

Original Article

Defining Molecular Phenotypes of Mesenchymal and hematopoietic Stem Cells derived from Peripheral blood of Acute Lymphocytic Leukemia patients for regenerative stem cell therapy

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Abstract:

Acute Lymphocytic Leukemia (ALL) is a clonal myeloid disorder affecting all age groups, characterized by accumulation of immature blast cells in bone marrow and in peripheral blood. Autologous Bone Marrow Transplantation is a present treatment for cure of ALL patients, which is very expensive, invasive process and may have possibility of transplantation of malignant stem cells to patients. In the present study, we hypothesized to isolate large number of normal Mesenchymal & Hematopoietic stem cells from peripheral blood of ALL patients, which will be further characterized for their normal phenotypes by using specific molecular stem cell markers. This is the first study, which defines the existing phenotypes of isolated MSCs and HSCs from peripheral blood of ALL patients. We have established three cell lines in which two were Mesenchymal stem cells designated as MSC_{ALL} and MSC_{Ns,ALL} and one was suspension cell line designated as HSC_{ALL}. The HSC_{ALL} cell line was developed from the lymphocyte like cells secreted by MSC_{ALL} cells. Our study also showed that MSC_{ALL} from peripheral blood of ALL patient secreted hematopoietic stem cells in vitro culture. We have characterized all three-cell lines by 14 specific stem cell molecular markers. It was found that both MSC cell lines expressed CD105, CD13, and CD73 with mixed expression of CD34 and CD45 at early passage whereas, HSC_{ALL} cell line expressed prominent feature of hematopoietic stem cells such as CD34 and CD45 with mild expression of CD105 and CD13. All three-cell lines expressed LIF, OCT4, NANOG, SOX2, IL6, and DAPK. These cells mildly expressed COX2 and did not express BCR-ABL. Overall it was shown that isolated MSCs and HSCs can be use as a model system to study the mechanism of leukemia at stem cell level and their use in stem cell regeneration therapy for Acute Lymphocytic Leukemia.

Keywords:

Acute Lymphocytic Leukemia, Stem cell markers, Peripheral blood cells, MSCs, HSCs

Introduction:

Acute leukemia is a clonal malignant disorder affecting all age groups with an average annual incidence rate of 4-7 % per 100000 people.^[1] It is characterized by the accumulation of immature blast cells in the bone marrow, which replaces normal marrow tissue, including hematopoietic precursor cells. This results in bone marrow

failure having peripheral blood cytopenias with presence of circulating blast cells.^[1] The acute forms of leukemia are the most common forms of leukemia in children. The Acute leukemia is subdivided into (a) Acute Lymphoblastic Leukemia (ALL), in which the abnormal proliferation is in the lymphoid progenitor cells i.e. immature lymphocytes and (b) Acute Myeloid Leukemia

(AML), which involves the myeloid lineages. The distinction between the two leukemia's is based on morphological, cytochemical, and immunological and cytogenetic differences and is of paramount importance as the treatment and prognosis differ with respect to the type of disease.

The Acute lymphoblastic leukemia (ALL) is a form of leukemia or cancer of white blood cells characterized by excess lymphoblasts. ALL is commonly observed in childhood with a peak incidence at 2-5 years of age and another peak in old age. The overall cure rate in children is 80% and about 35%-60% of adults have long-term disease-free survival.^[1] 12:21 is the most common translocation and portend a good diagnosis. 4:11 is the most common in children under 12 months and portends poor diagnosis.^[2, 3] The survival rate has improved from zero to 20-75 percent currently, largely due to clinical trials on new chemotherapeutic agents, steroids, radiation therapy, growth factors and intensive combined treatments include bone marrow transplantation or regenerative stem cell based therapy.^[4]

Stem cells are characterized by their ability to renew themselves through mitotic cell division and differentiating into a diverse range of specialized cell types. Two broad range of stem cells found are Embryonic stem cells and Adult stem cells. Even though Embryonic stem cells have a large potential to form different type of cells and their application is restricted due to ethical issues. A method for isolating adult stem cells from different sources like bone marrow, adipose tissue, skeletal muscle and even cardiac tissue has already been well established.^[5, 6] Isolating the tissue sample or bone marrow aspirate for extraction of stem cells is a very painful procedure and therefore there is a need to isolate these cells by a non-invasive method such as simple venous blood collection. We therefore thought of isolating Mesenchymal and Hematopoietic stem cells from peripheral blood of ALL patients, which can be easily collected in the required quantity without any harm to the individual. Molecular marker study is one of the important studies to characterize and establish any cell type. CD105, CD13 and CD73 are the molecular markers,

which are expressed by Mesenchymal stem cells (MSCs).^[7] CD34 and CD45 expression is most extensively used for identification isolation of Hematopoietic stem cells (HSCs).^[8,9] For understanding pluripotency and self-renewal capacity, various markers like OCT4, NANOG and SOX2 have been used most extensively by many researchers.^[10, 11] It has been established that there is change in expression of Molecular markers like DAPK, COX2 and LIF in cancerous cell types.^[12, 13, 14, 15] In present study, we mainly focused on establishing technology for development of MSCs and HSCs from peripheral blood of ALL patients and characterized the same by using 14 specific molecular markers for MSCs and HSCs cell types. This study is directed towards understanding the disease in better way and data can be utilized for treatment more specifically at stem cell level.

Materials & Methods:

Materials: Low-glucose Dulbecco's modified Eagle's Medium (DMEM), Penicillin Streptomycin (PenStrep), Phosphate Buffer Saline (PBS), Trypsin EDTA, Erythrocin B, Colchicine, were purchased from HIMedia (Mumbai India). Fetal Bovine Serum (FBS) from GIBCO BRL (Carboside, MA). Trizol reagent, cDNA preparation kit, Agarose purchased from Invitrogen (USA) Histopaque and Primers for CD105, CD13, CD73, CD34, CD45, OCT4, NANOG, SOX2, LIF, KERATIN18, COX2, DAPK, BCR-ABL, IL6 & β -ACTIN were purchased from Sigma Chemicals, USA.

Isolation of MSCs & HSCs from Acute Lymphocytic Leukemia Blood cells

Blood Sample was taken from Acute Lymphocytic Leukemia patient with consent of patient as per the guidelines of Ethical Committee of Jaslok Hospital and Research Center. Blood Sample was collected in sterile EDTA vacutainer tube and was processed for isolating blood cells using Histopaque density gradient. Whole Blood was diluted with 1XPBS in the ratio of 1:2. This diluted blood was overlaid on Histopaque and centrifuged at 4000rpm for 30 minutes. Middle buffy coat layer containing WBCs and other nucleated cells were taken out and washed with 1XPBS solution. Cell

pellet was then suspended in freshly prepared RPMI1640 growth medium with 10% FBS + 0.1% PenStrep and cultured in 65mm culture dishes. These dishes were kept in CO₂ Incubator at 37°C and Humidity of 90% for 5-7 days. After seven days many cells adhered to the bottom of petridish. These adhered cells were now fed with growth medium DMEM + 10% FBS + 0.1% PenStrep. The remaining suspension cells were washed with PBS and cultured in RPMI 1640 medium in another 65mm petridish. Many of these cells adhered to the bottom of the petridish and formed small colony of well growing MSCs like cells. These MSCs like adherent cells were fed twice a week with DMEM + 10 % FBS + 0.1% Pen Strep. These Adherent Cells started to multiply and became confluent within 15- 20 days. Confluent cells were plated into new flask by treatment with 0.25% Trypsin- EDTA. The same procedure was repeated after every confluency. The cells were observed every alternate day for their morphological changes under Inverted phase contrast Microscope and photographed.

Cryopreservation and Cell revival

Cryopreservation of cells was done using DMSO Cell freezing medium (HiMedia). Cells were

trypsinized and washed with PBS. Cell pellet was then suspended slowly in DMSO Cell freezing medium and transferred into 1.5 ml freezing Vial. Final freezing was done at -85 °C. For revival, vials were thawed and freezing medium was replaced with freshly prepared DMEM or RPMI1640 medium. The revival efficiency was found to be more than 80% with at least 95 % viability.

Phase Contrast Microscopy

The Morphology of cultured cells was observed using inverted Phase contrast Microscope (Carl Zeiss co.), which was attached to computer having TS View software for observing and capturing the images. Cells were regularly monitored using phase contrast microscope and images were also captured for analysis.

RNA extraction and RT-PCR

Total RNA of Adherent and Suspension cell was extracted using TRIZOL method (Invitrogen). cDNA was prepared from extracted RNA by using High capacity cDNA Reverse transcriptase kit (Applied Biosystem, USA) and was used for performing gene expression studies to characterize the cell phenotype. The Molecular markers and their Primers used were as follow

CD105 For-TGTCTCACTTCATGCCTCCAGCT Rev-AGGCTGTCCATGTTGAGGCAGT(378bp),
CD13 For-GTCTACTGCAACGCTATCGC Rev-GATGGACACATGTGGGCACCTTG(574bp),
CD73 For-CA CCAAGGTT CAGCAGATCC Rev-GTTCATCAATGGGCGACCGG(1007bp), **CD34**
 For-GCAAGCCA CCAGAGCTATTC Rev-GGTCCCAGGTCCTGAGCTAT(195bp), **CD45** For-
 ACCAGGGGTTGAAAAGT TTCAG Rev-GGGATTCCAGGTAATTACTCC(343bp), **OCT4** For-
 GAGCAAAACCCGGAGGAGT Rev-TTCTCTTTCGGGCCTGCAC(310bp), **NANOG** For-
 GCTTGCCTTGCTTTGAAGCA Rev-TTCTTGACCGG GACCTTGTC(256bp), **SOX2** For-
 GCCGAGTGAAACTTTTGTGTC Rev-GTTCATGTGCGCGTAACTGT (264bp), **LIF** For-
 GGCCCGGACACCCATAGACG Rev-CCACGCGCCATCCAGGTAAA(454bp), **KERATIN18** For-
 GAGATCGAGGCTCTCAAGGA Rev-CAAGCTGGCCTTCAGATTTTC(357bp), **COX-2** For-
 TTCAAATGAGATTGTGGGAAAATTGCT Rev-AGATCATCTCTGCCTGAGTATCTT(305bp),
DAPK For-TGACAGTTTATCATGACCGTGTTTCAG Rev-GTGCTGGATCTCCTTCAGGAT(231bp),
IL6 For-AGCT CAGCTATGAACTCCTTCTC Rev-CCAATCTGGATTCAATGAGGAGAC(328bp), **β-**
ACTIN For-AACCC CAAGGCCAACCGCGAGAAGATGACC Rev-
 GGTGATGACCTGGCCGTCAGGCAGCTCGTA(417 bp), **BCR-ABL** For-
 ACAGAATTCCGCTGACCATCAATAAG Rev-TGTTGACTGGCGTGATGTAGTTGCTT GG.

Thermal profile for ACTIN, OCT4, and NANOG PCR was set up at 95°C for 5 minutes with 35 cycles at 94°C for 30s, 55°C for 30s and 72°C for 1 minutes followed by final extension at 72 °C for 10 minutes. The reaction was terminated at 4°C. Whereas for LIF, SOX2, CD34, KERATIN18, and for CD105, CD13, CD45, CD73, the annealing temperature was kept at 57°C and 60°C respectively in above PCR program. The PCR program used for DAPK and IL6 was at 95°C for 3 minutes with 40 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min with an additional extension at 72°C for 5 minutes, finally reaction was terminated at 4°C. One step RT/PCR kit (Invitrogen, USA) was used to study expression of COX2, and BCR-ABL gene as per the manufacturer's instructions. The PCR products were analyzed by electrophoresis with 2% agarose gel and photographed under UV light.

Cell Growth Rate study

This study was mainly performed to find out the doubling time of cells by using Neubauer haemocytometer. Adherent Cells were plated at a density of 1×10^4 cells per 65 mm dish and fed with DMEM growth media and incubated in CO₂ incubator for 48, 96, 144, 192, and 240 hrs. At each time point, cells were washed with PBS and then trypsinized with 1ml of EDTA- Trypsin for 5-7 minutes. The trypsinized cells were then mixed with equal amount of Erythrocin B and cell count was done by using Neubauer haemocytometer. Suspension cells were plated at a density of 1×10^5 cells per dish and fed with RPMI 1640 medium

and incubated in CO₂ incubator for 24, 48, 72, 96, 120 and 144 hrs. At each time point cells were washed with PBS and cell count was done as described previously. Doubling time was calculated using the formula, **Doubling time** = $\ln 2 / \mu = 0.69 / \mu$ where, $\mu = 2.303 \times \text{slope}$ (calculated from graph)

Results:

Isolation of Mesenchymal Stem Cells (MSC_{ALL}) from Peripheral blood of Acute Lymphocytic Leukemia patient

The mononuclear cells were isolated and cultured as described above. Culture dishes were regularly observed under phase contrast microscope for any adhered cells. First Adherent cells were observed after 7- 9 days of culture. These cells were thin elongated and have morphology like that of Fibroblast cells. After observing these cells, the dishes were washed with 1XPBS and fed with DMEM medium for further expansion of these cells till their confluency which they attained within 25 days of culture as shown in figure1. Every time after confluency, these cells were passaged and transferred to 50mm Tissue culture flask by using Trypsin – EDTA treatment for 7 minutes. Presently these cells are at passage 6 and are growing well in culture. From growth rate study the mean doubling time of these cells was found to be 33hrs as shown in figure2. This cell line was designated as MSC_{ALL} cell line after confirming using molecular markers for their Mesenchymal phenotypes as described below.

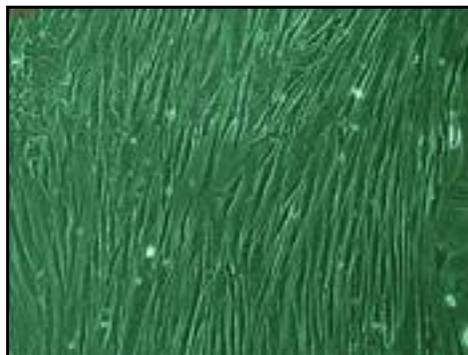


Figure 1 shows confluent MSC_{ALL} cells having spindle shaped morphology observed 20 days after first adherent cells were seen

Development of Suspension cell line (HSC_{ALL}) and MSC_{nsALL} cell lines from MSC_{ALL} cell line

After about a month of culturing MSC_{ALL} from peripheral blood cells of ALL patient, we found two different populations of adherent MSCs cells. One was growing normally designated as MSC_{ALL} (Figure 1) while, the other population produced mononuclear cells that remained in suspension as shown in figure 3 & 4. These suspension cells were then transferred into 50mm tissue culture flask and fed with RPMI1640 growth medium. These cells showed rapid growth and their mean doubling time was found to be just 11 hrs from

growth rate study (figure 2). These suspension cells also formed clones within few days of culture as shown in figure 4. These cells after reaching confluency were washed, centrifuged and the pellet obtained was suspended in RPMI growth medium and transferred to 50mm flask. The suspension cells were stained with Giemsa stain and it was found that these cells were spherical having a large nucleus and scanty cytoplasm having Lymphocyte like appearance as shown in Figure 5. At present, these cells are at passage 6 and designated as "HSC_{ALL} cell line". HSC_{ALL} cell line was characterized by using molecular stem cell markers as described below.

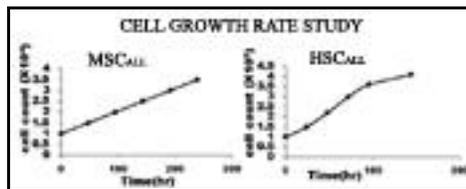


Figure 2 shows Cell growth rate study graph of MSC_{ALL} (left) and HSC_{ALL} (right) cell lines

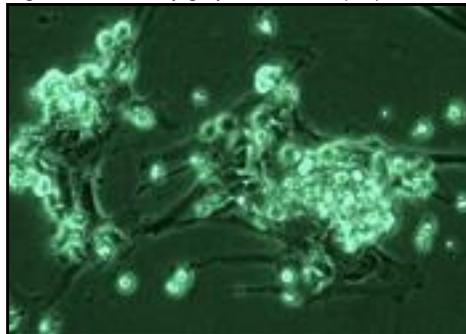


Figure 3 shows cells that are being produced (HSC_{ALL}) from adherent cells and could be seen coming into the suspension

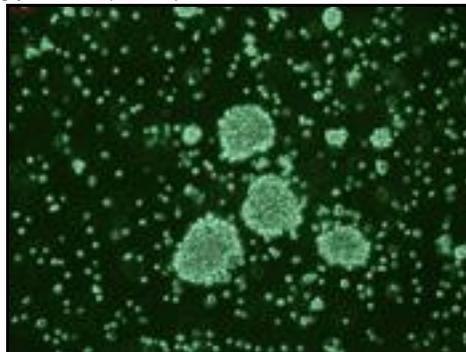


Figure 4 shows clones that were formed by HSC_{ALL} cells in suspension culture

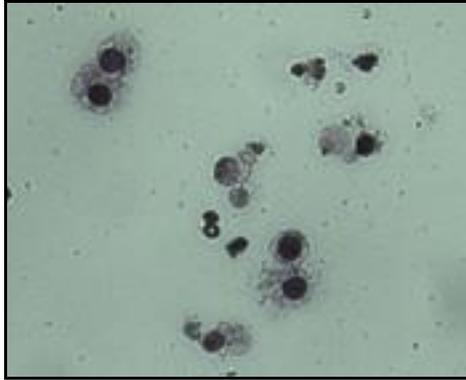


Figure 5 shows Geimsa Stained HSC_{ALL} cells, which shows mononuclear cells with large nucleus and scanty

The produced suspension cells were continuously removed from the flask and after 6 weeks it was observed that those MSCs were no more producing such kind of suspension cells and became non-secretary (ns). These non-secretary cells attained a large and flattened

morphology as shown in figure 6 and were very much different from the suspension cell producing stem cells. These cells were designated as “MSC_{nsALL} cell line” and were also characterized for their phenotypes by using specific stem cell markers as described below.

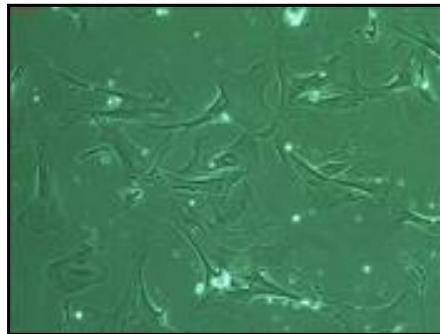


Figure 6 shows Large flattened MSC_{nsALL} cells, which stops secreting HSCs observed after 6 weeks in culture

Induction of MSC_{ALL} to secretory MSCs by co-culturing cell with HSC_{ALL} cell line:

We found that the secreted suspension cells have shown a distinct property of inducing non-secretary adherent stem cells to produce similar kind of suspension cells i.e. HSC_{ALL} cells. To prove this inducing phenomenon, HSC_{ALL} cells were grown in culture for 2 weeks to get proper growth of these cells. The supernatant of these cells with few suspension cells were co-cultured with well-grown non-secretary MSC_{ALL} cell line for

3 days in CO₂ incubator. After that these cells were washed with PBS, fed with DMEM growth medium and allowed to grow for 7-8 days. Treated adherent MSC_{ALL} cells started secreting mononuclear cells and showed similar morphological changes as seen in original HSC_{ALL} producing cells (Figure 3). This phenomenon was also supported by the fact that in a flask with small secretory cell patch, after some days most of the cells in that flask start producing HSC_{ALL} cells.

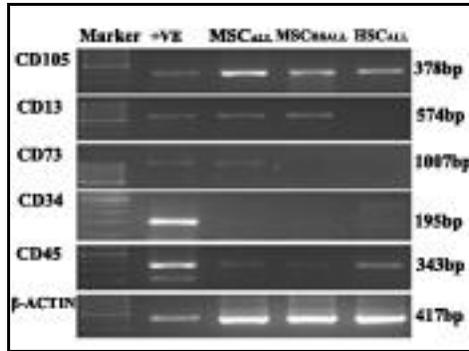


Figure 7 shows expression of molecular markers CD105, CD13, CD73, CD34 and CD45 in MSC_{ALL}, MSC_{NSALL} & HSC_{ALL} cell lines.

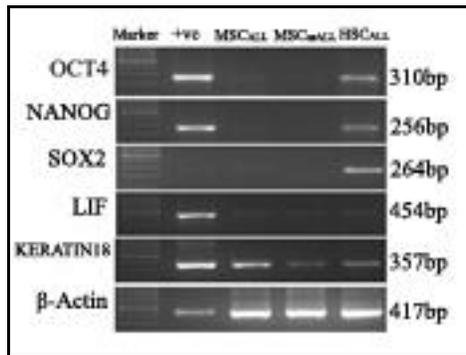


Figure 8 shows expression of molecular markers OCT4, NANOG, SOX2, LIF and KERATIN18 in MSC_{ALL}, MSC_{NSALL} & HSC_{ALL} cell lines.

Isolation of MSCs and HSCs from peripheral blood of normal individual

We have set up several similar experiments with peripheral blood of normal, non-leukemic individuals to isolate MSCs and HSCs. However, very few MSCs like cells were obtained after several days of culture which could not be expanded further using culture conditions utilized for isolating and expanding MSCs and HSCs from peripheral blood of ALL patient.

Molecular Marker Analysis for MSC_{ALL}, MSC_{NSALL} and HSC_{ALL} stem cell Lines.

Selection of Positive Controls: -

Our previous work has shown that CD105, CD45, CD34, and SOX2 genes were upregulated in patients with Chronic Myeloid Leukemia (CML). PCR for each of the above-mentioned genes was carried out using CML blood samples available in our laboratory. Amplified PCR products were sequenced for confirmation of required gene sequences using specific primers. The sequences obtained for each gene were confirmed from

NCBI database using BLAST software [data not shown]. We found 100% match for these specific genes reported here. Similar approach was used for OCT4, NANOG, LIF, KERATIN18, and DAPK, which were expressed in normal blood cells and CD13, CD73, COX2 and IL-6, were expressed by SCAT cell line developed by our laboratory. K562 cells were used as a positive control for BCR-ABL. On the basis of these results, positive controls were selected for this study.

Molecular characterization of MSC_{ALL} & MSC_{NSALL} cell lines

Molecular characterization of MSC_{ALL} & MSC_{NSALL} was performed using stem cell markers specific for Mesenchymal and Hematopoietic stem cells as shown in figure 7. MSC_{ALL} cell lines express CD105, CD13, CD73, CD34, and CD45 indicating these cells have both Mesenchymal and Hematopoietic phenotypes and it may be due to their origin from peripheral blood. Whereas, MSC_{NSALL} cell line mildly expressed CD105, CD13,

CD34, and CD45 but did not express CD73 gene. Further, to study pluripotency and differentiation of these cells, we have studied expression of OCT4, NANOG, SOX2 as markers of pluripotency & Self-renewability whereas LIF, KERATINI8 as differentiation markers. It was observed that MSC_{ALL} mildly expressed OCT4, NANOG, SOX2 & LIF whereas, KERATINI8 as differentiating marker expressed normally. MSC_{NSALL} cell line did not express OCT4, NANOG & SOX2 whereas, it mildly expressed LIF and KERATINI8 (Figure 8). This suggests that both these cell lines differ by their phenotypic expression. Morphological studies of MSC_{NSALL} cells showed that these cells were flat and larger than MSC_{ALL} and their property of duplication had reduced which was confirmed by molecular

findings as shown in Figure 8. DAPK expression of MSC_{ALL} and MSC_{NSALL} cell line is given in Figure 9, which shows that MSC_{ALL} and MSC_{NSALL} had normal expression of DAPK gene. As DAPK is expressed in normal cells and down regulates in the malignant cells, results indicate that both cell lines had normal phenotypes. Figure 9 shows COX2, IL-6 and BCR-ABL gene expression in MSC_{ALL} & MSC_{NSALL}. It was observed that both these Cell lines had low expression of COX2 and were negative for BCR-ABL confirming their normal phenotypes. Whereas, it was observed that MSC_{ALL} cell line significantly expressed IL-6, which may be important indication for use of these cells in regenerative stem cell therapy of ALL patients

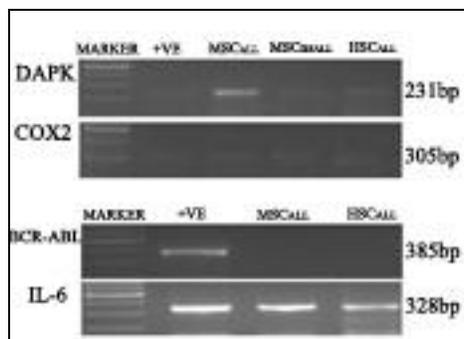


Figure 9 shows expression of molecular markers DAPK, COX2, BCR-ABL and IL6 in MSC_{ALL}, MSC_{NSALL} & HSC_{ALL} cell lines.

Molecular Characterization of HSC_{ALL}

HSC_{ALL} cell line was highly proliferative suspension cell line. The molecular characterization of these cells is shown in Figure 7, 8, 9. These cells show mild expression of CD105, CD13 whereas they prominently expressed hematopoietic markers CD34 and CD45 indicating their dominant origin as a hematopoietic cell line. This cell line did not express CD73 gene. Regarding pluripotency markers, this cell line significantly expressed

OCT4, NANOG, SOX2 and KERATINI8 with mild expression of LIF. Expression patterns clearly indicated the property of pluripotency and self-renewability of this cell line, which is also well correlated with growth rate study. Regarding Oncogenic phenotype, this cell line normally expressed DAPK and mildly expressed COX2 gene indicating their normal phenotype. It was also observed that this cell line highly expressed IL-6 and was negative for BCR-ABL gene as shown in figure 9.

Genes/ Samples	MSC _{ALL}	MSC _{ns} _{ALL}	HSC _{ALL}
CD105	++	+	+
CD13	++	+	+
CD73	+	-	-
CD34	+	+	+
CD45	+	+	+
OCT4	+	-	++
NANOG	+	-	++
SOX2	+	-	++
KERATIN	++	+	+
LIF	+	+	+
DAPK	++	+	+
COX2	+	+	+
BCR-ABL	-	NP	-
IL-6	+	NP	+
β-Actin	+++	+++	+++

Table I Shows comparative analysis of stem cell markers in MSC_{ALL}, MSC_{ns}_{ALL} and HSC_{ALL} cell lines. Key: (+++) Indicates strongly positive, (++) indicates positive, (+) indicates mildly positive and (-) Indicates Negative expression, (NP) indicates not performed

Discussion

Acute lymphocytic leukemia (ALL) is a heterogeneous group of malignancies arising from lymphocytic precursors. Epidemiological evidence suggests that genetics and environmental factors may play a role in its development. [16, 17] The important goal in the management of ALL is to identify patients that would preferentially benefit from either conventional chemotherapy or intensified therapy such as bone marrow transplant (BMT) or stem cell regenerative therapy. Studies have shown that there are higher relapse rates in recipients of autologous transplantation compared with allogeneic

transplant mainly due to contamination of the autologous marrow with residual leukemia cells. [18] Therefore, the role of autologous BMT for the treatment of ALL remains uncertain. In the present study we thought of overcoming these possibilities by isolating MSCs as well as HSCs stem cell lines of normal phenotype from peripheral blood of ALL patients for the future regenerative stem cell therapy for this disease.

The technology for isolating Mesenchymal and Hematopoietic stem cells from Bone marrow has already been well established. [4] As the procedure of Bone marrow aspiration is painful and may have complications during extraction, the use of

alternative methods for isolation of MSCs and HSCs from non-invasive sources become most important task for stem cell researchers. Despite considerable interest in the potential therapeutic applications of MSCs, there is currently no well-defined isolation or characterization protocol, which can isolate MSCs & HSCs from peripheral blood of ALL patients to be used for stem cell therapies.

In the present study, we have developed a technology to successfully isolate Mesenchymal stem cell lines MSC_{ALL} and Haematopoietic stem cell line HSC_{ALL} from peripheral blood of ALL patients and both these cell lines are at passage 6 and growing well in culture. We report here that MSC_{ALL} cell line was of two types, one is growing as a monolayer and other cells, which after 30-35 days in culture started secreting mononuclear cells in culture medium as suspension cells. These cells were then grown as an independent suspension cell line in RPMI1640 growth medium and designated as HSC_{ALL} cell line. We are the first to report a unique behavior of this MSC_{ALL} cell line. It was further noticed that after 45-60 days of secretion of HSC_{ALL} cells, the MSC_{ALL} cells stopped secreting mononuclear cells and grown as monolayer non secretory cells in DMEM growth medium. We designated this cell line as MSC_{NSALL} cell line. These cells were having slightly different morphological feature than parent MSC_{ALL} cell line, which was further confirmed at molecular level.

It was observed that all three-cell lines differ by their morphology and growth rate indicative of their different phenotypes. In this study, we have used 14 stem cell molecular markers to define their phenotypic and genotypic nature for their pluripotency, self-renewal capacity as well oncogenic properties. CD105 or Endoglin is a member of the TGF-beta receptor complex that modulates TGF-beta signaling was suggested as a putative stem cell marker. [5, 7] CD13 or aminopeptidase N and CD73 (Ecto-5-prime-nucleotidase) is also considered as a positive marker for MSCs. [7] MSC_{ALL} and MSC_{NSALL} cell lines prominently expressed CD105, CD13 and CD73 markers indicating their MSCs nature whereas, HSC_{ALL} cell line showed mild expression of these markers indicating difference from MSCs phenotype. Hematopoietic stem cells have long

been characterized using CD34 and CD45 expression. [8, 9] Both MSCs cell lines mildly expressed CD34 & CD45 genes whereas, HSC_{ALL} cell line prominently expressed CD34 and CD45 genes indicating hematopoietic nature of these cells.

Three transcription factors that are expressed at high levels in embryonic stem cells are NANOG, OCT-4 and SOX-2. [10, 11] The down regulation of these three transcription factors correlates with the loss of pluripotency and self-renewal. In the present study, MSC_{ALL} and HSC_{ALL} cell lineages were positive for OCT4, NANOG and SOX2, which mainly showed self-renewability of undifferentiated stem cells. Whereas, MSC_{NSALL} cell line was negative for both OCT4 and NANOG indicating its differentiated stage and loss of self-renewability. These cells showed different morphology and their doubling capacity was also very low. Metcalf group has shown that the leukemia inhibitory factor (LIF) maintains the developmental potential of embryonic stem cells, which can be regarded as a pluripotency and differentiating marker. [15] In the present study, MSC_{ALL} MSC_{NSALL} and HSC_{ALL} cell lines expressed LIF further confirming pluripotency & differentiating properties of these cell lines. Human keratin18 is type I IF protein subunits whose expression is restricted in adults to a variety of simple epithelial tissues. [19] MSC_{ALL}, MSC_{NSALL} and HSC_{ALL} cell lines were positive for Keratin18 indicating their epithelial phenotypes and thus confirmed that the MSCs isolated from peripheral blood of ALL patients were not fibroblasts.

After establishing the genotypes of all three cell lines, it was very essential to know whether the cell lines developed were of normal or malignant phenotypes before considering them for therapeutic purposes. We have utilized three markers viz. DAPK, COX2 and BCR-ABL genes to study the nature of cells in terms of malignancy. Many studies have identified the transcriptional silencing by DNA methylation as a mechanism responsible for tumor suppressor inactivation. [14] Several cancer cells show methylation of DAPK gene and thus showed down-regulation of this gene in malignant transformation. Similarly it has been well documented that COX2 gene is

upregulated in various cancer cells. [13] In our study we have observed that all three cell lines prominently expressed DAPK gene indicative of normal phenotypes whereas, mildly expressed COX2 gene indicative of high proliferative potential of these cell lines. It has been reported that BCR-ABL fusion protein is involved in some of the cases of ALL and we found that all three cell lines were negative for BCR-ABL gene indicating these cell lines had normal phenotype.

Recent clinical trials have focused almost entirely on the ability of MSCs to exert their biological function through topic mechanism including secretion of cytokines that might serve both paracrine and endocrine functions. [20, 21] This shift suggests that application of MSCs in therapy resulted in reduction of inflammation, apoptosis and fibrosis in numerous diseases despite a lack of MSCs differentiation in injured tissues. [21] In the present study, we found that MSC_{ALL} and HSC_{ALL} cell lines prominently secretes IL 6 cytokine therefore these cell lines may have a great importance in stem cell regenerative therapy which may improve innate immunity of ALL patients.

MSCs have been known to differentiate into various tissue cells in vivo and in vitro and contribute to the regeneration of bone, cartilage, muscle and adipose tissue. [5] We have utilized isolated MSCs to differentiate into cardiomyocyte with differentiating reagent Azacitidine, which differentiates MSCs into Cardiomyocyte cells. We observed that isolated MSC_{ALL} cells after treatment with azacitidine do not show any morphological changes, which could give rise to cardiomyocyte cells. Study with Cardiac specific molecular markers also revealed no expression of such markers (data not shown here). Therefore more in depth experimentation and study is needed to confirm whether these cells can differentiate into osteocytes, chondrocytes and other cells, which MSCs are known to differentiate. Similar study is also need to be performed to understand differentiation potential of HSCs isolated from ALL patient.

We have carried out several similar experiments with peripheral blood of normal, non-leukemic individuals to isolate MSCs and HSCs. It was found that such kind of cells couldn't be isolated from

normal individuals with the technique that we have used for isolation of MSCs & HSCs from peripheral blood of ALL patients. Domenech et al have shown that MSCs are mobilized into the peripheral blood in condition such as Hypoxia. [22] Similarly MSCs have been known to mobilized into peripheral blood after treatment with certain drugs. Therefore further modification in the technique is required to isolate such kind of cells from peripheral blood of normal individuals.

Overall, this paper describes the technology for isolation and characterization of MSCs and HSCs cell lines from peripheral blood of ALL patients. We are the first to report the secretary nature of isolated adherent MSCs giving rise to mononuclear cells, which were propagated as a suspension cell line. These suspension cells were capable of inducing other adherent MSCs to produce similar kind of suspension cells. Even though the cells isolated from peripheral blood of leukemia patient showed normal phenotypic expressions in the present study, it is not advisable to use these cells for any therapeutic purposes and further study is required to rule out any possibility of oncogenicity for therapeutic application.

Persons other than authors:

Mrs Naina Rane, Department of Molecular Medicine & Biology – Technical help during this project

Mrs Mamta, Department of Haematology, Jaslok Hospital and Research centre - provided blood samples for this work.

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