In Vitro Expansion of Fetal Liver Hematopoietic Stem Cells

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***In vitro* expansion of fetal liver hematopoietic stem cells**

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Abstract

Fetal liver hematopoietic stem cells because of their proliferative potential have been considered appropriate for management of aplastic anemia. Bone marrow recovery was possible in some cases; engraftment potential of these cells however, was unsatisfactory, possibly due to the availability of smaller number of these cells from a single fetus. Present study was undertaken to see if fetal liver hematopoietic stem cells could be expanded *in vitro*. Mononuclear cells were isolated and hematopoietic stem cells were identified and analyzed by cell surface marker CD34. CD34+ cells were separated by magnetic cell sorting positive selection method using flow cytometry. Hematopoietic stem cells (CD34+) were cultured by using 5 cytokines, stem cell factor, granulocyte macrophages-colony stimulating factor, interleukin-6, Fms-related tyrosine kinase 3 and erythropoietin, in 4 different combinations along with supplements, in serum free culture media for 21 days. Cell viability continued to be greater than 90% throughout 21 days of culture. The cells expanded best in combination of media, supplements and 4 cytokines, namely SCF, Flt-3, IL6 and Epo to yield large number of total (CD34+ & CD34-) cells. Even though the total number of nucleated cells increased in culture significantly, levels of CD34 antigen expression declined steadily over this period.

Keywords: CD34 +, Cell Culture, Fetal Liver, Hematopoietic stem cells, *In vitro*

1 Introduction

Hematopoiesis appears in the fetal liver (FL) at approximately 5 weeks of gestation and remains the primary site of hematopoiesis until mid-gestation [1]. The hematopoietic stem cells (HSCs) rapidly proliferate in the liver; undergo maturation and differentiation leading to erythropoiesis, myelopoiesis, B and T lymphopoiesis, and production of megakaryocytes [2]. Thereafter, the bone marrow replaces the fetal liver as the primary site of hematopoiesis [3]. HSCs have been used for the treatment of number of nonmalignant conditions such as aplastic anemia (AA), Severe combined immunodeficiency disease (SCID), congenital metabolic disorders, and also for malignant conditions such as acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), and multiple Myeloma (MM). Fetal liver cells have the advantage of better growth and differentiation due to their higher telomere content [4]. Also, the proportion of HSC (marked by surface antigen CD 34) in fetal liver are greater than those found in cord blood and bone

marrow; and are more or less equal to those found in mobilized peripheral blood [5]. Fetal liver hematopoietic stem cells have been found to exhibit greater proliferative potential compared to the adult bone marrow and cord blood. This was evidenced by increased capacity in serial transfer, greater tritiated thymidine uptake index, early appearance of peak number of colonies in semi-solid assays and greater plating efficiency [6] [7] [8] [9]. It was also found that these cells lack proper Human Leukocyte Antigen (HLA) expression [10]. With having all the above mentioned properties, fetal liver infusion has been utilized for the management of aplastic anemia [11]. Limited quantity of fetal liver cells obtained from single abortus however, creates problems in carrying out different experiments in laboratory. Regular supply of the fetal liver hematopoietic stem cells (FLHSC), probably can be obtained from expanded cells. Expanded fetal liver HSCs produced under “*in vitro*” conditions may provide large homogenous population of cells from a single fetal liver source; these HSCs may be cryopreserved and later used for multiple transplants/infusions as in the case of fetal liver infusion (FLI) in aplastic anemia patients.

2. Material and Methods

The study was approved by the Ethics Committee of All India Institute of Medical Sciences, New Delhi, India. Informed consent was obtained from patients underwent Medical Termination of Pregnancy (MTP). We hereby confirmed that all method were carried out with relevant guidelines and regulations

2.1 Preparation of cell suspension and cell viability

Human fetuses with gestation periods ranging from 8 to 20 weeks with no known chromosomal abnormality were obtained following medical termination of pregnancies (MTP) with Prostaglandin E or Emcradyl injection from the Department of Obstetrics and Gynecology at AIIMS. Immediately after expulsion, fetuses were collected on ice and brought to the Laboratory. The Fetuses were dissected under aseptic conditions to separate liver. The liver was put into Iscove’s Modified Dulbecco Medium (IMDM) containing 10% fetal bovine serum (FBS) and 10 IU/ml heparin. Further the liver was mashed into small pieces and allowed to pass through sterile muslin cloth repeatedly so as to obtain single cell suspension. 10µl of the fetal liver cell suspension was mixed with 90µl of Trypan blue dye and loaded on to a hemocytometer and the number of dye excluding cells (viable) were counted. Only those fetus samples which had 80% or more cell viability were taken up for further analysis.

2.2 Isolation of Fetal Liver Mononuclear Cells (MNC)

Mononuclear cells from fetal liver nucleated cell suspension were separated using density gradient centrifugation for their subsequent use in culture studies. Nucleated cells from fetal liver suspension were diluted with IMDM containing 10% FBS in the ratio of 1:3 (fetal liver suspension:IMDM with FBS) and 30ml of suspension was gently layered on 10 ml of Histopaque (Ficoll-Hypaque) in 50 ml sterile centrifuge tubes. The mononuclear cells were isolated by centrifugation (30 min, 1500 rpm, at room temperature) in a swinging-bucket rotor without brake. The white cell ring appearing at the interface of the medium and histopaque was carefully withdrawn with a fine pasture pipette. The mononuclear cells (MNC) so obtained were washed thrice in phosphate buffer saline (PBS) to remove any traces of Histopaque and cells were counted on a hemocytometer before labeling with magnetic bead or fluorochrome conjugated Antibodies.

2.3 Isolation of Fetal Liver Hematopoietic Stem Cells (CD34⁺ Cells) By MACS

The CD34⁺ fraction was isolated immunomagnetically using MS⁺ MiniMACS columns and the CD34 Direct Isolation Kit (Miltenyi Biotec). The cells were processed in the flow cytometer. Statistics were elaborated in 10000 events/sample by WinMDI software. Purified cells were used for cell culture

study. The excess of cells could be cryopreserved for future use.

2.4 Expansion of Fetal Liver Hematopoietic Stem Cells (FLHSC) Under ‘*In vitro*’ Conditions

Stroma free long term culture technique was followed to expand the FLHSC. Freshly isolated 1×10^4 FL CD 34⁺ cells (10^5 viable cells in 1ml) were cultured in a flat bottomed 24 well plates in serum-deprived culture medium STEM PRO-34-SFM (Gibco) supplemented with 100µg/ml insulin (Sigma Chemical Co.), 200µg/ml transferrin (Sigma Chemical Co.), 40 µg/ml low density lipoprotein (LDL) (Sigma Chemical Co.), 2mM Glutamine (Gibco BRL, Grand Island, NY), 10^{-4} M Mercaptoethanol (Sigma Chemical Co). Cytokine cocktail (Table 1) containing SCF, IL-3, IL-6, G-CSF, Flt-3 (Pepro Technologies) in Culture media IMDM and 10% FBS was used to expand CD34⁺ cells for a period of 21 days. Fetal liver CD34⁺ cultures were grown at 37°C in humidified 5% CO₂ incubator. Wells were semi- depopulated once a week. Harvested cells were counted and suitable aliquots assayed every seven days for the presence of specific surface antigen CD34⁺ cells by flow cytometry.

3 Results

3.1 Human Fetal Liver Characteristics

The age of the fetuses varied from 8-20 weeks (Median=12 weeks). The median number of total fetal cells without hepatocytes was 1.77×10^9 (Range; 0.02 - 5.2×10^9) with mean viability of $93.81 \pm 5\%$. Total viable mononuclear cells obtained from fetal liver nucleated (Non Hepatocytic) cell suspension using density gradient centrifugation were $2.27 \pm 1.8 \times 10^8$ (Range; 0.01 - 6.78×10^8) (Table 2).

3.2 Flow Cytometric Analysis

Fetal liver MNCs showed that the total CD34⁺ population ranged from 1.2% -12.8 % with a median value of 5.5%. Table 3 displays the percentage of CD34⁺ cells among FLMNCs according to gestational age. The peak values of CD34⁺ cells were observed during 8-16 weeks of gestational age (Figure 1). From 8th to 10th week, CD34⁺ percentage remained high ($8.50 \pm 2.4\%$ to $8.70 \pm 1.8\%$); subsequently it started declining, although difference between CD34⁺ cell percentage from 8th till 14th week of gestation period is statistically not significant ($P=0.097$); there was significant decrease ($P<0.05$) in number of CD34⁺ cells with subsequent increase in fetal age.

3.3 CD34⁺ Cell Purification by Magnetic Cell Sorting

The total no. of CD34⁺ cells separated by MACS positive selection method ranged from 5.20×10^5 - 4.34×10^6 to (median 2.79×10^6) with a median recovery of 62.3 % (range 31.8-75.3%). The median purity of CD34⁺ cells isolated from MiniMACS was 95% (Range 89%-98%). In all samples, the percentage cell viability of CD34⁺ isolated cells was greater than 95% as measured by Trypan blue dye exclusion methods. CD34⁺ cell fraction is nonadherent while CD34⁻ cells adhere to the flask in culture media and this fraction of cells forms fetal liver stromal cells.

3.4 Expansion of Fetal Liver Hematopoietic Stem Cells (HSCs)

Estimated cell counts at 7-days intervals are shown in Figure 2. Cell viability throughout 21 days of culture was greater than 90%. Expansion of total cells was expressed as fold increase over initial cell input; this was statistically significant ($P<0.05$) in all the tested combinations. Total nucleated cells expanded up to 137.24 ± 8.1 fold (M+S+M4) by day 21($p<0.01$).

3.5 CD34⁺ Expression on Expanded Cells

Expanded cells observed from day 0 to day 21 contained both CD34 positive and CD34 negative cells), however, the percentage of CD34⁺ cells declined quickly after day seven and continued to decline steadily over the twenty one days of culture. FACS analysis of CD34 expression of five

representative experiments at different time points is shown in Figure 3. The Percentage of CD34⁺ among the total nucleated cell count at day 21 was 15.42±4.9 in combination M+S+M1, 4.68±5.1% in M+S+M2, 18.24±4.2% in M+S+M3, and 13.08±2.3% in M+S+M4. Absolute number of total cells and CD34% cells at day 21 is given in Table 4.

Discussion

Hematopoietic stem cell (HSC) transplantation therapy has been effectively utilized for the treatment of several life threatening hematopoietic diseases. Bone marrow (BM) and mobilized peripheral blood are the major sources of HSCs; however, there is a paucity of human leucocyte antigen (HLA) matched donors. Hence, umbilical cord blood (UCB) has been used as an alternative to obtain HSCs for transplantation therapy. UCB transplantation therapy has the limitation of availability of low numbers of HSCs per unit of UCB. Possibility of *in vitro* expansion of HSCs has been studied by several researchers to address this issue. Combination of various growth factors have commonly been employed to expand HSCs *in vitro*. Mixture of stem cell factor (SCF), thrombopoietin (TPO), and FMS like tyrosine kinase 3 ligand (Flt-3L) was found to support the expansion of HSCs [12]. Human recombinant flt-3 ligand has been shown to be an early acting cytokine supporting the growth of very primitive HSCs only when synergized with other growth factors [13], including SCF [14], interleukin-3 (IL-3) [15], IL-6 [16] [17], granulocyte colony stimulating factor and erythropoietin (EPO) [18] and TPO [19] [13] [20]. With these different combinations, expansion of CD34⁺ cells could be observed by day 7; expansion ranged from 2.44 to as high as 25.11± 13.50 fold by day 7 [12] [13] [21] [22]. Long term expansion of human fetal liver primitive hematopoietic progenitor cells in stroma free cultures was successfully achieved [23]. Combination of 4 cytokines namely SCF, flt-3, IL-6 and EPO in a serum deprived culture medium STEM PRO-34-SFM supplemented with insulin, transferrin, low density lipoprotein, glutamine and mercaptoethanol in culture media IMDM and 10% fetal bovine serum (FBS) has been used by us for a period of 21 days, expansion of nucleated cells happened by 137.24±8.1 fold ($p<0.05$), levels of CD34 antigen expression however, declined steadily over this period. Sauvageau, Iscove and Humphries [24] observed net decline of 6.5 fold after four weeks, even though, there was demonstration of amplification of HSC's *in vitro*. This suggested that culture conditions were not optimal for the stimulation of HSC self-renewal and/ their continued survival. Growth factor dosing is another important issue [24]. At high levels of human IL-3, GM-CSF and SF, marked impairment of regeneration of human competitive repopulating cell (CRU) in NOD/SCID transgenic mice was noted by Nicolini *et al* [25]. Stimulation of HSC expansion by IL-11 was observed at a very narrow concentration range; for factors such as SF or FL, on the other hand, saturation effects were not achieved even at high concentration (> 300 mg/ml) [24]. Transforming growth factor beta 1 (TGF beta 1) at concentration of 1-50 pg/ml stimulated colony formation but at higher concentration, it had an inhibitory effect [26]. Stimulation of selective growth factor pathways, may yet be a crucial determinant of HSC self-renewal. For instance, regeneration of normal HSC's in recipients lacking TPO got impaired by 10-20 fold [27]. TPO has been implicated as a positive regulator of HOXB4 (part of a family of transcription factors) expression, a potent enhancer of HSC expansion. HOXB4 induced self -renewal *in vitro* even in conditions that were sub optimal for untransduced HSC's [24]. Over expression of another gene namely SALL4 has also been shown to have the capacity to substantially increase the number of HSCs *in vitro* [28]. More recently, Jing *et al* [29] demonstrated the expansion of both murine BM HSCs and human UCB HSCs with the combination of a moderate concentration of p38 inhibitor plus a GSK 3 inhibitor. Thus, it appears that despite the availability of various methods to expand HSCs *in vitro* [24] [28], an ideal method

to increase the number of HSCs *in vitro* is yet to be discovered.

Conclusion

Fetal liver, a major site of hematopoiesis during second trimester of pregnancy is a good source of hematopoietic stem cells. The higher cell number required for experimental studies is hindered by poor availability of fetuses and less number of cells obtained per fetus. To overcome this problem, magnetically sorted CD34+ cells populations have been subjected to expansion, using media supplements and different combinations of cytokines. Although the total number of nucleated cell increased in culture, levels of CD34 antigen expression declined steadily with time.

Data availability

All data generated or analyzed during this study are included in this “to be published” article.

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Conflict of Interest

There is no conflict of interest in between the authors.

Author contributions:

R. Bhardwaj : Collection and/or assembly of data, Data analysis and interpretation
 L. Kumar : Conception, Design, Data analysis and Interpretation,
 D. Chhabra : Manuscript writing
 N. K. Mehra : Interpretation/Discussion
 A. Sharma : Interpretation/Discussion
 S. Mohanty: Interpretation/discussion
 V. Kochupillai.: Conception and design, Manuscript writing, Final approval of manuscript

Figure 1: Percentage of CD34⁺ cells vs. Gestation age of the fetus

Figure 2: Kinetics of CD34⁺ cell expansion: Results are expressed as mean $\pm$ SEM of 5 experiments.

Figure 3: Percentage CD34⁺ cells among total FLMNCs during 21 days of expansion

Table 1: Combinations of Cytokines used for expansion of fetal liver hematopoietic stem cells.

Cytokine used in Combinations	Combination 1 (M)	Combination 2 (M+S)	Combination 3 (M+S+M1)	Combination 4 (M+S+M2)	Combination 5 (M+S+M3)	Combination 6 (M+S+M4)
Media	Media	Media	Media	Media	Media	Media
Supplements		Supplements	Supplements	Supplements	Supplements	Supplements
SCF			100ng/ml	100ng/ml	100ng/ml	100ng/ml
Flt-3			100ng/ml	100ng/ml	100ng/ml	100ng/ml
IL-6				20ng/ml	20ng/ml	20ng/ml
Epo					2U/ml	2U/ml
GM-CSF						200U/ml

M=Media, S=Supplements, M1=SCF+Flt-3, M2=SCF+Flt-3+IL6, M3=SCF+Flt-3+IL6+Epo, M4=SCF+Flt-3+IL6+Epo+GM-CSF

Table 2: Fetal liver characteristics at the time of collection.

Characteristics	Number
Total Fetuses collected	70
Sex Male: Female	39 (55.7%): 31 (44.2%)
Age (Gestation Period) Mean $\pm$ SD Median Range	13 $\pm$ 2.91 weeks 12 weeks 8-20 weeks
Time between expulsion and processing of Fetus Mean Range	1.00 $\pm$ 0.76 hr 0.25-3.5 hrs
Non Hepatocytic Cell Viability of Liver Cells Mean Range	93.81 $\pm$ 5.0 % 85-100%
Total viable Nucleated (Non Hepatocytic) Cells Mean $\pm$ SD Median Range	1.91 $\pm$ 1.31 X 10 ⁹ 1.77 X 10 ⁹ 0.02- 5.2 X10 ⁹
Total viable Fetal Liver Mononuclear Cells Mean $\pm$ SD Median Range	2.27 $\pm$ 1.81X 10 ⁸ 2.041 X 10 ⁸ 0.01- 6.78 X1 0 ⁸

Table 3: Percentage of CD34⁺ cells in FLMNCs at different gestational age groups

Gestational Age of the Fetus	Mean $\pm$ SD Value of CD34 ⁺ cells %age	Range of CD34 ⁺ cells %age
8 weeks	8.50 $\pm$ 3.8%	5.2%-12.8%
9 weeks	8.70 $\pm$ 1.8%	6.7%-10.2%
10 weeks	8.50 $\pm$ 2.4%	6.8%-11.3%
11 weeks	6.83 $\pm$ 1.2%	5.4%-7.9%
12 weeks	6.03 $\pm$ 2.2%	3.6%-8.0%
13 weeks	4.40 $\pm$ 1.3%	3.7%-5.5%
14 weeks	4.56 $\pm$ 2.0%	2.3%-6.2%
15 weeks	2.73 $\pm$ 0.9%	1.9%-3.8%
16 weeks	2.86 $\pm$ 1.5%	1.2%-4.3%

Table 4: Total cell expansion and percentage of CD34⁺ cells in different cytokine mixtures at Day 21

S. No.	M		M+S		M+S+M1		M+S+M2		M+S+M3		M+S+M4	
	Total cells $\times 10^4$	CD34 ⁺ cells (%)	Total cells $\times 10^4$	CD34 ⁺ cells (%)	Total cells $\times 10^4$	CD34 ⁺ cells (%)	Total cells $\times 10^4$	CD34 ⁺ cells (%)	Total cells $\times 10^4$	CD34 ⁺ cells (%)	Total cells $\times 10^4$	CD34 ⁺ cells (%)
1	21.2	4.1	35	3.1	51.4	22.1	61	23.2	90.9	12.8	130	10.7
2	23.6	2.3	24.2	0.8	56.8	19.2	70.2	13.5	99.2	15.3	147	13.8
3	22.9	1.2	44.7	1.3	62.3	13.2	61.6	10.9	87.4	18.9	141.9	12
4	18.7	1.7	42.2	1.2	45.3	12.3	61.2	15.4	90.3	23.2	127.8	16.7
5	21.6	0.9	30.9	2.1	41.8	10.3	59.4	10.4	84.6	21	139.5	12.2
Median	21.6	1.7	35	1.3	51.4	13.2	61.2	13.5	90.3	18.9	139.5	12.2
Mean	21.6 $\pm$ 1.8	1.7 $\pm$ 2.0	35.4 $\pm$ 8.4	1.7 $\pm$ 1.3	51.5 $\pm$ 8.3	15.4 $\pm$ 4.9	62.6 $\pm$ 4.2	14.6 $\pm$ 5.1	90.4 $\pm$ 5.4	18.2 $\pm$ 4.2	137.2 $\pm$ 8.5	13.8 $\pm$ 2.3

$\pm$ SD												1	
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Figures

Figure 1

Percentage of CD34+ cells vs. Gestation age of the fetus

Figure 2

Kinetics of CD34+ cell expansion: Results are expressed as mean $\pm$ SEM of 5 experiments.

Figure 3

Percentage CD34⁺ cells among total FLMNCs during 21 days of expansion