Identification of a Class of Hemopoietic Colony-Forming Cells from Human Umbilical Cord Blood in Culture.

Baydaa H. Mutlak *PhD.*

Abstract

Background: Human umbilical cord blood (UCB) contains hemopoietic stem cells (HSCs) which are regarded as valuable sources for cell transplantation.

Objective:This study is aimed to identify a class of human hemopoietic colony –forming cells and found the suitable concentration for replating experiments.

Methods: Cord blood (CB) was collected from placenta of newly delivered women in Al-Kadhemia Teaching Hospital in Baghdad for normal vaginal delivery. Isolation and culturing of cells took place in Medical Research Unit \ College of Medicine\ Al-Nahrain University.

The present study included two lines:-

A:-Immunocytochemistry analysis of mononucleated cells (MNC s) for CD34.

B:-Culturing MNCs in different concentrations in order to determine the suitable concentration for replating further experiments. Mononucleated cells were isolated by using density gradient centrifugation and the MNCs count and viability were determinated by using trypan blue.The MNCs were cultured in RPMI +10%FCS and the medium conditioned by 1%(v/v) phytohemagglutinin (PHA).The cultures were maintained in an environment of 37C°, 5%CO_2 and fully humidified atmosphere for 14 days.

Results: The results of immunocytochemical staining showed that MNCs were positive for CD34+,the conditioned medium gave rise to hemopoietic colonies containing colony forming unit –granulocyte-macrophage(CFU-GM) , burst forming colony- erythroid (BFU-E)and mixed colonies(CFU-GEMM).These colonies could be distinguished from other hemopoietic colonies *in situ* by the complete absence of signs of terminal differentiation.

Conclusions: The results of this study confirmed that UCB provides a great source of hemopoietic stem cells for using in medical applications.

Key words: Hemopoietic Colony-Forming Cells, Human Umbilical Cord Blood, in culture.

IRAQI J MED SCI, 2010; VOL. (4):71-77

Introduction

Adult stem cell sources like hemopoietic stem cells (HSCs) are currently identified and characterized in laboratories all over the world [(1)].

Umbilical cord blood (UCB) is the blood remaining in the umbilical cord and placenta; it is routinely discarded after delivery. In 1987,a child with Fanconi's anemia received an allogenic transplant ,the successful transplant took place in Paris[(2)].As a result of this report ,the potential of UCB as a source of HSCs for transplantation rapidly become an area of intense clinical and scientific interest [(3)].

It has been shown in early studies that UCB contains a significantly higher number of progenitor cells when compared with adult peripheral blood (PB) and bone marrow (BM). The number of colony – forming unit – granulocyte –macrophage (CFU-GM) is greatly increased in UCB compared with PB. The number of circulating colony – forming unit granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) also appears to be significantly increased in UCB compared with PB and BM [(2, 4)]. Moreover, *in vitro* studies have suggested that naïve UCB lymphocytes are potentially less immunologically active than those usually found in the PB and BM and may therefore produce

Dept. Biology, **College of Education / Ibn AL-Haitham / Baghdad University.**
Address Correspondence to: Dr. Baydaa H. Mutlak ,
E- mail: baida1970@yahoo.com
Received: 25th January 2010, Accepted: 2nd September 2010

fewer problems with graft versus host disease (GVHD) than functionally mature lymphocytes harvested from live donors[5,6].

CD34 antigen has been used as a marker of HSCs .In fact most colony – forming cells are found in the CD34+ cell fraction. CD34+stem cells can be isolated from UCB and BM [7].Most studies have indicated that UCB contains a significantly large number of colonies –forming cells than BM [8, 9]. Fourteen day expansion of CD34+cells isolated from UCB stimulated with stem cell factor (SCF) and granulocyte- colony stimulating factor (G-CSF) was significantly greater than from stimulated CD34+cells isolated from BM [8].Isolated UCB CD34+cells yield approximately the same number of CFU-GEMM, twice the number CFU-GM and three times of BFU-E as similar cell populations isolated from BM [9].

Purification and characterization of HSCs are, therefore important not only for studies of the biological properties of HSCs ,but also for clinical applications so this study was aimed to identify the class of human hemopoietic colony –forming cells and found the suitable concentration for replating experiments .

Materials and Methods

Umbilical cord blood samples were obtained freshly from discarded placenta of full term normal vaginal deliveries from AL-Kadhimyia Teaching Hospital in Baghdad. The specimens were transferred to Medical Research Unit \ College of Medicine\ Al-Nahrain University. CB was diluted 1:1with phosphate buffer saline (PBS) then carefully overlaid on Ficoll-paque at a ratio of 3:1 in 10ml sterile conical tubes. The specimens were centrifuged in cooled centrifuge at 2000 rpm for 25 min at 4C°. After density gradient centrifugation,the resulted mononucleated cells (MNCs)were retrieved from buffy coat layer by pipetting and washing 2-3 times with PBS at 2000 rpm for 10 min at 4C°[10]. The final product was used in immunocytochemistry analysis for CD34 and culturing MNCs in different concentration.

The MNCs were resuspended in 1ml of PBS and the suspension was applied to slides by spinning on a cytocentrifuge. The slides were left to dry and fixed with 4% para-formaldehyde in PBS, so the slides were ready for immunocytochemistry staining. The first step was the addition of 4% hydrogen peroxide for 15 min. The second step was the addition of primary antibody (Mouse anti- Human CD34) for 1h., then the addition of secondary antibody (anti-Mouse IgG biotin) for 1h.The streptavidin conjugated to horse radish peroxidase was added to the slides for 1h.The slides in all the above steps were incubated in a humidified chamber at 37C° and the slides were washed extensively with PBS after each step. For visualization the peroxide, liquid Diamino benzidine (DAB) chromogen solution was added to the slides for 15 min then washed with PBS and counter stained in Harris hematoxylin for 2-3 min then washed with distilled water then with PBS.The slides were mounted with glycerol and were inspected by light microscope and photographed [11].

For culturing the MNCs ,the final product of MNCs was resuspended in 1ml of RPMI 1640 supplemented with 10%fetal calf serum (FCS).The cell number and viability were determined by using trypan blue solution[10] .

The cell suspension was cultured in tissue culture plates in five groups at final concentration as follow:-

Group A:-$4x10^5$ cell/ml

Group B:-$6X10^5$ cell/ml

Group C: - $8x10^5$ cell/ml

Group D:-1X10^6 cell/ml
Group E:-2X10^6 cell/ml

The cells were cultured in 1 ml of RPMI 1640 +10%FCS and the medium conditioned by 1% vol /vol phytohemagglutinin (PHA)for each well .The plates were incubated at 37 ˚C in a humidified atmosphere flushed with 5% Co_2 in air.

The numbers of hemopoeitic colonies were determinate by direct cell counting *in situ* by using inverted microscope [(2)].

The hemopoietic colonies were scored and photographed on day 14 of culture

Results

-Immunocytochemical analysis for CD34

The results of immmunocyto-chemistry staining showed that the UCB –derived MNCs were positive for CD34+. The expression of cell surface marker that appeared on the UCB derived HSCs after purification ,showed that the deep brown color for Diamino benzidine (DAB) stain represents the positive cells while the blue color for the counter stain (hematoxylin)represent the negative cells (Figure.1)

-Culturing the MNCs

After two days of culturing, some of the MNCs were adherent to the culture plates. The adherent cells began to form homogeneous population of small rounded cells with high nucleus to cytoplasm ratio, and the clusters of HSCs began to appear in culture plates 5 days after primary culture plating. As the cells proliferate, some of them detached from the plastic and remained floating in suspension; however, they stayed viable and gave rise to new clusters (Figure.2).

On the days 12-14 of culture , only three types of HSCs colonies revealed no signs of degeneration ,these colonies showed signs of terminal differentiation for example , the erythroid progenitors large megakaryocytes ,and granulocytes recognizable by their polygonal shape . When the plates were examined with an inverted microscope , three types of HSCs colonies were recognized by their distinct color and morphology *in situ* .The first type is colony forming unit-granulocyte-macrophage (CFU-GM) which were represented with a flat arrangement of non hemo-globinized cells (Figure.3 A). The second type represented by burst forming unit-erythroid (BFU-E) which appeared with densely packed configuration of hemoglobinized cells (Figure.3 B). The third type represented the mixed colonies or colony forming unit –granulocyte, erythroid, monocyte ,megakaryocyte (CFU-GEMM)which appeared as a compact colonies ,usually ,central hemoglobinized small and large cells (Figure.3 C)

The results of culturing in different concentration (Table 1) demonstrated that at low densities the assay efficiency is decreased .Also, at higher plating densities was the problem of colony crowding and the difficultly distinguishing of the overlapping granulocytic and erythroid colonies from true mixed colonies .For this reason, cultures were best plated at cell concentration of 1x10^6 cell/ml because it is a suitable concentration for re plating experiments.

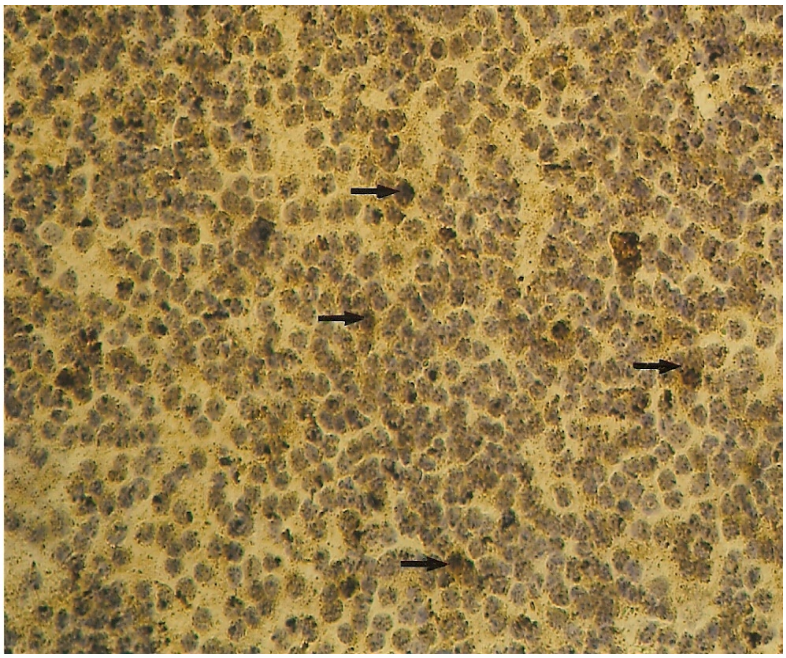

Figure 1: Expression of CD34 marker on HSCs after purification of the cells. The deep brown colors represent the CD34 positive cells (arrows) (X100.8).

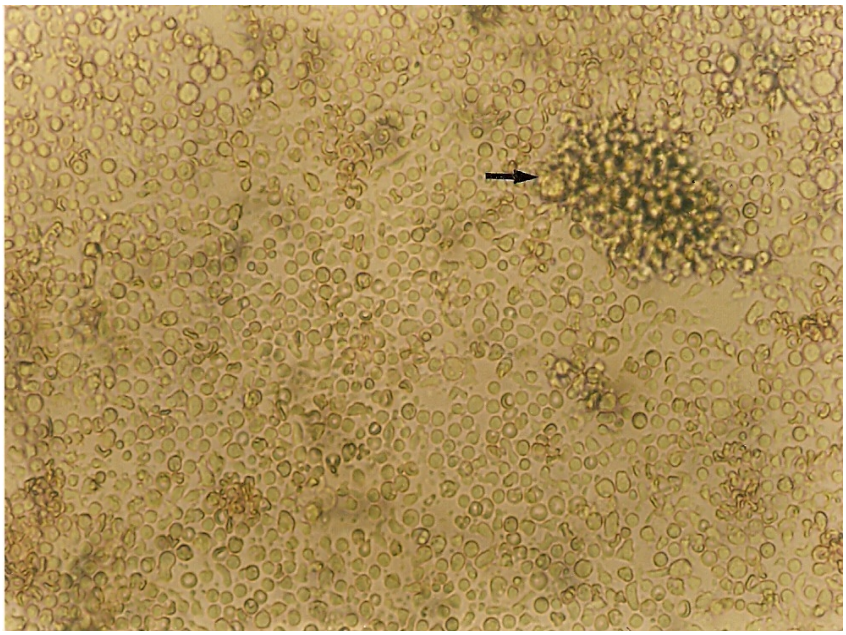

Figure 2: After 5 days in culture the cells began to proliferate and formed clusters of HSCs (arrow) which detached from the plastic surface, and it stayed viable (X160).

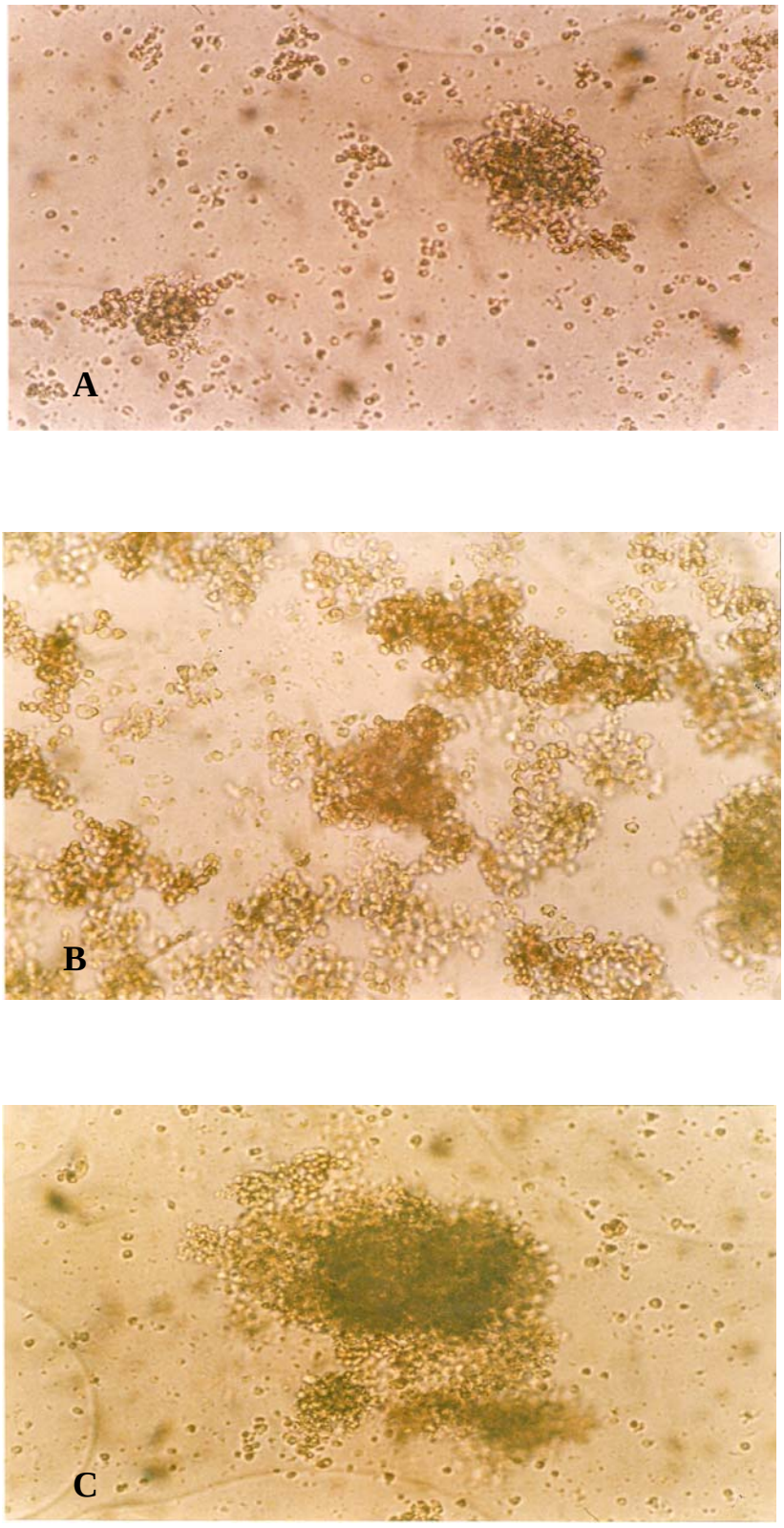

Figure 3: Hemopoietic colony cells types cultured in RPMI media+10%FCS +PHA examined under inverted microscope and photographed *in situ*. A: Colony forming unit-granulocyte-macrophage (CFU –GM), B: Burst forming unit-erythroid (BFU–E), C: colony forming unit –granulocyte, erythroid, monocyte ,megakaryocyte (CFU– GEMM)(X100.8).

Table 1:Cytologic analysis of hemopoietic stem cells colonies cultured in different concentration .Individual colonies were determinate on day 14 by direct cell counting *in situ* by using inverted microscope .

Cell concentration	CFU-GM	BFU-E	CFU-GEMM
$4X10^5$ cell/ml	20	14	25
$6x10^5$ cell/ml	37	30	48
$8x10^5$ cell/ml	48	41	57
$1x10^6$ cell/ml	55	50	65
$2x10^6$ cell/ml	59	57	80

Discussion

Until recently, blood that remained in the umbilical cord and placenta after delivary was routinely discarded. Human UCB is now considered a valuable source for stem cells ,this blood is known to contain both HSCs and pluripotent mesenchymal stem cells (MSCs).There has been a substantial increase in the clinical use and research investigation of UCB in hemopoietic transplantation and regenerative medicine [(12)].

Hemopoietic stem cells colonies in this study were identified as acompact colonies of stem cells having a high nucleus to cytoplasmic ratio. These properties are identical to HSCs colonies which previously described by different workers [(2, 13)].

The allogenic transplantation was used to treat thousands of patients, who have life threatening hematological diseases .The principal limitations of BM transplantation for majority of patients are the lack of suitable HLA-matched donors and the complication of Graft Versus Host Disease(GVHS) associated with HLA –mismatching . The expected advantages of using CB transplantation are enrichment of immature progenitor HSCs and the immaturity of immune system at birth, which should decrease the incidence and severity of GVHD [(14)]. Cord blood–derived HSCs have distinctive proliferative advantages factors. The small number and the relative immaturity of CB-T lymphocyte could reduce the risk and severity of GVHD.Also studies of *in vitro* cultures of $CD34^+$ cells from UCB suggested that CB may have a greater ability to generate new blood cells than BM; there are nearly ten times as many blood producing cells in CB. This fact suggests that a smaller number of CB cells are needed for successful transplantations than PB and BM transplantation [(15,16)].

In the present study, the immune-cytochemistry staining showed that UCB-derived MNC s having a good percentage of positive cells for $CD34^+$.The isolation method which was used in this study is unable to purify the HSC s at a high level. However, the yield of $CD34^+$ cells which were obtained by this isolation method would allow us to proceed to further steps of HSCs culture. Moreover, the cell count and viability which were detected by trypan blue method were satisfying and reflect the success of this isolation method.

In this study, a class of HSCs that had extensive self –renewal capacities and very high incidences for early hemopoietic progenitors have been described. These human HSC s exhibit at least some features similar to those characterizing the pluripotent stem cell of murine [(4)].

The conditioned medium may contain agents capable of supporting the growth of hemopoietic progenitors. The dependence of HSCs on the presence of PHA may be indicative of the presence of adistinct hemopoietic

factor in PHA whose activity is permissive for the growth of HSCs colonies .The role of PHA is probably in the stimulation of a population of cells among PB leukocytes, which are capable during preincubation of producing various stimulatory factors [2,8].

The results of this study confirmed that UCB provides a great source of hemopoietic stem cells for cellular therapy and using in medical applications and as an alternative source to BM.

References

1. Prentice DA.Adult stem cells.The published record, prepared for the president's council on bioethics. (2004) Appendex K (Downloaded from http:// bioethics.gov/on April 29, 2007).

2. Broxmeyer H, Douglas G, Hangoc G, Cooper S, Bard J, English D, et al.. Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proc. Natl. Acad. Sci. USA, 1989; 86: 3828-3832.

3. Hows JM. Status of umbilical cord transplantation in the year 2001. J. Clin. Pathol. 2001; 54: 428-434.

4. Nakahata T and Ogawa M. Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. Proc. Natl. Acad. Sci. USA. 1982; 79: 3843-3847.

5. Rabian-Herzog C, Lesage S, Gluckman E and Charron D. Characterization of lymphocyte sub population in cord blood. J.Hematol. 1993; 2:255-257.

6. Will A M. Umbilical cord blood transplantation. Arch. Dis. Child. 1999; 80: 3-6.

7. Berenson RR, Anderews G and Bensiger B.Antigen CD34+ marrow cells engraft lethally irradiated baboons. J.Clin.Inves. 1988; 81:951-955.

8. Ash RC, Detrick RA and Zanjani ED. Studies of human pluoripotential hemopoietic stem cells (CFU-GEMM) in vitro.Blood.1980; 58(2):309-316.

9. Kim DK, Fujiki Y, Fukushima T, Ema H, Shibuya A and Nakauchi H. Comparison of hematopoietic activities of human bone and UCB CD34 positive and negative cells. Stem Cells.1999; 17 (5): 286-294.

10. Cardoso AA, Batard P, Hatzfeld A, Brown EL and Hatzfeld J. Release from quiescence of CD34 CD38 human UCB cells reveal their potentiality to engraft adults. Proc. Nat. Acad. Sci. USA.1993; 90:8707-8710.

11. Lorette C. Immunocytochemistry methods and protocols. 2nd edition. Human press, Totowa, New Jersey. 1999.

12. Moise KJ .Umbilical cord stem cells. Obst. Gyne. 2005; 106 (6): 1393-1407.

13. Smith C. Hematopoietic stem cells and hematopoiesis. Can. Con. 2003; 10 (1): 9-16.

14. Gluckman E, Rocha V and Chastong C.Use of cord blood cells for banking and transplant .Oncol.1997; 2(5):340-343.

15. Gluckman E. Ex vivo expansion of cord blood cells. Exp. Hematol.2004; 32: 410-412. [Abstract].

16. Rienberg S. Umbilical cord blood stem cell transplantation. Report for federal standards for cord blood banks. 2001(Downloaded from http://discover.bio-rad.com on September 5, 2007).