CHAPTER III

Result

3.1 Analysis of freshly isolated PBMCs from whole blood

PBMCs freshly isolated were spherical shape composed of two subpopulations as monocytes and lymphocytes. Figure 3.1 demonstrated that very small amounts of PBMCs were positively staining with anti-$CD34^{+}$-FITC. As clearly seen that the anti-$CD34^{+}$-FITC positive staining cells were found in both suppopulations; 1.1% (Figure 3.1c), and 1.4% (Figure 3.1d), were found in lymphocyte and monocyte fraction, respectively. Cellular DNA content of PBMCs was measured using propidium iodide (PI) staining and analyzed by flow cytometer. As indicated in Figure 3.2a, most of PBMCs were positively staining with PI. It should be noted that initially of PBMCs isolation the cells were found in different phases including G0/G1, S and G2/M (Figure 3.2b and c). The cellular DNA content was measured at every 24 hours after the PBMCs fraction was cultured. The amounts of PBMCs isolated from 12 donors were indicated in Table 3.1. In our experimental conditions, the mean value of total PBMCs in male and female was equal to $3.7 \times 10^5 \pm 1.3 \times 10^5$, $3.6 \times 10^5 \pm 1.3 \times 10^5$cell/ml for adult, and $5.5 \times 10^5 \pm 2.7 \times 10^5$, $5 \times 10^5 \pm 1.72 \times 10^5$ cell/ml for elderly group, respectively. The result obtained from both groups was no-significantly difference (P= 0.28). In particularly, the anti-CD34+-FITC positive staining cell and the number of cell found in phase S and G2/M of male and female was equal to 8.64 ± 5.18, 3.29%for adult, and 2.81 ± 0.13, 2.11±1.77%for elderly with P-values equal to 0.31 signified that all subjects have similar amounts stem cells in blood. The histogram of cellular DNA content was changed. For example, at day 6 an amount of cells were found in sub G0/G1 phase and the cells found in S and G2/M were increased (figure 3.2d).

3.2 Expansion of PBSCs in culture

3.2.1 Effects of different origin of serum on PBSCs culture

We have verified that the colony forming of stem cells determined in our system was dependence on the density of PBMCs; only the density of cells lower degree than 5×10^6 cell/mL can observe cell proliferation and colony forming units of the stem cells. The behavior of PBMCs when cultured using the medium completed with 10% serum of their own donors compared with the series using fetal bovine serum was indicated in Figure 3.3. It was clearly shown that afterward the PBMCs were suspended in the medium and placed in a CO_2-incubator for 24 hours, almost of cells were suspended and aggregated among them throughout the flask. Few of cells with spindle shape were found to attach on the bottom of the flask at day-6, however only few colonies were developed even after day-24, while the aggregation of cells were bigger proportionally to the incubation time. The attached cells at day 24 were majorly foam like cells (Figure 3.4). Contrary, the series using fetal bovine serum the PBSCs were found as single distributed throughout the culture flask. At day-6, the colonies of mesenchymal stem cells were abundantly found attached onto the bottom of culture flask. When the cell cultures were prolonged to day 24, a variety of cell types were observed including, adipocytes, osteocytes, chondrocytes, neurons, leucocytes, stromal cells, etc (Figure 3.5).

3.2.2 Effects of sex and age on the potency of PBSCs culture

In order to compare the behavior of PBSCs obtained from adult and elderly age, the cells were cultured using the medium completed with 10% fetal bovine serum. Figure 3.6 showed that PBSCs grew and underwent differentiation similarly in all donors independently neither sex nor age. H&E staining of cells allowed identifying the specific cell type as indicated in Figure 3.5. The potency of PBSCs growth and differentiation in the presence of biocompatible 3D-nanofibrous PVDF scaffold was indicated in

figure 3.7. The PBSCs of all donors able to grow and undergo differentiation which yielding a tissue when they were cultured using the scaffold.

3.2.3 Analysis of cellular kinetics of PBSCs

On the microscopic analysis point of view, PBSCs composed of two subpopulations; one was adherent cell and the other is suspension cell. Both of cell types were founded the colony forming units of stem cells in the culture system but it was difficult to enumeration of the stem cells. The study rigorously isolated only the suspension cells then the cells were analyzed by measuring their DNA content as a function of time. Figure 3.8a shows that these cells were found in different cell phases including G0/G1, S, G2/M and sub G0/G1 that corresponding to the apoptotic cells. The results of this study allow writing the diagram (Figure 3.8b).Explaining the homeostasis which is the characteristic of normal stem cells. The parameters of cell kinetics including the mean rate of cell to re-entering to the cell cycle (γ), move from S to G2/M (k2), move from G2/M to G0/G1 (k1) undergone apoptosis (β) and differentiation (α) were determined as indicated in Table 3.2.

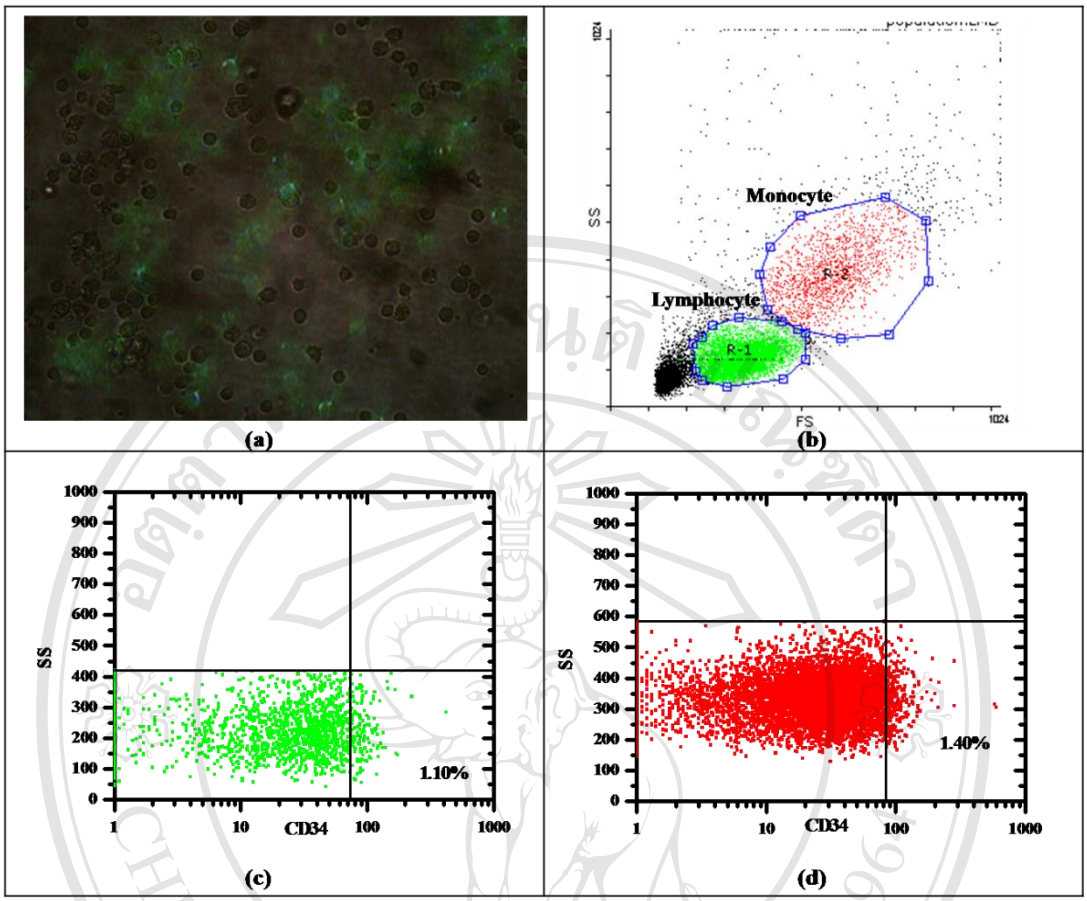

Figure 3.1 Characterization of peripheral blood stem cells by fluorescence micrograph (a), and cell counts using flow cytometer (b, c, d).

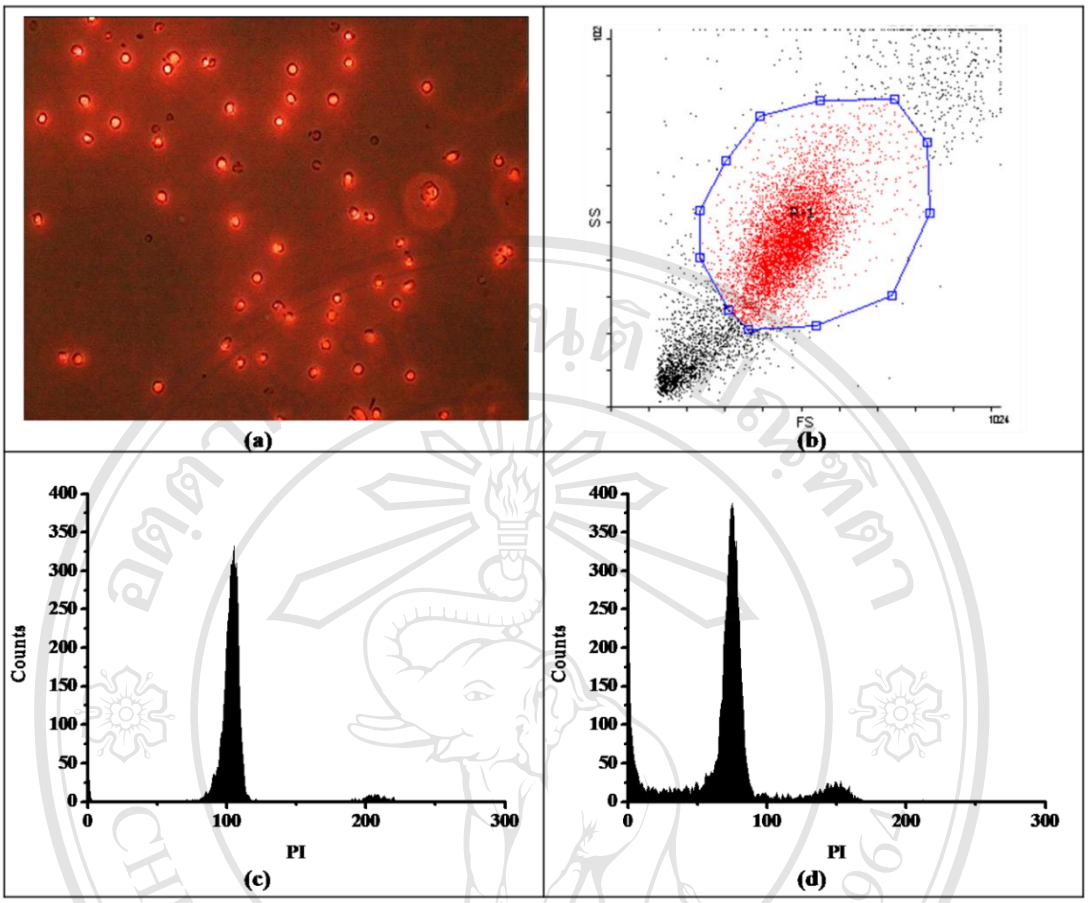

Figure 3.2 Characterization of peripheral blood stem cells by fluorescence micrograph (a), and cell counts using flow cytometer (b), and number of proliferating cells as a function of culture time (c, d).

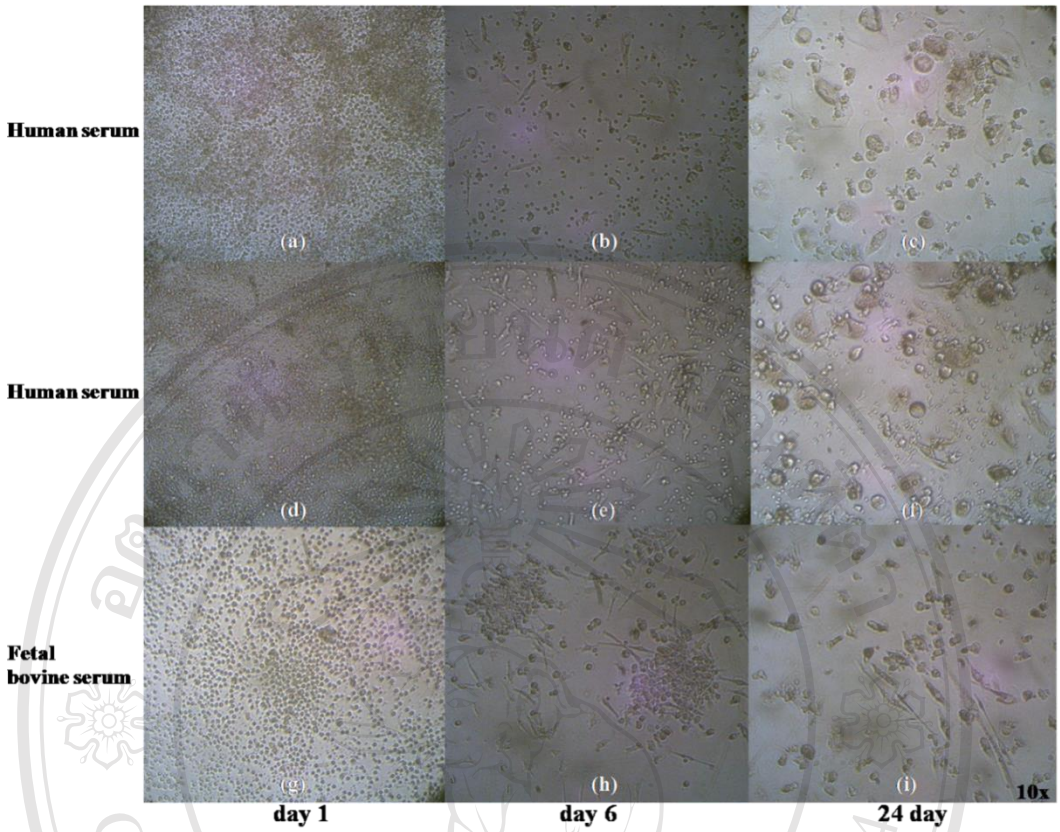

Figure 3.3 Micrograph of peripheral blood stem cells at day 1, day 6, day 24 after seeded. Serum of their own donor compared with the series using fetal bovine serum.

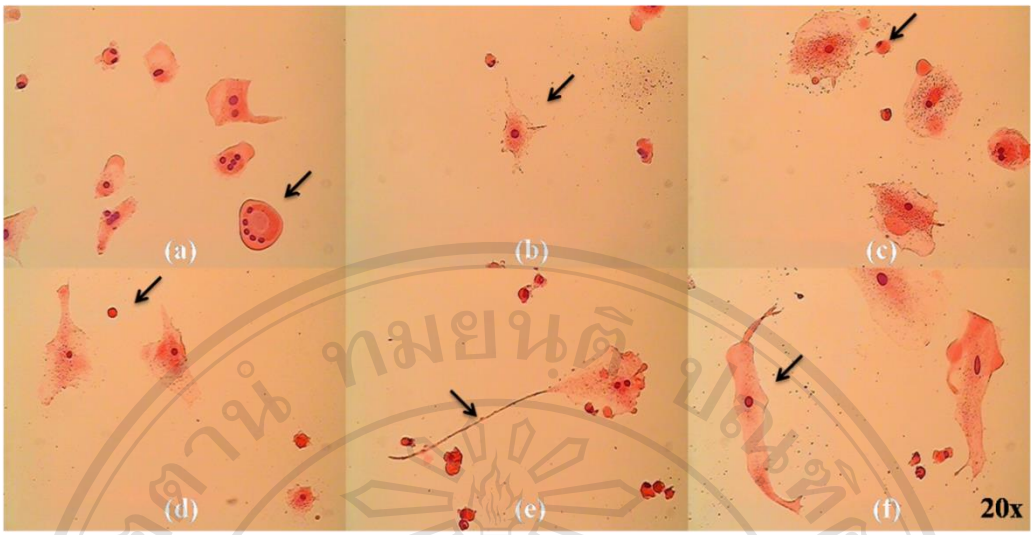

Figure 3.4 Micrographs of H&E staining cell in human serum; (a, f) keratinocytes, (b, c, d,) foam cells and (e) dendritic with the objective lens 20x

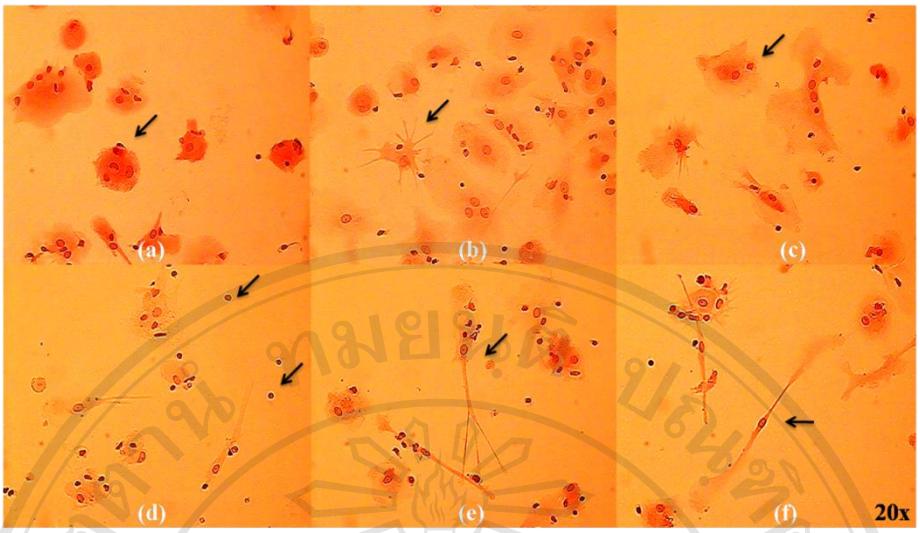

Figure 3.5 Micrographs of H&E staining cell in bovine serum; (a) Adipocyte, (b) Osteocyte, (c) Chondrocytes, (d) Leukocyte, (e) Neuron, (f) Stromal cell at 24 day with the objective lens 20x.

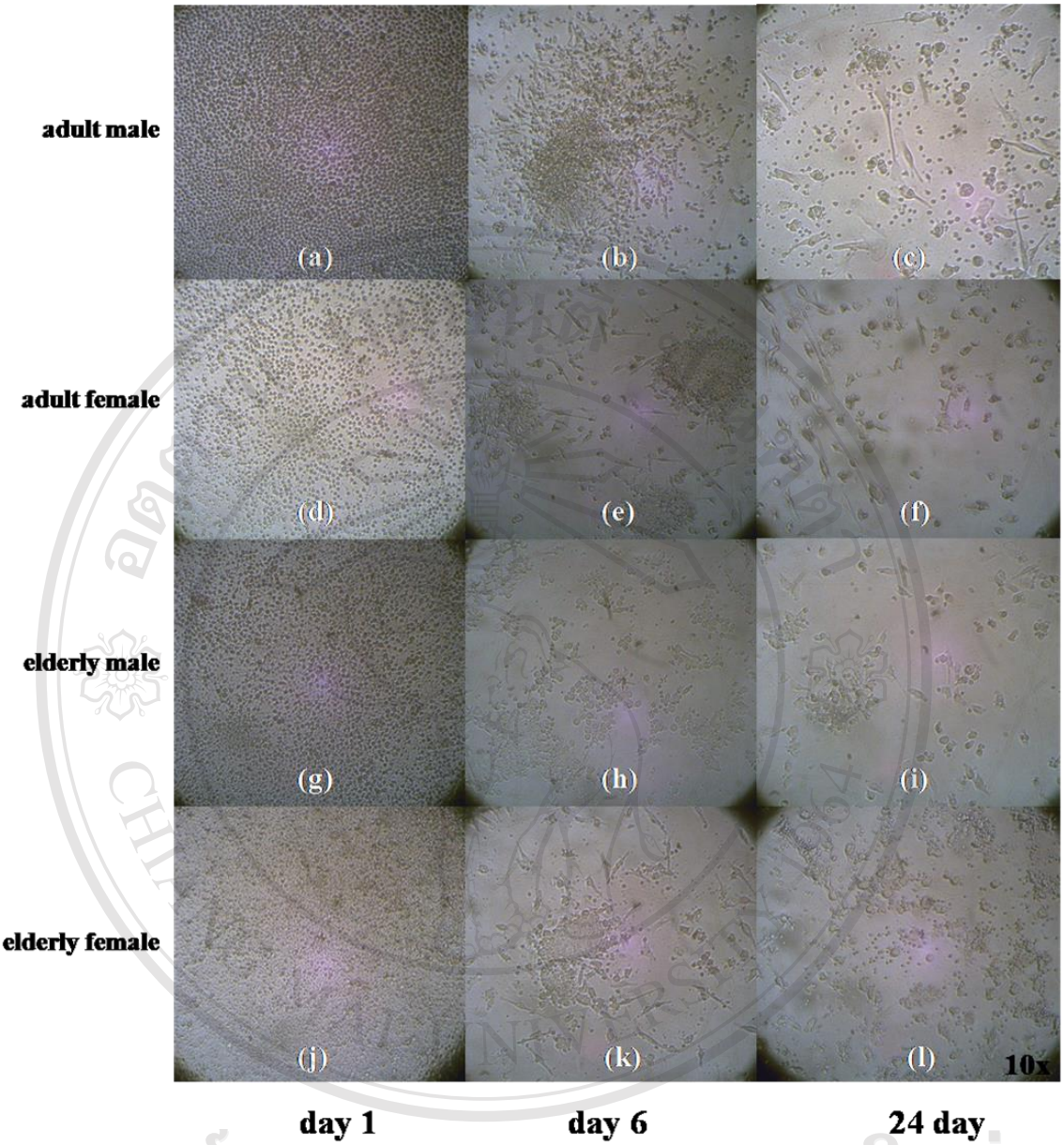

Figure 3.6 Micrograph of peripheral blood stem cells at day 1, day 6, day 24 after seeded**.** The cells were cultured using the medium completed with 10% fetal bovine serum.

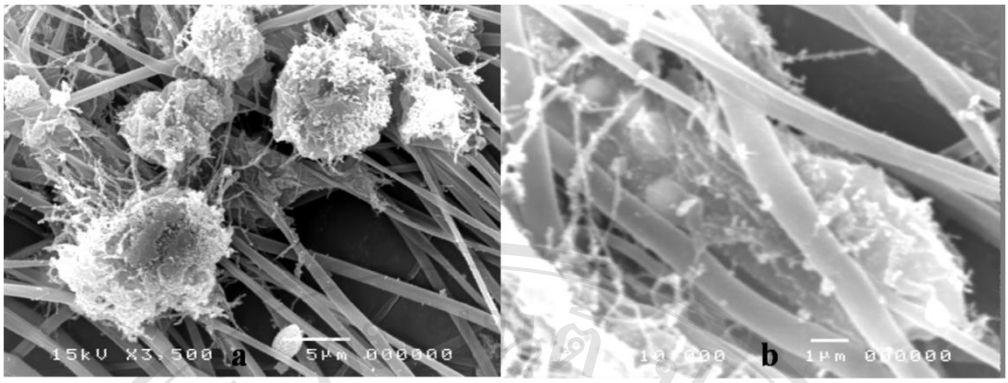

Figure 3.7 SEM demonstrated that the irregular forms of cell organizations (a), and the cells used the fibers as support for hanging themselves in the space (b).

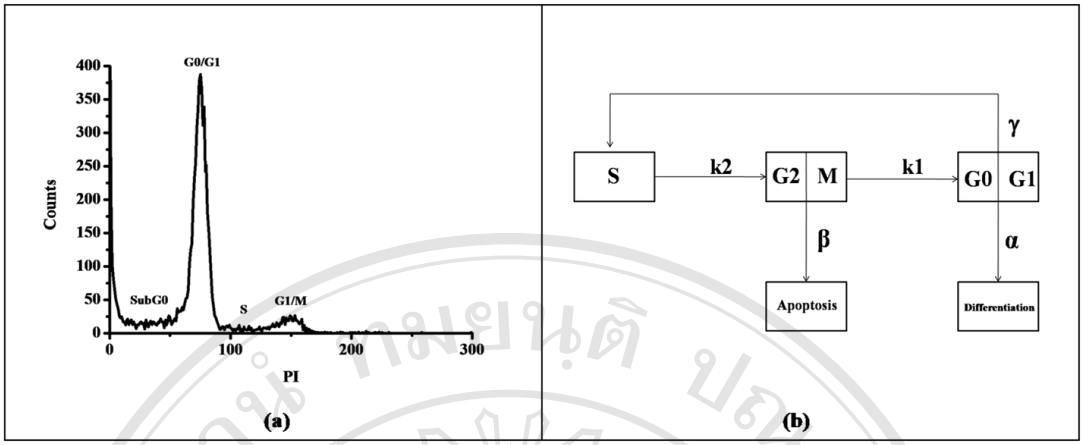

Figure 3.8 Number of proliferating cells as a function of culture time were found in different cell phases including G0/G1, S, G2/M and sub G0/G1 (a), Diagram explaining the homeostasis (b).

Table 3.1 Characteristics of PBMCs isolated from 12 donors number of PBMCs and number of S=G2/M, ($p = 0.059$ and 0.44).

Donor	Numbers of PBMCs, cell/mL	Number of S +G2/M, % of PBMCs
Adult		
AM1	**$3.7 \times 10^5 \pm 1.3 \times 10^5$**	**11.79**
AM2	**$5.0 \times 10^5 \pm 1.3 \times 10^5$**	**11.48**
AM3	**$2.4 \times 10^5 \pm 1.3 \times 10^5$**	**2.61**
AF1	**$2.5 \times 10^5 \pm 1.3 \times 10^5$**	**-**
AF2	**$2.8 \times 10^5 \pm 1.3 \times 10^5$**	**-**
AF3	**$5.4 \times 10^5 \pm 1.3 \times 10^5$**	**3.29**
Elderly		
EM1	**$4.4 \times 10^5 \pm 12.7 \times 10^5$**	**2.94**
EM2	**$3.5 \times 10^5 \pm 12.7 \times 10^5$**	**2.83**
EM2	**$8.6 \times 10^5 \pm 12.7 \times 10^5$**	**2.6**
EF1	**$6.8 \times 10^5 \pm 1.72 \times 10^5$**	**0.65**
EF2	**$4.7 \times 10^5 \pm 1.72 \times 10^5$**	**4.08**
EF3	**$3.5 \times 10^5 \pm 1.72 \times 10^5$**	**1.61**

Table 3.2 The determined parameters of cell kinetics including the mean rate of cell to re-entering to the cell cycle (γ), move from S to G2/M (k2), move from G2/M to G0/G1 (k1) undergone apoptosis (β) and differentiation (α).

Donor	**Kinetic parameter of Homeostasis**				
	k2(s)	**k1(G2/M)**	**β(SubG0)**	**α(Diff)**	**γ(G0/G1)**
AM1	2.1	4.9	0.8	1.25	4.95
AM2	24.8	8.16	0.61	24.35	8
AM3	3.32	7.7	0.45	21.82	32.4
AF1	1.1	4.2	0.8	18.9	23.4
AF2	8.8	0.002	5.4	1.702	1.7
AF3	0.175	0.084	0.2	37.541	37.6
EM1	1.2	1.04	1.518	19.678	20.4
EM2	2.36	0.35	0.72	26.81	28.8
EM3	1.72	0.51	0.18	16	18.05
EF1	2.2	6.87	0.6	0.37	8.1
EF2	0.182	0.12	2.42	3.038	0.92
EF3	3.84	0.735	3.9	15.525	16.2
Mean	**4.3±6.8 cell/day (0.17±0.28 cell/hr)**	**2.8±3.2 cell/day (0.11±0.13 cell/hr)**	**1.4±1.6 cell/day (0.05±0.06 cell/hr)**	**15.58±11.82 cell/day (0.64±0.49 cell/hr)**	**16.71±12.25cell/day (0.69±0.51 cell/hr)**