CHAPTER III

RESEARCH METHADOLOGY

Materials

1. Chemicals, instruments, plastic and glasswares

The chemicals, materials and instruments employed in the present studies were summarized in Tables 1 and 2

Table 1 List of chemicals equipments and plastic wares

Name	Source
Ringer solutions	
NaCl	Merk Germany
KCl	Merk Germany
$MgSo_4.7H_2O$	Merk Germany
HEPES (N-2-hydroxethyl sulfuric acid)	Sigma Sweden
$CaCl_2.2H_2O$	Merk Germany
D-Glucose	Merk Germany
NaOH	Merk Germany
Inducing agent	
Tertiary-butylhydroperoxide (tBOOH)	Sigma Sweden
CH11 Fas-activating antibody	Millipore Germany
Ionomycin	Sigma Sweden
Dimethyl sulfoxide (DMSO)	Sigma Sweden

Table 1 (Cont.)

Name	Source
Insulin	Novo nordisk Denmark
Lymphoprep™	NYCOMED Norway
Phosphate buffered saline (PBS)	Sigma Sweden
RPMI 1640	Gibco USA
Penicillin and streptomycin solution	Sigma Sweden
Annexin V-FITC apoptosis detection kit I	BD Biosciences Germany
Equipments and plastic wares	
24-well plate flat, bottom	BD Biosciences Germany
Culture Flask	BD Biosciences Germany
Centrifuge and Test Tubes 15mL	BD Biosciences Germany

Table 2 List of instrument used in the studies

Name	Source
Centrifuge machine	Sokuzan
CO2 incubator	Memmert Germany
FACScalibur cell analyzer (model FACSCaliburTM Flowcytometry)	BD Biosciences Germany
Automated chemistry analyzer	Roche (Hitachi 912) Germany

2. Collection of blood samples

Blood samples to be studied should be fresh EDTA (Ethylenediaminetetra-acetate) blood of thalassemia patients and healthy from Laboratory of Naresuan University Hospital.

2.1 Purification of red blood cells

Fresh EDTA blood samples were white blood cells removed by centrifugation through Ficoll-Hypaque (Lymphoprep^{TM}, NYCOMED AS, Norway). After centrifugation for 10 min at 1000g, plasma and buffy coat were removed, and red blood cells were washed three times with isotonic phosphate-buffered saline (PBS), pH 7.4.

3. Cell lines

Human T cell leukaemia cell line Jurkat was used for apoptotic control cell.

3.1 Culture of Jurkat cells

Human T cell leukaemia cell line Jurkat were cultured and maintained in RPMI 1640 with L-glutamine (Gibco/BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillins and 100 µg/ml streptomycins. Cells were grown at 37°C with 5% CO2 in humidifies air.

Methods

1. Functional impact of CD95 on the induction of eryptosis

1.1 Stimulation of red blood cells phosphatidylserine exposure by CH11 Fas-activating antibody

Purified human erythrocytes were suspended in Ringer solution (in Mm): 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 HEPES / NaOH(pH 7.4), 5 glucose, 1 $CaCl_2$. Eryhtrocyte activated CD95 system for the induction of PS exposure in erythrocytes treated with increasing concentrations of 0, 100, 300 and 500 ng/ml agonistic antibody CH11(Millipore, Billerica, MA) were incubated for 24, 48 and 72h in an atmosphere of 5% CO_2 at 37 °C. As a positive control for induction of PS exposure in exposure in erythrocytes were incubated in Ringer solution containing 1µM of ionomycin for 2h.

1.2 Apoptosis Induction in Jurkat cells

Culture of Jurkat cells:

Human T cell leukaemia cell line Jurkat were cultured in RPMI 1640 with L-glutamine (Gibco/BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillins and 100 µg/ml streptomycin. All cultures were in an atmosphere of 5% CO_2 at 37 °C for24h initiated with 1×10^6 Jurkat cells / well, in 1 ml volumes, in 24- well plates, the cells were exposed to different apoptotic stimuli.

CH11, a Fas- activating antibody, at concentration of 0, 30 and 50 ng /ml were used to initiate the receptor death domain dependent apoptotic process.

2. Influence of insulin to reduce eryptosis

2.1 Determination of glycolysis induction by insulin in red blood cells

Measure stimulated lactate production, lactate concentration was determined using the automated chemistry analyzer by Hitachi 912, Roche.

Resuspend purified red blood cells (8-10% hematocrit) in 40 ml of Ringer solution and aliquot of 10 ml red blood cells suspension for 4 samples. An aliquot of 10 ml red blood cells suspension was added insulin to abtain the final concentration of 0, 10, 100, and 1,000 Mm insulin. Each insulin-treated samples take 2 ml of the suspension and centrifuge it at 1, 2, 3, and 4 h after incubation and measure lactate concentration of the supernatant according to automated chemistry analyzer.

2.2 Determination of the optimal concentration of tBOOH /H_2O_2 for oxidative stress induce of red blood cells eryptosis.

Resuspend purified red blood cells (8-10% hematocrit) in 8 ml of Ringer solution and aliquot of 1 ml red blood cells suspension for 8 samples. The aliquot of 1 ml red blood cells suspension of sample 1 - 4 were added tBOOH solution to abtain the final concentration of 0, 0.33, 0.66, and 1.0 mM tBOOH. After that mix and incubated at room temperature for 15 min, then centrifuge the suspensions, remove supernatant and replace it with fresh Ringer solution.

The aliquot of 1 ml red blood cells suspension of sample 5 – 8 were added H_2O_2 solution to abtain the final concentration of 0, 1, 2, and 4 mM H_2O_2. After the treatment incubated at 37 °C for 24 h and measured phosphatidylserine exposure by FACS. Positive control was treated with 1 μM ionomycin.

2.3 Effect of insulin to reduces thalassemic red blood cells eryptosis

Resuspend purified red blood cells (8-10% hematocrit) in 8 ml of Ringer solution and aliquot of 1 ml red blood cells suspension for 8 samples. The aliquot of 1 ml red blood cells suspension of sample 1 - 4 were absent of 10 mM insulin and aliquot of 1 ml red blood cells suspension of sample 5 – 8 were added 10 mM insulin. The treated and untreated insulin samples were incubated at 37 °C for 3 h and induced eryptosis by tBOOH solution to abtain the final concentration of 0, 0.02 and 0.1 mM tBOOH in treated and untreated insulin samples. After that,

all samples were incubated at 37 °C for 17 h and measured phosphatidylserine exposure by FACS.

3. Analysis of phosphatidylserine exposure

Phosphatidylserine is a phospholipid component, usually kept on the inner-leaflet, the cytosolic side, of cell membranes by an enzyme called flippase. When a red blood cell undergoes apoptotic cell death or eryptosis, phosphatidylserine exposure at the outer membrane leaflet.

3.1 Annexin V-FITC method

AnnexinV-FITC (BD Bioscience) was used as a marker for phosphatidylserine (PS) detection. The annexin V-FITC apoptosis detection kit (BD Biosciences) was used. Briefly, cells were washed once with PBS and twice with binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2), and were resuspended in 100 µL binding buffer at concentration of 1×10^6cells/ml and 5 µL annexin V were added and incubated at room temperature for 15 minutes in the dark. After incubation, cells were resuspended in 400 µL of binding buffer and and measured using flow cytometric analysis (FACSCalibur;BDBiosciences, Heidel berg, Germany). Cells were analyzed by forward scatter, and annexin fluorescence intensity was measured in fluorescence channel FL-1with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

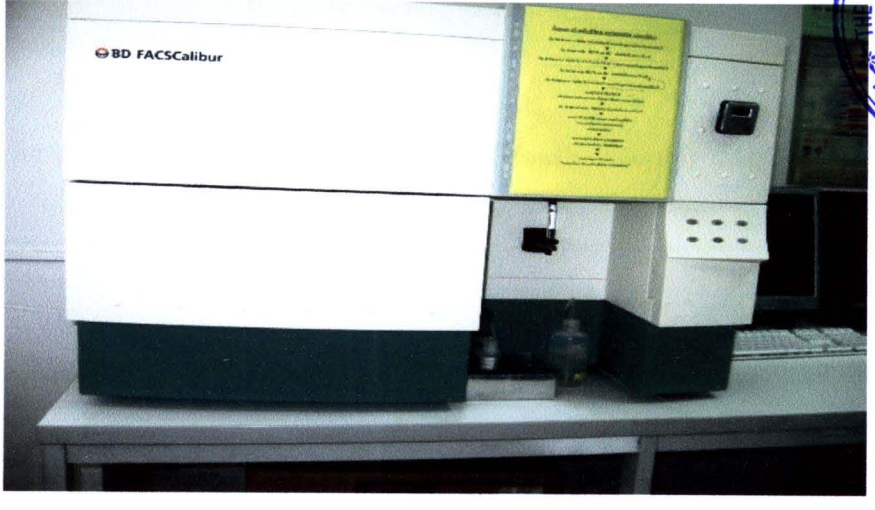

Figure 6 BD FACSCalibur Flow Cytometer; BDBiosciences, Heidel berg, Germany