CHAPTER III

MATERIALS AND METHODS

Research Instruments

1.Sterile blood bag containing the anticoagulant CPDA-1 (Kawasumi laboratories., Thailand)

2.MS separation column (Miltenyi Biotec GmbH, Germany)

3.Separation filter (Miltenyi Biotec GmbH, Germany)

4.Magnetic stand with column (Miltenyi Biotec GmbH, Germany)

5. Hemocytometer (Bright-line, USA.)

6. Centrifugator (Eppendorf, France)

7.15 and 50 ml. tube (Falcon, USA.)

8. Conical tube (Falcon, USA.)

9. Cryopreserve tube (Corning, USA.)

10.1.5 ml. microcentrifuge tube (Falcon, USA.)

11.25, 75 cm^2 flask (Falcon, USA.)

12.1, 5, 10, 25 ml. and transfer pipette (Falcon, USA.)

13.10, 100, 1000 µl tip (Axygen scientific, USA.)

14.6-well culture plate (Falcon, USA.)

15.Light microscope (Olympus, Japan)

16.Inverted microscope (Nicon, Japan)

17.Slide (Sail brand, China)

18.Cytospin (Shandon, USA.)

19.Automatic adjustable micropipette : P2 (0.1-2 μl), P10 (0.5-10 μl), P20 (5-20 μl), P100 (20-100 μl), P1000 (0.1-1 ml) (Gilson, France)

20.Pipetette boy (Drummond, Japan)

21.Vortex (Scientific industry, USA.)

22.Parafilm (Pechiney, USA)

23.Waterbath (Memmert, Germany)

24.35 mm. dish (assay dish) (Stem cell technologies, Canada)

25.60 mm. gridded scoring dish (Stem cell technologies, Canada)

26.100 mm. Dish (Corning, USA.)

27.CO_2 incubator (Lab-lines, USA.)

28.Laminar flow (Holten LaminAir, Denmark)

29.FAScan (Becton-Dickinson, USA)

30.Camera set (Olympus, Japan)

31.Refrigerator 4 ^{0}C (Frigidarire, USA.)

32.Deep freeze -20 ^{0}C, -80 ^{0}C (SHARP, Japan)

33.Autoclave (Stermite, Japan)

Reagents

1.General reagent

1.1 Phosphate buffer saline; PBS (GIBCO, USA)

1.2 Tryphane blue (Merck, USA.)

1.3 Giemsa and giemsa buffer (Polysciences, USA.)

1.4 Distilled water (Medical Product Public, Thailand)

2.Cell isolation and culture reagents

2.1 Ficoll Hypaque (Robbin scientific, USA)

2.2 CD133 cell isolation kit (Miltenyi Biotec GmbH, Germany)

2.3 Dulbecco's modified eagles medium-high glucose ; DMEM-HG (BioWhittaker, USA)

2.4 Fetal calf serum; FCS (BioWhittaker, USA)

2.5 Stemline II medium (Sigma-Aldrich Corporation, USA)

2.6 L-glutamine (GIBCO, USA)

2.7 Trypsin-EDTA (GIBCO, USA)

2.8 Complete methylcellulose medium with recombinant cytokine; Methocult GM H4434 (Stem cell technologies, Canada)

2.9 Iscove's modified eagles medium; IMDM supplement with 2% FCS (Stem cell technologies, Canada)

2.10 Gentamycin (M&H Manufacturing, Thailand)

2.11 Recombinant human interleukin-1alpha; rhIL-1α (R&D systems, USA)

2.12 Recombinant human thrombopoietin; rhTPO (R&D systems, USA)

2.13 Recombinant human flt3-ligand; rhFL (R&D systems, USA)

2.14 Freezing medium (GIBCO, USA)

3.Flow cytometry reagent

3.1 CD133/2 conjugated with PE (Miltenyi Biotec GmbH, Germany)

3.2 CD34 conjugated with FITC (Caltag Laboratories, USA)

3.3 CD34 conjugated with PE (Becton Dickinson, USA)

3.4 CD38 conjugated with FITC (Caltag Laboratories, USA)

3.5 CD45 conjugated with TriColor (Caltag Laboratories, USA)

3.6 HLA-DR conjugated with FITC (Caltag Laboratories, USA)

3.7 CD19 conjugated with PE (Immunotech-Coulter, USA)

3.8 CD7 conjugated with FITC (Becton Dickinson, USA)

3.9 CD5 conjugated with PE (Immunotech-Coulter, USA)

3.10 CD15 conjugated with FITC (Immunotech-Coulter, USA)

3.11 CD33 conjugated with PE (Immunotech-Coulter, USA)

3.12 GlycophorinA conjugated with PE (Immunotech-Coulter, USA)

3.13 CD41 conjugated with FITC (Immunotech-Coulter, USA)

3.14 Isoton II sheath fluid (Immunotech-Coulter, USA)

3.15 1% paraformaldehyde (Becton Dickinson, USA)

Procedure

1. Human donor cell preparation

Heparinized human BM was obtained by aspiration from the posterior iliac crest of hematologically normal donors who had given informed consent. UCB was collected immediately after delivery at the end of full-term pregnancies in a sterile blood bag containing the anticoagulant CPDA-1. Informed consent of the mother was obtained.

2. Human bone marrow mesenchymal stem cell (MSC) culture

Heparinized BM was mixed with an equal volume of PBS, gently layered on top of Ficoll-Hypaque gradients and centrifuged at 1300xg for 20 min. without breaking. MNC collecting at the interface were recovered, wash twice with PBS, resuspended in human MSC medium and plated at a density of $1x10^5$ cells/$25cm^2$ flask. Human MSC medium consisted of DMEM-HG supplemented with 10% FBS, 200mM L-Glutamine and 1% gentamycin . Culture were incubated at 37°C with 5% humidified CO_2. MSC layer were obtained after culture for 10-14 days, harvested with 0.25% trypsin and 1mM EDTA for 5 min at 37°C, and the replated at about 25,000 cells / $25cm^2$ in a new flask. Fresh medium was replaced at 48 h. and thereafter every 3-4 days. Cells from passage 2 were harvested with 0.25% trypsin/EDTA, suspended at $1-2x10^6$ cell/ml in freezing medium , and frozen at 1-ml aliquots in liquid nitrogen. To expand a culture, a frozen stock of MSCs was thawed, plated at 5,000 cell/cm^2, and incubated for 7-10 days. The cells were harvested and diluted for further expanding by plating at initial densities of about three cell/cm^2 and culturing in the human MSC medium. The cell numbers were counted with a hemocytometer. Cells at passage 3-5 were trysinized and seeded at $3x10^4$ cells/well in 6-well culture plate 1-2 days before coculturing with CB CD133-enriched cells.

3. Cord blood $CD133^+$ cell purification

MNC were isolated from CB using Ficoll-Hypaque density centrifugation by centrifuged at 1300xg for 20 min without breaking. The MNC band at the interface was removed, washed twice with PBS supplemented with 2mM EDTA and resuspend in 300 μl of PBS supplemented with 2mM EDTA and 0.5% FBS (referred to as MACS buffer)

The $CD133^+$ MNC fraction was isolated with superparamagnetic microbead selection using high-gradient magnetic field and miniMACS columns according to the manufacturer's instruction. Briefly, UCB MNC were incubated for 30 min at 4 °C with 100 μl FCR blocking reagent to block the Fc receptors and 100 μl anti-CD133 antibody with microbead. Cells were washed with MACS buffer. Labeled cells

were applied to magnetic column, unbound cells washed out, and $CD133^+$ cells eluted from the column with MACS buffer. To improve the purity of $CD133^+$ cells, a second purification cycle was performed. The efficiency of the purification was verified by flow cytometry countertaining with CD133-phycoerythrin antibody. In the cell fraction containing purified, $CD133^+$ cells ranged from 89.12% to 97.29%.

4. Recombinant human cytokines

The following recombinant purified human cytokines were used in these studies: recombinant human Flt3-L (FL); recombinant human thrombopoietin (TPO) and recombinant human interleukin-1alpha (IL-1α), all purchase from R&D systems.

5. Ex-vivo expansion of CB $CD133^+$ cells

CB $CD133^+$ -enriched cells ($0.8\text{-}3\times10^5$) were cultured in 6-well plate (4 ml) for 28 days in Stemline II medium at 37 °C under 5% humidified CO_2, in following condition: MSC + IL-1α (10 ng/ml), MSC + FL (35 ng/ml) + TPO (35 ng/ml) and FL (35 ng/ml) + TPO (35 ng/ml). Culture were half-fed every 4 days with half of the cultures being harvested and used for analysis, and the same volume being replaced with fresh media and cytokine. Cultures were passage into the new flask if necessary.

6. Proliferative and phenotypic analysis

The ex vivo expansion of the $CD133^+$- enriched population was determined at each time point by counting the content of hematopoietic cells in each well using tryphan blue stain 0.4% solution and analyzed for stem cell and lineage content by flow cytometry using monoclonal antibodies against CD5, CD7, CD19 and CD45 to evaluate lymphoid lineage differentiation; CD15, CD41, CD33 and Glycophorin A (GlyA) to evaluate myeloid differentiation; and CD34, CD38, CD133 and HLA-DR to assess the percentage of stem and progenitor cells remaining in culture. Flow cytometric analysis was performed at each time point of culture, by incubating harvested cells with different fluorescent conjugated monoclonal antibodies for 30 min at 4 °C in dark room. After washing with PBS, the cell were fixed in 300 µl PBS with 1% paraformaldehyde. The antibodies were labeled as follow: FITC-conjugated antibodies (CD5, CD15, CD34,

CD38, CD41, HLA-DR), PE-conjugated antibodies (CD7, CD19, CD33, CD34, CD133, GlyA) and TriColor-conjugated antibodies (CD45). Five thousand to 10,000 events were counted. Analysis was performed at a FACScan using CellQuest software.

7. Clonogenic assays

Assays for clonogenic progenitors were performed in duplicate in MethoCult GF H4434 with unexpanded $CD133^+$- enriched cells and expanded popualtion. Cultures were incubated at 37°C with 5% humidified CO_2. After 10-14 days colonies were counted and categorized ascording to standard criteria.

8. Morphology studies

The morphology of isolated $CD133^+$ and expanded cells were assessed using the Giemsa stained cytospin preparations.

9. Statistical analysis

The results was compared with an independent Kruskal-Wallis Test. Bonferroni were used for multiple comparison tests. P value of less than 0.05 were considered statistically significant. Graphed data were expressed as mean.