colony was selected, and the spawn was inoculated into 5 mL of a 2xYT medium containing 100 μg/ml of ampicillin.

For generation of mouse constructs, A DNA fragment corresponding to the protein sequence was synthesized in Genewiz (No. 80-383034849). The corresponding DNA fragment was amplified through PCR, and introduced using a linearized pcDNA3.3 expression vector. The construction thereof was verified through sequencing, and then a sufficient amount of DNA to perform cell transfection was obtained through a large-scale plasmid preparation process.

Transfection and human fusion protein expression

For expressing human proteins, The 293F seed strain containing a complete medium was maintained in an incubator shaker at 130 rpm, 37°C, and 8% CO_2. The medium was replaced every 2 to 3 days. 24 hours before transfection, freshly subcultured 293F cells were prepared at an optimal density. The prepared cells were cultured in an incubator shaker at 130 rpm, 37°C, and 8% CO_2. On the day of transfection, the density of the cells was adjusted using a fresh medium. The adjustment was performed with a total volume of 1 L in a 3-L shaker flask.

HC and LC plasmid were diluted with 50 ml of OPTI MEM I, and filtered with a filter of 0.22 μm. Then, 2 mg of PEI was diluted with 50 ml of OPTI MEM I to prepare a transfection reagent. The diluted PEI was added to the DNA mixture, and then immediately mixed. Subsequently, culturing was performed at room temperature for 15 minutes. The DNA-PEI mixture was added to the 293F cells. The cells were then continuously cultured for 24 hours in an incubator shaker at 130 rpm, 37℃, and 8% CO_2. 24 hours after transfection, 10% peptone was added to 1/20 of the culture medium so that the final concentration was 0.5%. The cells were then continuously cultured in an incubator shaker at 130 rpm, 37℃, and 8% CO_2. The density/viability of the cells was daily measured and recorded, over a period of 2 to 5 days after the transfection. The cells were collected for purification 7 days after the transfection or when the cell viability was less than 70%.

For expressing mouse proteins, Expi293F cells were prepared at an optimal density. Plasmid DNA and an EXPIFECTAMINETM 293 reagent were first diluted in Opti-MEM, then mixed, and added to the cell culture medium. Cell culturing was performed in a platform shaker at a stirring speed of 150 rpm. The temperature was maintained at 37℃ and the concentration of CO2 was maintained at 8%. 18 to 20 hours after transfection, an enhancer 1 and an enhancer 2 were added to the cell culture medium. After 6 days of the cell culturing, the cells were centrifuged at 4,000 rpm at 25℃ for 10 minutes. The supernatant was collected for purification and gel electrophoresis. The supernatant was loaded onto SDS-PAGE gel according to the instructions for NUPAGETM 4% to 12% Bis-Tris Protein Gels (ThermoFisher). The remaining supernatant of each protein was used in the subsequent purification process.

Purification human and mouse fusion proteins

The human fusion proteins were purified using a Mabselect sure column. Specifically,

the supernatant was collected through centrifugation at 4℃ and 2,000 xg for 20 minutes. Subsequently, the supernatant was filtered with the Sartopore 2 filter. The clarified supernatant was loaded onto a 5-ml MabSelect Sure column equilibrated with the buffer A (25 mM Tris, 150 mM NaCl, pH 8.0). The column was then washed with the buffer A until the A280 absorbance reached a baseline. The column was washed with 10 CV of the buffer B (25 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 0.1% Triton X-114, pH 8.0). The column was washed with 10 CV of the buffer A. The bound proteins were eluted with 6 CV of the buffer C (100 mM Sodium Citrate, 150 mM NaCl, pH 3.0), 1/6 volume of the buffer D (1 M Arginine, 400 mM Succinic acid, pH 9.0) was added to neutralize the eluents, and SDS-PAGE analysis and SEC-HPLC analysis were performed. Thereafter, the proteins were purified through a HIC column. The proteins were then dialyzed against the buffer E (20 mM PB, pH 6.5, 1 M $(NH_4)_2SO_4$) at 4℃ overnight. The supernatant was loaded onto a HIC column equilibrated with the buffer E. The column was then washed with the buffer E until the A280 absorbance reached a baseline. The bound proteins were eluted through gradient elution (10 CV of buffer F(20 mM PB, pH 6.5, 25% isopropyl alcohol), 0% to 40%). The bound proteins were eluted with 2 CV of a 100% buffer F, and SDS-PAGE analysis was performed. After purifying the proteins, the proteins were collected in one place, and then dialyzed against the final buffer (20 mM HEPES, pH 7.5, 240 mM sucrose, or 20 mM His acetate pH 5.5, 240 mM sucrose) at 4℃ overnight. Subsequently, SDS-PAGE analysis and SEC-HPLC analysis were performed.

To purify the mouse fusion proteins, a protein A column was prepackaged together with a MabSelect Sure resin. Before loading the cell culture medium, the column was equilibrated with 0.1 M Tris (pH 7.0). After loading the cell culture medium, the column was washed with 0.1 M Tris (pH 7.0), and then eluted with 0.1 M glycine (pH 3.5). The eluents were neutralized by adding 0.1 M Tris (pH 9.0). Subsequently, the samples were dialyzed in PBS buffer (Sangon Biotech, B548117-0500). Before loading the samples, an SEC column

(GE lifesciences, Superdex 200 increase 10/300) was equilibrated with PBS. After the loading, the samples were eluted with PBS and collected through chromatography. SDS-PAGE was performed in order to analyze each peak thereof. Each sample was dialyzed in a formulation buffer (10 mM sodium phosphate, 0.3 to 0.4 M NaCl, pH 6.8). A CHT column was prepackaged together with a CHT resin (Bio-rad, MPCTM Ceramic Hydroxyfluoroapatite), and equilibrated with the buffer A (10 mM sodium phosphate, 30 mM NaCl, pH 6.8) before loading the samples. After the loading, the column was eluted with a 30% buffer B (10 mM sodium phosphate, 1 M NaCl, pH 6.8), and then eluted with a 30% to 90% linear gradient buffer B and a final 100% of buffer B. The eluents were characterized by SDS-PAGE, and the samples were dialyzed in a formulation buffer (10 mM sodium phosphate, 0.3 to 0.4 M NaCl, pH 6.8). The final proteins were filtered with a filter of 0.2 μm, and aseptically dispensed in an amount of 0.5 mL into each of 1.5 mL tubes.

Purification C3 of rabbit and rat

For purification of C3 of rabbit and rat, a step for removing high-molecular-weight substances from plasma was performed. The volume of the plasma was measured, and then 10% (ratio of weight/volume) Na_2SO_4 (anhydrous) of the powdered salt crystals was slowly added to the protein solution while stirring. Subsequently, stirring was performed at 4℃ for 2 hours. After centrifugation was performed at 4 ℃ and 26,892 xg for 30 minutes using a Sorvall Ultracentrifuge, the supernatant was obtained and the volume thereof was measured. The obtained supernatant was dialyzed against a DEAE buffer A (10 mM Potassium phosphate (KH_2PO_4), 5 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 1 mM benzamidine, pH 7.8), and after 2 hours, replacement with a new solution was performed. For the best results, the dialysis was performed at 4℃ overnight. In order to confirm whether the dialysis was

completed, the volume, A280, and conductivity of the dialysate were measured.

The protein purification was performed in the order of anion exchange chromatography, cation exchange chromatography, and size exclusion chromatography. Before loading the dialysate, a DEAE column (GE healthcare, HIPREPTM DEAE Fast Flow 16/10) was equilibrated with the buffer A (10 mM KH_2PO_4, 5 mM EDTA, 1 mM benzamidine, pH 7.8). After the loading, the column was washed with the buffer A, and eluted with a 0% to 50% linear gradient buffer B (10 mM KH2PO4, 5 mM EDTA, 1 mM benzamidine, 1 M NaCl, pH 7.8). The fractions of the eluent were analyzed through SDS-PAGE, the fractions containing C3 were collected, and the A280 thereof was measured. The obtained eluted substance was dialyzed in 2 L of a Mono S buffer A (50 mM sodium phosphate) at 4℃ for 2 hours.

A Mono S column (GE healthcare, MONO S® 5/50 GL) was equilibrated with the buffer A (50 mM sodium phosphate) before loading the dialysate. After loading a DEAE eluate, the column was washed with the buffer A, and eluted with a 0% to 35% linear gradient buffer B (50 mM sodium phosphate, 1 M NaCl, pH 5.5). The fractions of the eluted substance were analyzed through SDS-PAGE, the centrally located peak fractions were pooled, and the A280 thereof was measured.

Before loading the samples, an SEC column (GE lifesciences, Superdex 200 increase 10/300) was equilibrated with PBS. After the loading, the samples were eluted with PBS and collected through chromatography. SDS-PAGE was performed in order to analyze each peak thereof.

Purification C3b of rabbit and rat

C3 was prepared by diluting with 0.5 mg/mL of PBS. The C3 was converted into C3b

by adding a 0.4 μM factor B, a 0.05 μM factor D, and 5 mM MgCl2 and culturing the resultant at 25℃ for 30 minutes. The C3b was further purified through a Superdex 200 (60 mL) gel filtration column. Subsequently, SDS-PAGE analysis and SEC-HPLC analysis of rabbit C3, rabbit C3b, rat C3, and rat C3b were performed.