CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and instruments used in this study are shown in Appendix A

2.2 Preparation of immunogen

2.2.1 Preparation of recombinant hAHSP

The lyophilized human alpha hemoglobin stabilizing protein, hAHSP (kindly provided by Dr. Mitchell J. Weiss, The Children's Hospital of Philadelphia, Philadelphia, USA) was reconstituted in 600 µl of distilled water. Then protein was dialyzed in 1x PBS, pH 7.2 and stored at -20°C.

2.2.2 Preparation of recombinant hAHSP-BCCP

2.2.2.1 hAHSP gene amplification

A pair of specific primer, AHSP_*Nde*I (5'GAG GAG GAG GTC ATA TGA TGG CTC TTC TTA AGG CC 3') with underlined *Nde*I restriction site and AHSP_*Eco*RI (5'GAG GAG GAG CTG AAT TCG GAG GAG GGC GGT GGG TG 3') underlined *EcoR*I restriction site, was synthesized in order to amplify hAHSP domain coding sequence from plasmid vector pGEX2T-hAHSP (kindly provided by Dr. Mitchell J. Weiss, The Children's Hospital of Philadelphia, Philadelphia, USA). The hAHSP cDNA was annealed with 250 ng of each described primer in 100 µl of reaction mixture containing of 25 ng pGEX2T-hAHSP, 5U ProofStart DNA

polymerase, 0.3 mM of each dNTP and ProofStart amplification buffer with 1 mM $MgSO_4$. The PCR cycling condition starts as one cycle at 95°C for 5 minutes followed by the three steps of PCR amplification: denaturation at 94°C for 50 seconds, annealing at 50°C for 50 seconds, and extension at 72°C for 1 minute. After 35 amplification cycles, the mixture was incubated at 72°C for 10 minutes. Briefly, the amplified product was loaded onto 1% agarose gel and electrophoresed at 120 volt. In order to visualize the DNA in the agarose gel, the gel was stained with 1% ethidium bromide (EtBr) for 20 minutes. Then the gel was destained with distilled water for 5 minutes. Consequently, the DNA bands were observed by UV transilluminator.

2.2.2.2 Restriction enzyme digestion of amplified hAHSP

To isolate the desired PCR product from nonspecific amplification products, the reaction products were separated by electrophoresis in a 1% agarose gel. The PCR product as described in 2.2.2.1 was purified by QIAquick PCR purification kit (QIAGEN), following the manufacturer's instruction, prior to be treated with restriction enzymes. Subsequently, 4 μl of purified PCR product was digested with 20U of both *NdeI* and *EcoRI* (Fermentas) at 37°C for 18 hours. Then the digested DNA was analyzed by 1% agarose gel electrophoresis. The digested hAHSP fragment was further purified by QIAquick PCR purification kit (QIAGEN) before subcloning into plasmid vector.

2.2.2.3 Preparation of pAK400CB plasmid cloning sites

Five nanogram of plasmid vector pAK400CB (previously described by Santala and Lamminmaki, 2004) was digested with 25U of both *NdeI* and *EcoRI* (Fermentas) at 37°C for 18 hours. To purify the digested pAK400CB vector, the plasmid was firstly separated by agarose gel electrophoresis and the desired fragment

of approximately 4 kbp was cut out of the gel and the gel pieces were transferred to clean using QIAquick Gel Extraction kit in accordance with the recommended protocol. Then digested pAK400CB plasmid was retrieved by QIAquick Gel extraction kit (QIAGEN). The purified plasmid vector was further confirmed by agarose gel electrophoresis.

2.2.2.4 Construction of plasmid containing hAHSP-BCCP gene

The digested pAK400CB plasmid vector was ligated with digested hAHSP fragment by T4 DNA ligase (Fermentas). Five units of T4 DNA ligase was introduced into the ligation mixture containing 50 ng of digested hAHSP and 100 ng of digested pAK400CB vector. The ligation mixture was subsequently incubated at 4°C for 18 hours. The constructed recombinant plasmid was named pAK400CB-hAHSP.

2.2.3 Introduction of plasmid DNA pAK400CB-hAHSP into the bacterial cells

2.2.3.1 Bacterial cell transformation

For plasmid amplification, the ligated product was firstly transformed into *E. coli* Nova Blue [*endA1 hsdR17*(r_{K12}^{-} m_{K12}^{+}) *supE44 thi-1 recA1 gyrA96 relA1 lac* F' [*proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Z*△*M15*::Tn*10*]] (Novagen). The pAK400CB-hAHSP was co-incubated with 200 μl of $CaCl_2$-treated Nova Blue competent cells on ice for 1 hour. The mixture was transferred into cooled screw cap tube and subsequently shocked at 42°C for 90 seconds, then abruptly chilled on ice for 1 minute. Three milliliters Luria Bertani (LB) broth without antibiotic was added and further cultured with shaking (120 rpm) at 37°C for 3 hours. The transformed cells were centrifuged (3,000 rpm) at

room temperature for 10 minutes and plated on LB agar containing 25 μg/ml chloramphenicol. The plates were then incubated at 37°C overnight.

2.2.3.2 Purification of plasmid DNA by alkaline lysis method

The isolated colonies were randomly picked and grown in 3 ml of LB broth containing 25 μg/ml chloramphenicol with shaking (180 rpm) at 37°C for 18 hours. Thereafter, 1.5 ml of culture media containing transformed *E.coli* was centrifuged at 13,000 g for 5 minutes at 4°C. The supernatant was discarded and 100 μl of 1x glucomix-lysozyme was added to the cell pellet, followed by vigorous shaking. Two-hundred microlitres of freshly prepared NaOH/SDS was added and mixed by inverting. Then, 150 μl of potassium acetate was added and gently mixed by vortex. The solution was centrifuged at 10,000 g for 5 minutes at 4°C and the supernatant was collected. To precipitate DNA, 900 μl of absolute ethanol was added and the solution was kept on ice for 2 minutes. The DNA was spun down at 10,000 g for 5 minutes at 4°C and the supernatant was discarded. The DNA pellet was reconstituted by adding 100 μl of sterile distilled water, followed by adding 50 μl of 7.5M ammonium acetate, and incubated at -70°C for 10 minutes. The supernatant was collected by centrifugation at 10,000 g for 5 minutes at 4°C. Then, 300 μl absolute ethanol was added to the supernatant and incubated at -70°C for 10 minutes. The solution was spun down and the pellet was cleaned up with 1 ml of 70% ethanol by centrifugation at 10,000 g for 5 minutes at 4°C. The DNA pellet was dried at 37°C, reconstituted with 30 μl of sterile distilled water and stored at -20°C.

2.2.3.3 Characterization of recombinant clones

To verify the inserted gene in the purified plasmid DNA, the purified plasmid from individual clone was characterized by restriction fragment analysis. The purified

plasmids were analyzed by fractionating on a 1% agarose gel electrophoresis. To verify the correct *E.coli* clones, the purified plasmid from the individual clone was characterized by digested with restriction enzymes, *NdeI*, *EcoRI*, and by PCR re-amplification of hAHSP fragment using a pair of primer as described above to identify the correct band. After the corrected clone was identified, the purified plasmid retrieved that clone was used to transform *E.coli* strain Origami B, Nova Blue (Novagen), TG1 (Pharmacia) or XL-1 Blue (Stratagene) according to the described protocol in 2.2.3.1, except transformed Origami B that was plated on LB agar containing 25 μg/ml of chloramphenicol, 10 μg/ml of tetracycline and 15 μg/ml of kanamycin. The antibiotic-resistant colonies from individual clone were picked and purified by alkaline lysis method. The *E.coli* clone harboring pAK400CB-hAHSP was identified by restriction analysis and PCR re-amplification, as described above.

2.2.3.4 Expression of biotinylated hAHSP-BCCP fusion protein

The selected clone of *E.coli* harboring pAK400CB-hAHSP from each strain was grown in 10 ml of SB medium containing antibiotic and supplemented with 0.05% glucose and 4 μM biotin. The culture was shaken 300 rpm at 37°C until an optical density (OD) at 600 nm (OD_{600}) reached 0.5. The bacteria culture was subsequently transferred to 20 ml of the same media and further shaken at 37°C until the OD_{600} reached 0.8. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the culture at a final concentration of 100 μM. The culture was further cultivated for 22 hours at 24°C with 200 rpm shaking.

2.2.3.5 Total bacteria protein extraction

The bacteria cultures were harvested by centrifugation at 400 g, 4°C for 10 minutes. The culture supernatant was discarded and the wet weight of bacterial pellet was determined. The cell pellet was resuspended in BugBuster Protein extracting reagent (Novagen) by gentle vortexing, using 5 ml reagent per gram of wet cell pellet. The mixture was incubated at room temperature for 10-20 minutes at 200 rpm shaking. The insoluble cell debris was removed by centrifugation at 16,000 g, 4°C for 20 minutes. The clarified bacterial extract containing biotinylated hAHSP-BCCP was collected and stored at -70°C until used. .

2.2.4 Detection of hAHSP and biotinylated hAHSP-BCCP fusion protein

2.2.4.1 Detection of hAHSP recombinant protein by indirect ELISA

ELISA plate was coated with 50 μl of 0.5 μg hAHSP in carbonate/bicarbonate buffer, pH 9.6 at 4 °C for 18 hours. The coated wells were blocked with blocking buffer (2% skimmed milk in PBS, pH 7.2) by incubation for 1 hour at room temperature. After washing with washing buffer (0.05% Tween 20 in PBS pH 7.2), 50 μl of the rabbit anti-hAHSP polyclonal antibody (pAb) at various dilutions in blocking buffer were added. Then, antigen-antibody complexes were monitored by adding 50 μl of swine anti-rabbit immunoglobulins conjugated HRP diluted at 1:3,500. After 1 hour of incubation and three times washing, 50 μl of tetramethylbenzidine (TMB) were added into each well and incubated at room temperature in the dark. The reaction was stopped by adding 100 μl of 1N HCl. The intensity of developed color was determined by measuring absorbance using ELISA reader at 450 nm.

2.2.4.2 Detection of biotinylated hAHSP-BCCP protein by indirect ELISA

To determine biotinylated hAHSP-BCCP in bacterial extract from each strains, indirect ELISA was employed. ELISA plate was coated with 50 µl of 10 µg/ml avidin and incubated at 4 ^{o}C for 18 hours. The coated well were blocked with blocking buffer by incubation for 1 hour at room temperature. After four times washing with washing buffer, 50 µl of various concentrations of bacterial extract containing hAHSP-BCCP or CD147-BCCP in blocking buffer were added. After 1 hour of incubation, the plate was washed for four times using washing buffer followed by adding 50 µl of rabbit anti-hAHSP pAb diluted at 1:2,500 or 10 µg/ml CD147 mAb (M6-1B9) or anti-BCCP mAb (BCCP-2). The plate was then incubated at room temperature for 1 hour. The antigen-antibody complexes were monitored by adding 50 µl of swine anti-rabbit immunoglobulins conjugated-HRP at dilution 1:3,500 or rabbit anti-mouse immunoglobulins conjugated-HRP at dilution 1:2,000. After 1 hour of incubation and three times washing, 50 µl of tetramethylbenzidine (TMB) were added into each well and incubated at room temperature in the dark. The reaction was stopped by adding 100 µl of 1N HCl. The intensity of developed color was determined by measuring absorbance using ELISA reader at 450 nm.

2.2.4.3 Detection of hAHSP and hAHSP-BCCP protein by Western blot analysis

Bacterial extract containing hAHSP-BCCP or CD147-BCCP or recombinant hAHSP were resolved on a 10% SDS–PAGE gel and then transferred to a nitrocellulose membrane by semi-dry blotter. The membranes were then blocked with 5% skimmed milk in PBS. The blocked membranes were incubated with anti-hAHSP

pAb or anti-BCCP mAb. After five times washing with 0.1% Tween 20 in PBS, the membranes were incubated with HRP-conjugated swine anti-rabbit immunoglobulins at dilution 1:3,500 or HRP-conjugated rabbit anti-mouse immunoglobulins at dilution 1:2,000 or HRP-conjugated streptavidin at dilution 1:20,000 for 1 hour at room temperature. Thereafter, the membranes were washed three times with 0.1% PBS-Tween 20 and twice with PBS. The reactive bands were visualized by the enhanced chemiluminescence detection system.

2.2.4.4 Magnetic bead sorting

To separate biotinylated hAHSP-BCCP from bacterial components, the magnetic bead sorting was performed. Five hundred microliters of bacterial extract were incubated with 2.5×10^{8} streptavidin-coated magnetic beads for 30 minutes at room temperature. After three times washing with sterile PBS, the hAHSP-BCCP beads were resuspended in 500 µl of sterile PBS and stored at 4^{o}C until used as an immunogen.

To determine whether hAHSP–BCCP was presented on streptavidin magnetic beads, indirect immunofluorescence staining and flow cytometry analysis were performed .

For indirect immunofluorescence staining, 2.5×10^{6} hAHSP-BCCP beads or CD147-BCCP beads were incubated with rabbit anti-hAHSP pAb diluted at 1:2,500, 10 µg/ml CD147 mAb (M6-1B9) or anti-BCCP mAb (BCCP-2) for 30 minutes at room temperature. After twice washing with 1%BSA-PBS-0.02%NaN_3, FITC-conjugated mouse anti-rabbit immunoglobulins or FITC-conjugated sheep anti-mouse immunoglobulins were added and incubated for 30 minutes at room temperature. The

beads were then washed for three times and resuspended in 500 µl PBS containing 1% paraformaldehyde. The fixed beads were further analyzed by flow cytometer.

2.3 Production of monoclonal antibodies

2.3.1 Mouse immunization

Female BALB/c mice at 6 weeks of ages were used. Before immunization, blood sample was collected by tail-bleeding as pre-immunized serum. Mouse was intraperitoneally immunized with 100 µg of hAHSP or $5x10^7$ hAHSP-BCCP magnetic beads in 500 µl sterile PBS. The immunization was repeated every 2 weeks and blood sample was collected every 2 weeks before immunization.

2.3.2 Determination of antibody response in the immunized mouse

Antibodies against hAHSP in mice sera were determined by indirect ELISA as described in 2.2.4.1 and 2.2.4.2 using hAHSP or hAHSP-BCCP bacterial extract as coated antigen.

2.3.3 Hybridoma production

The BALB/c mouse that response for immunization was intraperitoneally boosted with 100 µg of the corresponding antigen. Five days after boosting, mouse was sacrificed and spleen was removed. Then, splenocytes were carefully isolated and homogenized. The obtained splenocytes were counted with Turk's solution using hematocytometer. While myeloma cells were collected from cultured flasks and counted by 0.2% trypan blue. Then, the splenocytes were fused with mouse myeloma cells at the ratio 2:1 using 50% polyethelene glycol (PEG) by standard hybridoma techniques. The mixture was centrifuged at 300g for 10 minutes. Thereafter, supernatant were removed, the cells pellet was gently mixed and warmed at 37°C for

5 minutes. The detail of fusion procedures are as followed. The 1.5 ml of 50% PEG was dropped into the cells mixture within 1 minute and gentle mixed for 1 minute. Then, cell mixture was immediately diluted with IMDM medium. The mixture containing fused cells was then centrifuged at room temperature at 300g for 5 minutes and warmed at 37 ^{o}C for 5 minutes. After the supernatant was removed, the pellet containing fused cells was resuspended in HAT selection medium and 100 µl of solution was gently transferred into 96-well culture plates. The plates were incubated at 37 ^{o}C in 5%CO_2 incubator. After 5 days of cultivation, 150 µl of HT supplement medium was added into each well. The hybridoma clones were monitored by inverted light microscope.

2.3.4 Screening of hybridomas producing mAb against hAHSP by indirect ELISA

Antibody reactivity against hAHSP in culture supernatant from each well was determined by indirect ELISA as described in 2.2.4.1 and 2.2.4.2 using the optimal dilution of bacterial extract as antigen.

2.3.5 Single cell cloning by limiting dilution

In order to get antibody from single clone, the limiting dilution was carried out. Hybridoma producing positive reactivity antibody was counted and diluted in IMDM medium containing 10% FCS and 10% BM condimed at concentration of 4 cells/150 µl. After that hybridoma cells were diluted in serial two-fold dilution as 2 cells/150 µl and 1 cell/150 µl, respectively. Then, 150 µl of cell suspension were added into 96-well culture plates. The culture plates were incubated at 37 ^{o}C in 5%CO_2 incubator for 2 weeks. Culture supernatant was collected from each well that

containing single clone and determined antibody reactivity by indirect ELISA as previously described.

2.4. Characterizations of anti-hAHSP monoclonal antibodies

2.4.1 Characterization of anti-hAHSP mAbs by ELISA

Fifty microlitres (50 µg/ml) of hAHSP or AHSP-BCCP were coated into ELISA plate, then plates were incubated at 4 ^{o}C for overnight. The specificity of mAbs were determined by indirect ELISA as was described in 2.2.4.1 and 2.2.4.2.

2.4.2 Characterization of anti-hAHSP mAbs by Western blotting

Monoclonal antibody against hAHSP were characterized by Western blot analysis as described in 2.2.4.3