CHAPTER I

INTRODUCTION

Background and Rationale

Postnatal bone marrow transplantation (BMT) has long been used successfully in treatment of various disorders, including inherited metabolic, hematologic and immunologic diseases. More recently, in utero hematopoietic stem cell (HSC) transplantation has became an theoretical alternative to postnatal stem cell transplantation for the treatment of congenital hematologic disorders [1] as shown in Table 1.

Table1 : Congenital hematologic diseases successfully treated by postnatal HSC transplantation and potentially amenable to in utero HSC transplantation. [2]

β- Thalassemia
Sickle cell anemia
Severe aplastic anemia
Severe combined immunodeficiency*
Wiskott – Aldrich syndrome*
Chronic granulomatous disease
Kostman's syndrome
Infantile malignant syndrome

* Successfully treated by in utero transplantation.

The Thalassemias are a collection of genetic defects of hemoglobin associated with a reduced synthesis of one of the two adult (α and β) globin chains. This leads leading to the accumulation of another chain and toxicity, resulting in the death of erythrocytes in the bone marrow (BM).

Both α and β - thalassemias are prevalent in Thailand (figure1). [3] These abnormal genes with different combinations can lead to over 60 thalassemia syndromes, ranging in severity from asymptomatic conditions to total lethality. Since thalassemia and hemoglobinpathies prevalence is high in Thailand (Table 2)[4] thus they are not only medical but also socioeconomic problem of the country.

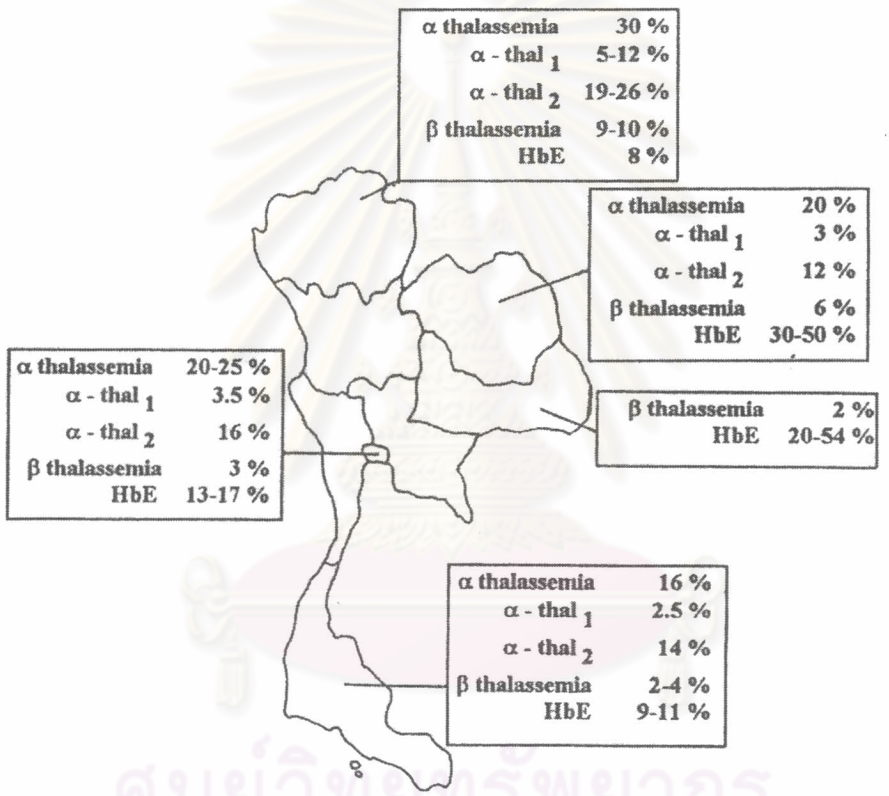
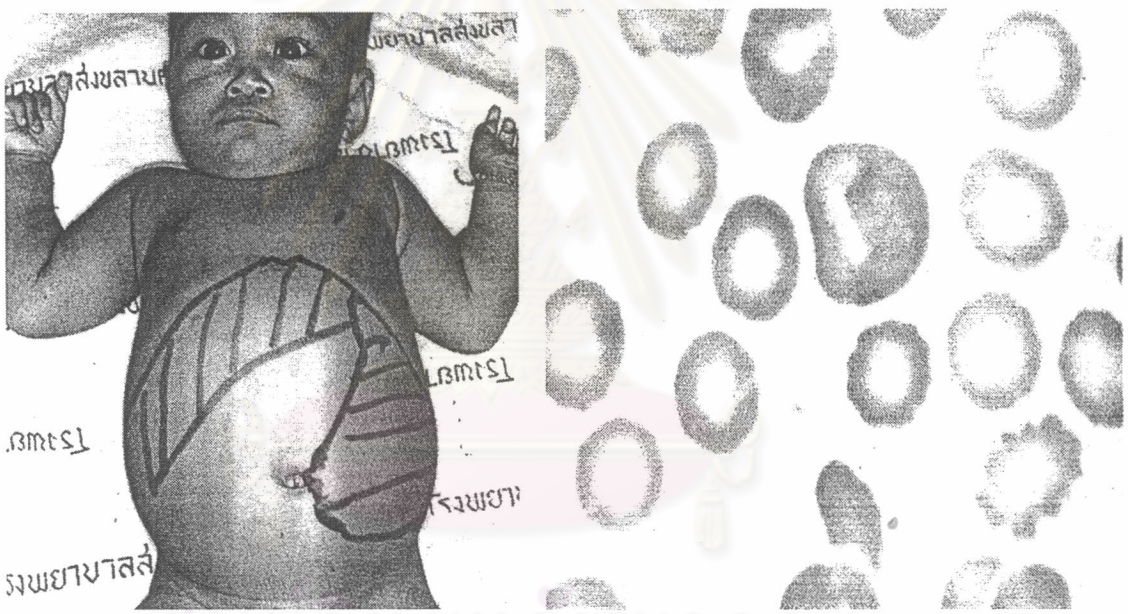


Figure 1 Prevalence of Thalassemia and Hemoglobinopathies in Thailand (1998) [3]

Table 2 : The incidence of major thalassemia in Thailand [4]

Geographic locations	α -Thalassemia (Percentage)	β - Thalassemia (Percentage)	HbE (Percentage)
North	30	9 - 10	8
Northeast	20	2 - 6	32-60
Central	20-25	3	13-17
South	16	2 - 4	9 -11



A B

Figure2 A) Both liver and spleen are enlarged in β - Thalassemic patient at 6 months of age

B) Blood smear of β - Thalassemia [5]

Early treatment of major thalassemia diseases includes blood transfusion.[6] Adequate blood transfusion has been shown to prevent defective physical development and abnormal bones, and also leads to general well – being to sustain life. Splenectomy is often performed to increase the survival of endogenously produced and transfused red cell.[7] The constant complication of multiple transfusion are associated with development of infection, anti- red blood cell (RBC) and platelet antibodies, and increased iron deposition in the body which ordinarily leads to organ damages and death. This excess iron can be reduced by giving iron chelating agent such as Deferoxamine or desferrioxamine which used require daily infusion for up to 12 hours.[8] This leads to a lack of compliance which may reverse the clinical outcome and increase in death rate from complications of iron overload.[9]

In the past 30 years since the first successful bone marrow transplantation, the field of stem cell transplantation from bone marrow have been given to more than 80 thalassemia patients.[10] For patients underwent transplantation with bone marrow from a match sibling, the probability of cure rate might be as high as 75-80%, depended on the compatibility of the donor, host immune system and their disease status at the time of transplantation. The suitable donors, fully matched for the human leukocyte antigen (HLA) system could be obtained in approximately 20-30% of patient needing allogeneic transplantation. [11-12] Due to the morbidity and mortality associated with bone marrow transplantation, few hemoglobinopathy patients have received this treatment.

In utero transplantation, hematopoietic stem cell (HSC) transplantation is a theoretical alternative to postnatal bone marrow transplantation (BMT) for the treatment of congenital hematologic disorders which can be diagnosed early in gestation. The rationale for consideration of In utero transplantation is based on normal developmental ontogeny. The early gestation fetuses may be immunologically immature and uniquely tolerant to foreign antigen (Ag) enough to allow the acceptance of allogeneic or xenogeneic cells without the risk of marrow ablation,graft rejection and graft- versus host disease (GVHD).[13] It has also resulted in the consideration of new strategic approaches for the therapeutic application of in utero HSC transplantation.

Early attempts to obtain experimental hematopoietic chimerism after in utero transplantation into normal animal models can be very useful for the study of basic transplantation biology and GVHD prevention as described in Figure 3. [14]

Congenital disorders

Several models of congenital disorders correctable by HSC transplantation have been studies

Stem cell studies

Studies stem cell engraftment after HSC transplantation.

Graft versus host disease

Syndrome is similar in its distribution and pathology. Best model of human skin GVHD is pig.

Growth factor

Most human growth factors are active in rodents and pigs.

Late effects

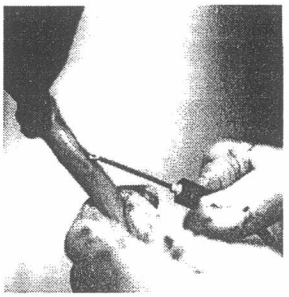
The short natural life span of animal model makes them useful for the studies of effects of HSC transplantation.

Figure 3 Animal models of HSC transplantation and their applicability to human transplantation [14]

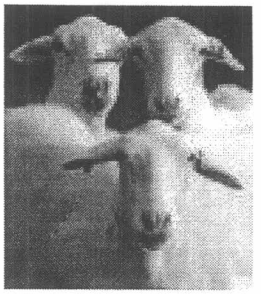
There are several potential sources of donor HSC for transplantation. Human umbilical cord blood (UCB) has been increasingly interest and becomes a choice as an alternative sources of HSC other than fetal liver, adult bone marrow or peripheral blood. [15] The benefit of UCB include wide availability, easy and low cost of procurement, unbias ethnic participation, low incidence of GVHD and a decrease in risk of infection.[16-17]

Donor HSC obtained from human UCB were transplanted into preimmune sheep fetuses by intraperitoneal injection under ultrasound guidance. After in utero transplantation, the donor HSC were expected to get into the recipient circulation and homing into specific environment of bone marrow. Following after donor cell engraftment, recovery of the stable reconstitution of hematopoiesis would be indicated by functional levels of both human donor myeloid and lymphoid cells. The presence of significant numbers of donor cells in host peripheral blood following in utero transplantation could be used as definitive marker of successful engraftment and chimerism (Figure 4). [18]

Our goal in this study was to compare the sensitivity and accuracy of Flow Cytometry and FISH analysis in detection of human cord blood lymphocytes in *in vitro* mixed human - sheep lymphocytes. These techniques would be used to analyze the donor human cells in the newborn sheep after in utero transplantation.



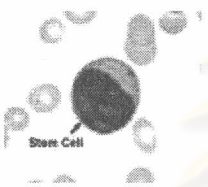
Donor HSC from human UCB



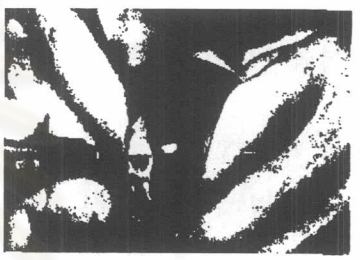
Pregnant sheep

CD 34 + Progenitor cell isolation

sheep fetus recipient



Intraperitoneal in utero transplantation



Infusion of donor HSC into the circulation

The mechanism of homing

Donor cell engraftment

Detection of donor cell engraftment from peripheral blood

Figure 4 The human – sheep model for in utero transplantation

The wide variety of techniques had been reported regarding detection of donor cell engraftment.These included HLA- mismatch, immunohistochemistry, Flow Cytometry, [19] Fluorescence *in situ* hybridization (FISH), [20] Restriction fragment length polymorphism

(RFLP) and polymerase chain reaction (PCR) [21] etc. Therefore, the techniques and protocolsensitive enough for detection of donor cells engraftment after in utero transplantation would be required.

The level of human $CD45^+$ cells (human lymphocytes) which represents a nuclear hematocyte would be used as a marker for engraftment and hematopoietic chimerism in this human – sheep in utero transplantation model. The donor HSC engraftment and appearance of donor cells would be sequentially determined by analysis of peripheral blood (PB) of sheep recipients after birth by both methods including Flow Cytometry (FCM) analysis for CD 45 antigen and Fluorescence *in situ* hybridization (FISH) with DNA probes (CEP16) specific for repeated satellite sequence on human autosome.

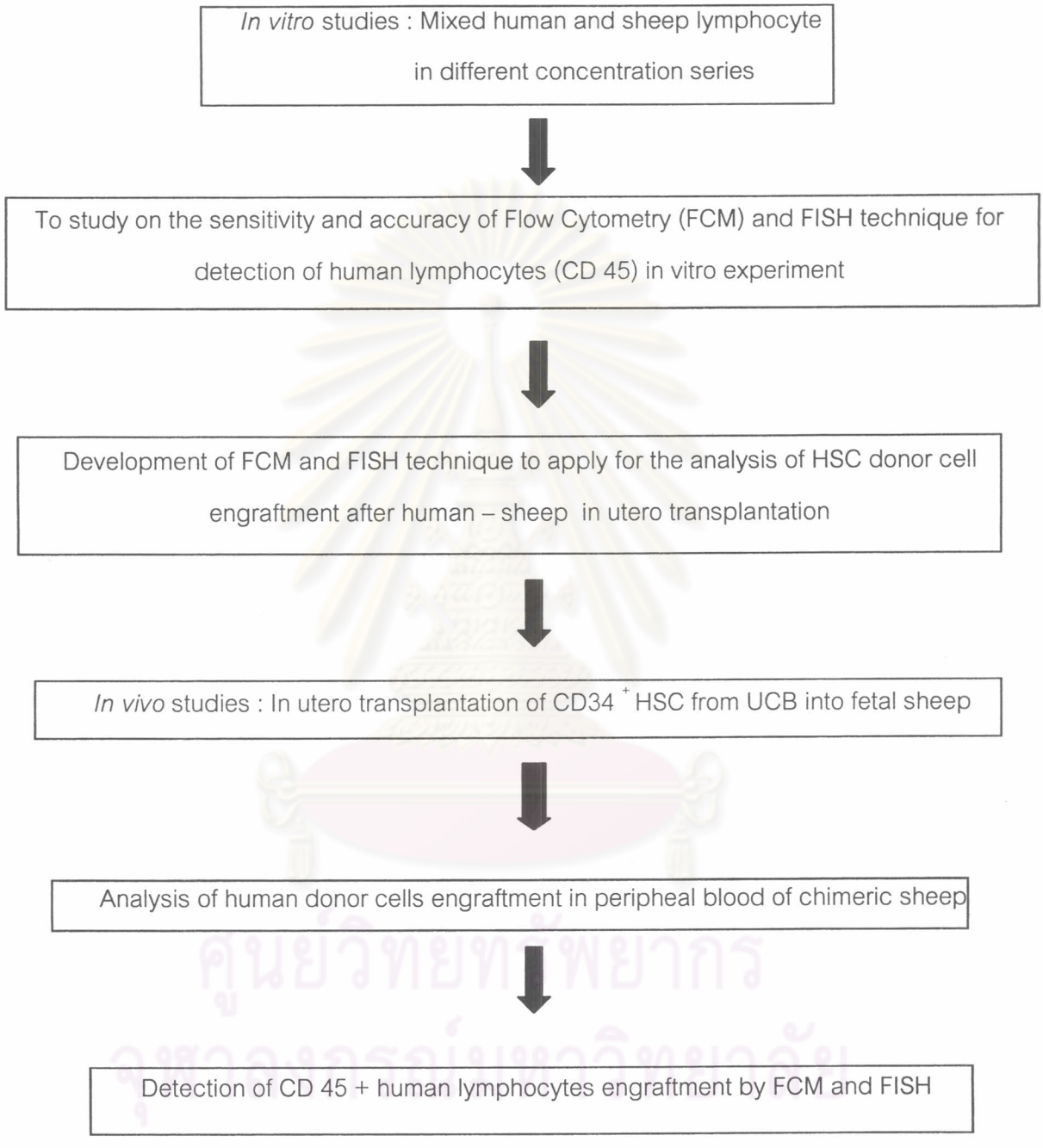
In the present study we used mix human-sheep lymphocytes *in vitro* experiments for the development of FCM and FISH techniques. In addition, *in vivo* studies, we applied these techniques to investigate the engraftment and chimerism of human donor HSC following the human – sheep in utero transplantation model.

Objectives

1.1 *In vitro* studies on the sensitivity and accuracy of Flow cytometry (FCM) and FISH technique to detect human lymphocytes in different concentration series of mixed human- sheep lymphocytes

1.2 *In vivo* studies to detect human - sheep engraftment after in utero transplantation of human umbilical cord blood (UCB) into fetal sheep by Flow cytometry and FISH

Conceptual Framework



Assumption

In this research mixed *in vitro* human and sheep lymphocytes in different concentrations were used to test the sensitivity and accuracy to detect human lymphocytes by FCM and FISH analysis. In order to analyze the donor cell engraftment after in utero transplantation of human UCB into fetal sheep, the need for *in vitro* experiment as a basis of standardized method was modeled samples.

Keywords

Human – sheep chimerism

Human umbilical cord blood

Flow Cytometry

FISH

Operational Definition

$$\text{Mononuclear cell count} = \frac{\text{The number of lymphocytes} \times \text{concentration} \times 10^3}{\text{The Volume of chamber hemocytometer}}$$

Flow Cytometry : Gate Percentage = A numerical percentage or peripheral boundary (region) that defines the cell populations of interest to select the data from cytometry

$$\text{FISH} : \frac{\text{The number of fluorochrome human lymphocyte} \times 100}{\text{The total of cell lymphocyte}}$$

Expected Benefit & Application

1. Establish a basis data on sensitivity and accuracy of FCM and FISH technique to detect human lymphocytes in mixed human – sheep lymphocytes experiment.
2. Development of a laboratory model which can be applied to assess human lymphocytes engraftment after in utero human UCB transplantation into fetal sheep.
3. Original basis on scientific fact for human- sheep model (the xenograft chimera) may provide an insight into many areas of research including the physiology of hematopoiesis stem cell biology, gene transfer to HSC before transplantation, etc.
4. Applied to clinical research on transplantation of UCB in treatment of a variety of diseases such as thalassemia, leukemia, and other life threatening diseases.

Research Methodology

1. Target population

   1.1) The collection of umbilical cord blood was performed in the delivery and operating suite of the Department of Obstetrics and Gynecology at King Chulalongkorn Memorial Hospital in 2000-2002.

   Inclusion case - Normal UCB samples collected from full term healthy pregnant mothers without genetic disorders and infectious diseases.

   Exclusion case - UCB samples infected with common blood transmitted Infectious disease such as Hepatitis virus : HbsAg, Retroviruses : HIV and Syphilis - VDRL.

   1.2) Sheep PB collection was obtained from the Faculty of Veterinary, Chulalongkorn University and the National Institute of Animal at Kasetsart University.

2. Sample Collection

2.1) Human umbilical cord blood sampling (donor) 20 ml were collected in heparinized vaccutainer from full- term healthy pregnancies with informed consent.

2.2) Sheep blood sampling (recipient) 20 ml were collected in heparinized vaccutainer from the external jugular vein of adult sheep.

3. Process to *In vitro* study

3.1) Heparinized human umbilical cord blood donor (UCB) was collected after informed consent and then centrifuged in Histopaque (Ficoll – hypaque) to separate the peripheral blood mononuclear cell (PBMC) or lymphocytes out. Counted human lymphocytes in chamber of hemocytometer and calculate the absolute number of lymphocyte per microliter of sample.

3.2) Heparinized sheep blood (recipient) was centrifuged to separate the buffy coat at the interphase between serum and erythrocytes. Collected the buffy coat into PBS then it was separated on 60% Percoll solution. Then counted sheep lymphocytes in chamber of hemocytometer to calculate the absolute number of lymphocyte per microliter of sample.

3.3) The lymphocytes from step 3.1 and 3.2 were mixed for establishing a dilution series between human lymphocyte (donor) and sheep lymphocytes (recipient) into 5 ratios 1:100 ,1:500 , 1:1,000, 1:5,000 and 1:10,000.

3.4) The dilution series of human and sheep blood cells was divided into 2 aliquots for the analysis of human lymphocytes by FCM and FISH analysis.

3.5) Flow cytometry (FCM) analysis : Each of the dilution series was incubated with 10 μl of human lymphocyte CD 45 – PerCP antibody for 30 min. Then the cells were washed with PBS and centrifuged for 5 min. The cell pellet was fixed by the addition of 500 μl paraformaldehyde (1%w/v) and immediately measured by FCM of human lymphocytes.

3.5) FISH analysis : Each of the dilution series was fixed and smeared on slides to detect human lymphocytes by Fluorescence *in situ* hybridization (FISH) analysis. Then, the cells were hybridized with CEP 16 probe specific to human autosome. The slides were covered with a standard glass coverslips and examined under a fluorescence microscope for detection of the percentage of human lymphocytes.

4. Process of *in vivo* study

4.1) Human UCB was obtained from full – term healthy pregnancies with informed consent. The required screening test include HBsAg, anti-HIV, VDRL and complete blood count.

4.2) The isolation of hematopoietic stem cell (HSC) from UCB (donor cell) is performed by immunomagnetic beads positive selection of $CD34^+$ expressing cells.

4.3) Creation of human – sheep models were performed by in utero transplantation of CD34 + HSC from human UCB into preimmune fetal sheep at 48-54 days of gestation . Fetal sheep recipient was transplanted with CD 34 + HSC by transcutaneous intraperitoneal injection under ultrasound guidance.

4.4) Detection of donor cell engraftment was done by analysis of recipient peripheral blood (PB) after birth by a combination methods including Flow Cytometry (FCM) analysis of CD 45 antigen (associated with human lymphocytes) and Fluorescence *in situ* hybridization (FISH) with DNA probe, CEP 16 (specific for repeated satellite sequences on human autosomes).