

Expansion of CD133⁺ umbilical cord blood derived hematopoietic stem cells on a biocompatible microwells

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Abstract

Umbilical cord Blood (UCB) as a source of Hematopoietic Stem / Progenitor cells (HSPCs) used for Umbilical cord blood transplantation (UCBT). The main obstacle in application of this source as an appropriate source of HSPCs is low volume of this product. So ex vivo expansion of these cells in a microenvironment which mimic body condition is important. In current study we designed biocompatible microwells in which collagen type I is coated by softlithography method. Our findings designated that in 3-Dimensional (3D) microenvironment CD133⁺ UCB derived HSC expanded significantly compared to 2-Dimensional (2D) microenvironment.

Keywords: CD133⁺ umbilical cord blood derived, Biocompatible microwells,

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Introduction

Hematopoietic stem cells (HSCs) defined as multipotent self-renewing cells with the ability to give rise to all blood cell types.¹ HSCs can be collected from different sources such as bone marrow (BM), Umbilical cord blood (UCB) as well peripheral blood. Since the first successful umbilical stem cell transplantation (UCBT) in 1989 to a patient with fanconi's anemia,² UCB served as an attractive alternative source to BM for HSCs transplantation.^{3, 4} Despite the advantages of using UCB as source of hematopoietic stem / progenitor cells (HSPCs) for transplantation such as low incidence of GVHD and HLA mismatching,⁵ the main disadvantage of this source to use for UCBT, is the low cell dose due to small volume of obtained samples.⁶ To conquer the problem of low number of HSCs, ex vivo expansion of HPSCs from UCB has been challenged.⁷ CD133 (Also Known as

AC133) counted as a hematopoietic stem cell markers,⁸ CD133 mostly is co expressed with CD34. Ex vivo expansion with maintaining HSCs unique specifications could be performed in both 2-Dimensional (2D) and 3-Dimensional (3D) microenvironment. In 2D culture condition in plastic dishes, large amount of various cytokine cocktails are needed for expansion of HSPCs. So, fabrication a microenvironment which mimicking bone marrow structure bring to attraction. Extracellular matrix (ECM), is a complex ultra structure, which is not only a environment to sustain tissue structure, but also controls cell proliferation, growth, migration and differentiation.⁹ So, developing and environment which could mimicking BM niche is so important. Softlithography, a approach based on self assembly and replica modeling, used as a low cost technique for fabrication of nano- and micro-structures.¹⁰ Here, We

developed a microwell which is coated with collagen type I for ex-vivo expansion of CD133 UCB-derived cells. We compared the ability of expansion of seeded cells in this microwells and routine 2D cells culture condition in the presence of expansion cocktails.

Methods and Materials

Sample preparation

Umbilical cord blood samples collected from normal and C-section full term deliveries after obtaining informed consent. Collected samples were diluted with hydroxyethyl starch (HES) in the ratio of 1:4 to deplete red blood cells (RBCs). The diluted samples were layered onto Ficoll-Paque (Pharmacia-Amersham, Piscataway, NJ; $\rho = 1.077 \text{ g/mL}$) gradient and centrifuged at $400 \times g$ for 30 minutes at 20°C . Following that mononuclear (MNC) interface layer was collected and washed twice with Phosphate buffer saline (PBS) / EDTA.

CD133 Cell Purification

CD133 cells were purified using magnetic cell sorting (MACS) technology (Miltenyi Biotec, CA, USA) according to manufacturer's instruction. MNCs were incubated with $50 \mu\text{l}$ of CD133 microbeads (Miltenyi Biotec, CA, USA) for 1 hour at 4°C in dark. Incubated MNCs with CD133 microbeads were centrifuged at $400 \times g$ for 5 minutes at 4°C . Positive selection was done by LS columns. Purity of isolated CD133+ assessed by flowcytometry analysis.

Preparation of microwells

Microwells were fabricated using softlithography method. PDMS (Polydimethylsiloxane), a biocompatible elastomer, used for biomolecular patterning. Circular pattern of microwells with $200 \mu\text{m}$ diameter was fabricated with defined thickness by spin coater on glass by using a photoresist polymer. Spin coating was performed for 90 seconds at 1500 rpm. Polymerization of the photoresist glass coated wafers were exposed to UV light of 420nm for 6 minutes through a photomask. The UV exposed patterns was developed using developer, rinsed with isopropyl alcohol to fix the pattern and neutralized the developer. PDMS molds were fabricated by adding 10:1 mixture of PDMS solution and the initiator.

Consequently, prepared microwells were coated with collagen type I to mimic bone marrow microenvironment.

Cell culture

The prepared microwells were sterilized with 70% ethanol and finally washed with PBS. Purified CD133+ cells were seeded onto microwells at a concentration of 5×10^4 cells / microwell. Then, culture medium containing 100ng/ml SCF, 100 ng /ml FLT-3 ligand, 100 ng/ml TPO were added to microwells. The medium was changed every 48 hours. CD 133+ cells were cultured in 24 well plates as a 2D cell culture condition. Cells in 2D condition cultured in stemspan (stem cell technologies, USA) supplemented with 100ng/ml SCF, 100 ng /ml FLT-3 ligand, 100 ng/ml TPO. The medium was changed every 48 hours. At day 7 and 14 cells were harvested from microwells and counted by Hemacytometer.

Microscopic assay

Microscopic assay of cultured cells were performed on day 7 and 14 then compared with day 0 of 3D microenvironment. Increasing in cell count was observed by light microscopy. Cells were harvested from microwells and counted by hemacytometer. Finally, cell count were compared to 2D culture condition.

Flowcytometry analysis

Cells were extracted from the collagen coated microwells at defined times (days 7 and 14). Also, cells in 2D cell culture were harvested at the same time points. Flowcytometry analysis was performed for expression of CD133. Cells were exposed to Phycoerythrin (PE) conjugated CD 133 monoclonal antibody (mAb) (Miltenyi Biotec, Auburn, CA). After incubation at 4°C in dark for 30 minutes with mAb, cells were fixed with 2% paraformaldehyde. CD133 labelled fixed cells analysis were performed by a FACSCalibur (Becton Dickinson, NJ, USA).

Statistical analysis

Statistical analysis was performed using repeated measure method in SPSS software.

Results

Isolation of CD133 cells form cord blood

Purity of isolated CD133 cells were assessed by Flowcytometry the day after isolation. Purification of isolated cells was 98 % (Figure 1).

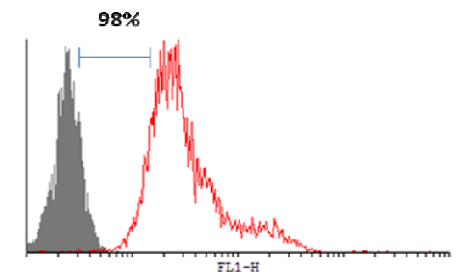


Figure 1 . CD 133 expression of isolated cells on Day 1 .

Expansion of cultured cells in 2D and 3D microenvironment

Expansion of seeded cells in 3D culture were significant after 14 days of cell culture in the presence of cytokine cocktail (Figure2). Expanded cells were shown at day 7 in routine cell culture dishes (Figure 3). Results from statistical analysis were significant ($p \leq 0.05$) (Figure4).

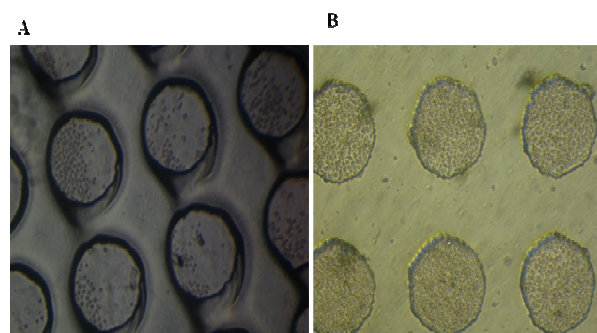


Figure 2 . Cells in microwells. A) Day 7 B) Day 14

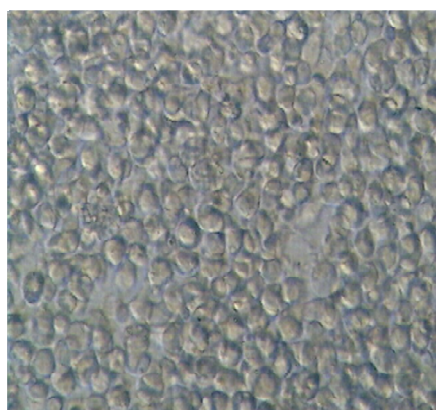


Figure 3 . Expanded CD133 isolated cells in 2D microenvironment at day 7

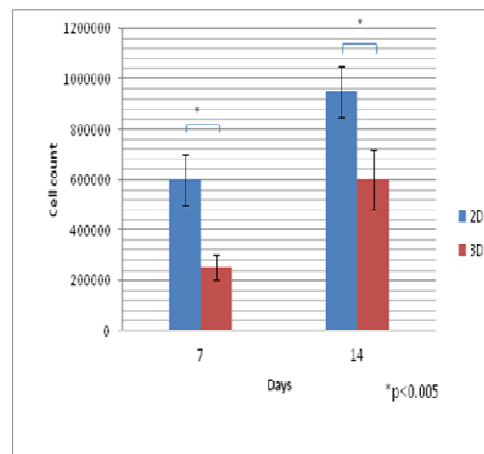


Figure 4 . Statistical analysis of cell count in 2D and 3D microenvironment.

Expression of CD133 analysis

CD133 expression was assessed in both 2D and microwells at mentioned time points. Flowcytometry analysis showed higher expression level of CD133 in 3D environment compared to plastic petri dishes at day 7 and 14 of expansion ($p < 0.05$) (Figure5 and 6). Interestingly, our results showed that the collagen coated microwells could support the expansion of CD133 cells with lower rate of differentiation.

Discussion

In this study , a collagen coated 3D scaffold was used which is able in expanding CD133⁺ hematopoietic derived umbilical cord blood stem cells with lower rate of differentiation in comparison to 2D environment . CD133⁺ cells are immature potential source of hematopoietic stem cells which could be isolated and expanded from various sources. Many clinical applications described for expanded CD133⁺ including using in situation which low amount of stem cells provided from BM and peripheral blood. Also, expanded hematopoietic stem cells could be used in gene therapy. Viral gene delivery mediated by cells which are in synthesis phase (S phase). Stem cells could also apply in umbilical cord blood transplantation. By establishment of cord blood banks and records of successful transplantation, UCBT is progressively used in treatment of malignant and non-malignant hematologic disease. Expansion potential of umbilical cord blood derived stem cells is higher than BM (ability of expansion for 30 weeks compared to 10 weeks in BM). In order to achieved sufficient and

appropriate stem cells which maintained their pluripotency and multilineage differentiation potential, cells must be expanded in various cytokines and different cell culture conditions in vitro. We demonstrated that, prepared biocompatible collagen coated 3D scaffold resulted in a higher expansion of CD133 derived UCB compared to routine cell culture conditions ($p < 0.05$). Moreover, lower rate of differentiation were observed in CD133 cells in 3D environment by flowcytometry. (31 % in 2D culture compared to 50 % in 3D culture condition after 14 days). So, these results showed that using collagen coated microwells had the higher ability to maintain seeded cells in undifferentiated state compared to 2D environment. In contrary to three-dimensional cell culture, 2D environment only provides a monolayer of cells. So, 2D cell culture didn't count as a suitable model of cellular interactions. The main goal of using 3D environment is mimicking physico-chemical, cellular and natural microenvironment of body.¹¹ 3D architecture is used to increasing the surface to volume ratio in order to increase cellular interactions. In 2D cell culture condition this interactions is very low and only a paracrine interaction is between cells. We demonstrated that 3D structure had higher capacity in expansion with lower rate of differentiation compared to 2D culture condition. Recently, scientist found that fibroblast culture in 3D environment caused significant alterations in structure and function of these cells. Cukierman and co-workers demonstrated that cultivated fibroblasts in 3D environment expanded and migrated faster than cells in 2D culture conditions.^{12, 13} In 2005 Liu and Colleagues indicated that culturing mouse embryonic stem cell on a 3D highly porous tantalum based

scaffold resulted an increased in differentiation of embryonic stem cells into hematopoietic precursors in 3D scaffolds compared to traditional 2D cell culture conditions.¹⁴ In current study, we demonstrated that hematopoietic stem cell derived from UCB could maintained and expanded for 14 days in the biocompatible microwells coated with collagen type I and a mixture of expansion growth factors. These cells significantly expressed higher level of CD133 surface marker compared to 2D culture ($p < 0.05$). Also, they demonstrated that cultivation of embryonic stem cells on fibrin and fibrin with PEG scaffolds affected expression of pluripotency markers.¹⁵

It is reported that cultured hematopoietic stem cells from cord blood in the absence of serum and cytokines in a 3D biocompatible scaffold called Cytomatrix which is coated with fibronectin. They shown that their 3D scaffold expand cell numbers better than traditional 2D environment.¹⁶ Our developed collagen based microwells result in higher expansion of UCB-derived CD133 cells. Feng et al. seeded CD34⁺ cells from umbilical cord blood on PET scaffold coated with full-length fibronectin. they reported that seeded cells had 100 fold over expansion after 10 days.¹⁷ Our microwells supported expansion of CD133 hematopoietic stem cells in the presence of expansion cytokines cocktail.

We showed that our developed collagen coated microwells is able to support expansion of CD133⁺ cells from umbilical cord blood better than plastic culture dishes. This 3D scaffold was designed to enhanced surface to volume ratio.

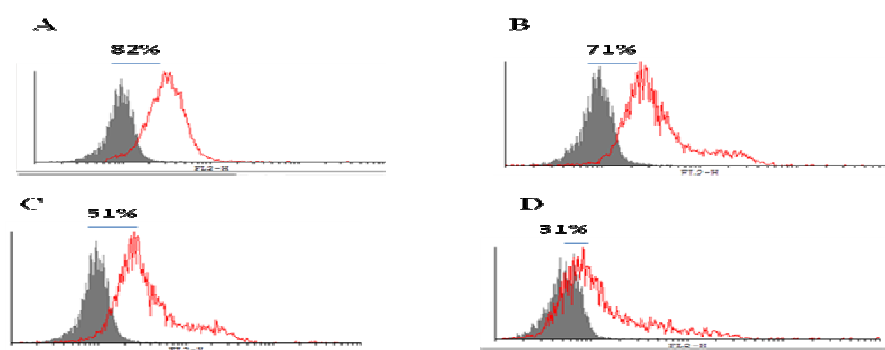


Figure 5 . A) Expression of CD133 cells in 3D micro-environment at Day 7 . B) Expression of CD133 cells in 2D micro-environment at Day 7 . C) Expression of CD133 cells in 3D micro-environment at Day 14 . D) Expression of CD133 cells in 2D micro-environment at Day 14

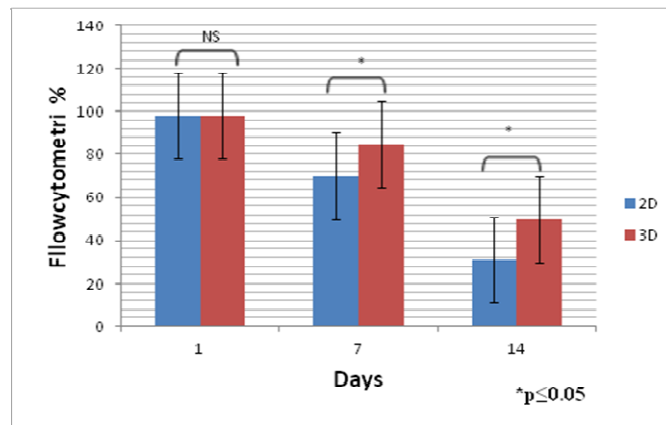


Figure 6 . Statistical analysis of expression CD133 at defined time points .

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