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| Original Article |

Osteoblast Differentiation of Umbilical Cord Blood-Derived Mesenchymal Stem Cells and Enhanced Cell Adhesion by Fibronectin

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Abstract : Mononuclear cells isolated from human umbilical cord blood were differentiated in to spindle shaped fibrous mesenchymal stem cells (MSCs) in DMEM with 10% fetal bovine serum. These progenitor cells were further examined for their ability to differentiate into osteoblasts by culturing them in osteogenic differentiation media (Stem Pro®). Calcium mineralization assay, alizarin red S, von kossa staining confirmed that MSCs from cord blood were capable of mineralization when they were cultured in osteogenic medium. The adhesion studies shown that MSCs have greater affinity towards fibronectin (FN) coated dishes, compared to albumin (Alb) coated dishes due to CD29and CD44 surface receptors.

Key words: *cord blood cells, fibronectin, mesenchymal stem cells, osteogenic differentiation, tissue engineering.*

1. Introduction

Human mesenchymal stem cells (hMSCs) are multipotent stem cells, which naturally gives rise to connective tissue such as bone, cartilage, making bone regeneration one of the most investigated therapeutic areas for hMSCs today.[1] Osteogenic differentiation of these hMSCs from various sources in combination with biocompatible scaffolds make the bone regeneration a possible task.[2, 3] During the *in vitro* bone regeneration studies, it is important to understand that the degree of bone cell adhesion to the scaffold influences the osteointegration followed by the development of new bone tissue structures.[4, 5]

The strategy we adopted to improve osteointegration had been pre-coating the implants with an extracellular matrix protein (fibronectin) for use as a scaffold, which enables specific cell-extracellular matrix interactions[6, 7]; along with the above we used the growth factors to facilitate the differentiation of the osteoblasts.[8] The extracellular matrices (ECM) like fibrinectin (FN) and collagen type-I are rich in RGD (arginine-glycine- aspartic acid) sequences, which are well recognized by the integrin receptors that mediate the cell adhesion with the extracellular matrix (ECM).[9] Apart from these direct effects, the ECM is also considered to play an important role in the presentation of cytokines and other growth factors to neighboring cells.[10]

In this case study, we would like to monitor the adhesion patterns of umbilical cord blood derived hMSCs on to FN coated plastic and their differentiation in osteogenic medium. Based on these results, we will further proceed with fibronectin coated implants for 3 dimensional (3D) cell culture studies.

2. Materials and Methods

2.1 Isolation of Mesenchymal Stem Cells from Umbilical Cord Blood (UCB)

Freshly collected cord blood was diluted with 3x Phosphate Buffer Saline (PBS). 35 mL of the diluted blood was carefully layered over 15 mL Ficoll® (Sigma Aldrich, St. Louis, Mo, USA) and centrifuged at 500 × g for 15min in swinging bucket centrifuge. Once we obtained the density gradient separation of cord blood contents (Fig 1), the mononuclear cell (MNCs) fraction was carefully aspirated in to sterile tube. This MNC fraction was washed once with PBS and further cultured in DMEM with low glucose (Sigma Aldrich) containing 10% Fetal Bovine Serum (FBS) (Sigma Aldrich), L-glutamine (Sigma Aldrich) and penicillin/ streptomycin (Sigma Aldrich). These culture plates were incubated in a humidified CO_2 Incubator with 5% CO_2 (Shell Lab, India).

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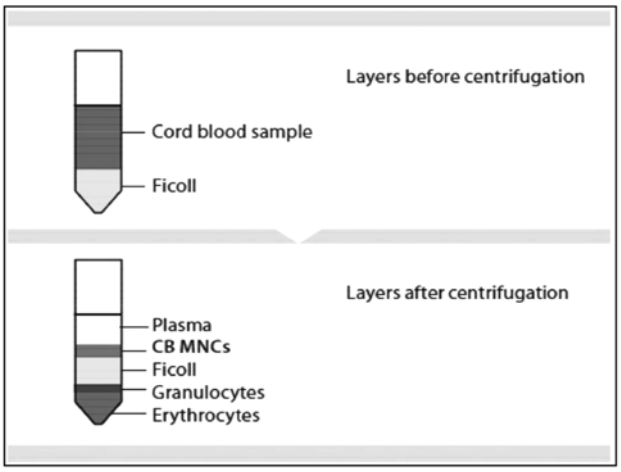

Figure 1. Schematic diagram of density gradient centrifugation.

2.2 Osteogenic Differentiation

For osteogenic differentiation studies, human mesenchymal stem cells (hMSCs) were initially cultured in DMEM (3.5×10^3 cells/cm^2). After 24 hr, the media is changed with osteogenic differentiation media which includes, Stem Pro® osteogenesis differentiating kit (Invitrogen, Grand lsland, NY, USA) consisting of growth supplements for osteoblast differentiation, MesenPRO RS™ (Invitrogen) basal media, L-Glutamine, Penicillin/ Streptomycin and dexamethasone. This experiment is continued for 20 days and media is changed for every 2 days. We used DMEM supplemented with 10% FBS, l-glutamine, Penicillin/ Streptomycin and dexamethasone as control medium. Fig 2 will give us the schematic of this experiment.

2.3 Microscopic Observations

For growth and morphology of hMSCs from cord blood, we monitor the culture flask at regular intervals by phase contrast microscope (Leica, Cambridge, England) and pictures are recorded using digital camera.

2.4 Fibronectin Coating

Fibronectin (FN) (Sigma Aldrich) was reconstituted in sterile distilled water (1 mg/mL). This protein solution was further diluted in sterile balanced salt solution (10 μg/mL) and coated to culture dishes and allowed for overnight incubation at 37°C. After incubation dishes were washed with sterile PBS and stored at 4°C for cell seeding. In this case study we used Albumin (Alb) (Sigma Aldrich) as control.

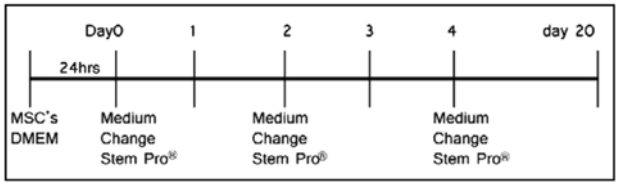

Figure 2. Schematic representation of ostogenic differentiation studies.

2.5 Cell Adhesion

For cell adhesion studies, 5×10^4 hMSCs/well were added in to each FN and Alb coated dish and grown in DMEM with L-glutamine and penicillin/streptomycin. For every 20 min interval cells are detached from each culture dish by trypsinization (0.025% trypsin) (Sigma Aldrich). Cell numbers were counted with neubauer chamber (Fuchs-Rosenthal).

2.6 Histochemical Studies

Phosphate and sulfate mineralization in hMSCs cultured both in "DMEM and Stem Pro® media" was studied by alizarin red staining. The cells were washed with PBS and fixed with neutral buffered formalin (NBF) for 20 min. Fixed cells were washed once with distilled water and stained with 1% alizarin red S (Sigma Aldrich) for 10 min. The remaining dye was washed out with distilled water, and the cells were air-dried for observation. We too carried out von Kossa staining for calcium phosphate deposition in 20 days old hMSCs. Briefly, cell lines were immersed in a solution of 5% silver nitrate and kept under ultraviolet light for 20 min. Excess stained was washed with sterile distilled water followed by sodium thiosulphate washing. These cell lines are further counter stained with nuclear fast red and observations are made under microscope.

2.7 Calcium Mineralization Assay

Calcium concentration was determined by *o*-cresolphthalein colorimetric assay. hMSCs grown both in DMEM and Stem Pro® were washed twice with PBS, and calcium was extracted by overnight incubation with 0.5M HCl on a shaker. Once we collect the cell lysate, calcium was quantified by mixing the 50 μl of cell lysate with 950 μl of *o*-cresolphthalein assay solution containing the calcium binding reagent (0.024wt% *o*-cresolphthalein and 0.25wt% 8-hydroxyquinone sulfate in water) (Sigma Aldrich) and calcium buffer (500 mM 2-amino-2-methyl-1, 3 propenediol in water) in 1:1 ratios. The absorbance of each solution was measured at 570 nm (as per Cayman chemical kit, NY, USA).

2.8 Alkaline Phosphatase Assay (ALP)

ALP activity was measured by quantifying a soluble yellow colored *P*-nitrophenol from P- nitrophenol phosphate (PNPP) (Thermo Chem, VG, West Sussex, UK). In brief, 10^5 cells

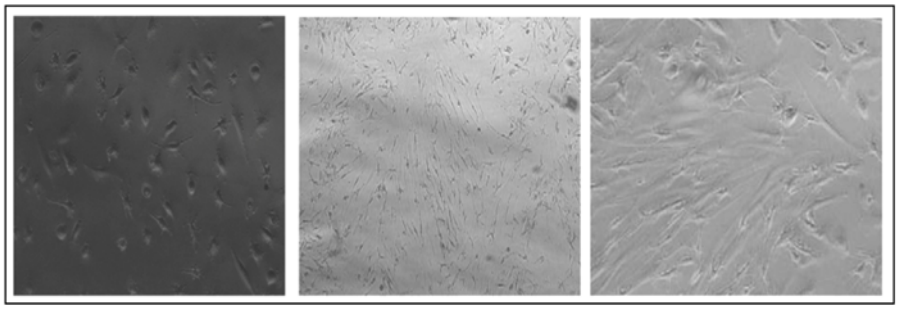

Figure 3. Mesenchymal stem cells at different stages of their growth.

(hMSCs) were cultured in 64 well plates and washed with PBS. These cells are fixed with 4% paraformaldehyde/PBS for 10 min at 4^0C and subsequently cells were incubated with PNPP for 15min at 37^0C. Reaction was terminated by adding 1 M NaOH and the absorbance of *P*-nitrophenol was measured at 415 nm. The ALP activity is determined by plotting our values in *P*-nitrophenol standard curve.

3. Results

3.1 Isolation of Mesenchymal Stem Cells

After an incubation period of two weeks, we were successful in isolating mesenchymal stem cells (MSCs) from the mono nuclear cells (MNCs). The adherent cell population with spindle shape, fibroblast like morphology confirms them as mesenchymal progenitors (Fig 3).

3.2 Osteogenic Differentiation

The mesenchymal stem cells were further examined for their osteogenic differentaition, by growing them in osteogenic differentiation medium Stem Pro®. By the end of twenty days, most of the adherent cells started to form a uniform monolayer with cuboidal cells (Fig 4), resembling the osteoblasts. These morphological findings were further confirmed by histochemical studies. Growth of adherent cells was comparatively slow in control dishes and cells retain their fibroblast like morphology.

3.3 Cell Adhesion

The hMSCs were tested for their adhesion and growth in fibronectin (FN) coated dishes and compared with albumin (Alb) coated dishes as controls. We observed better cell adhesion to FN-coated dishes when compared to Alb-coated dishes. As per Fig 5, the hMSC adhesion rate also rapid in case of FN-coated dishes when compared to Alb-coated dishes.

3.4 Histochemical Studies

The hMSCs cultured with osteogenic differentiation medium (Stem Pro®) started showing mineral deposition by day 10 and the deposition rate increases till day 20. Fig 6 confirms our experimental results, and the mineral deposition in hMSCs cultured with DMEM was comparatively less and time taking. Simultaneously, von Kossa staining confirms (Fig 7) an

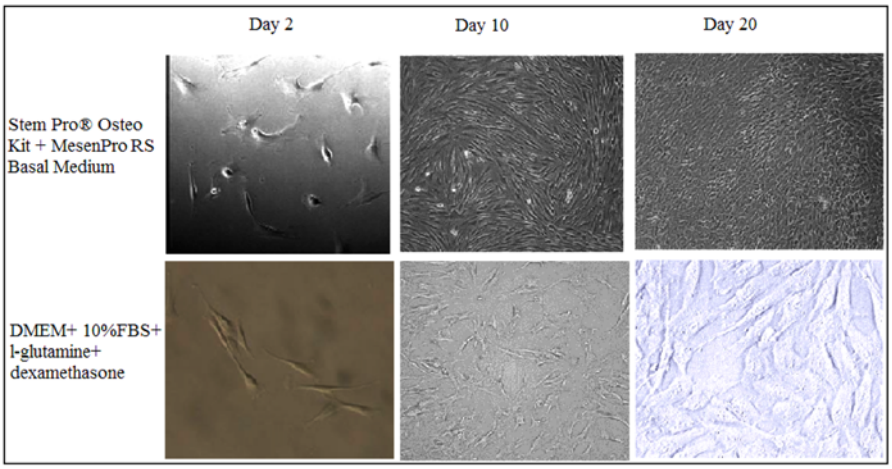

Figure 4. Osteogenic differentiation of hMSCs

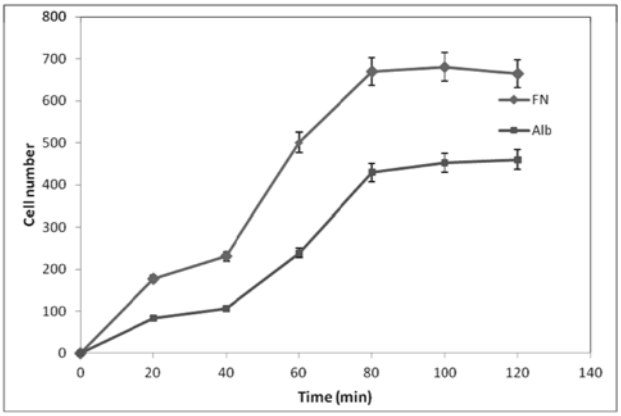

Figure 5. Number of hMSCs adhered to FN and Alb coated dishes. Values represent the means±SD of the results from three samples.

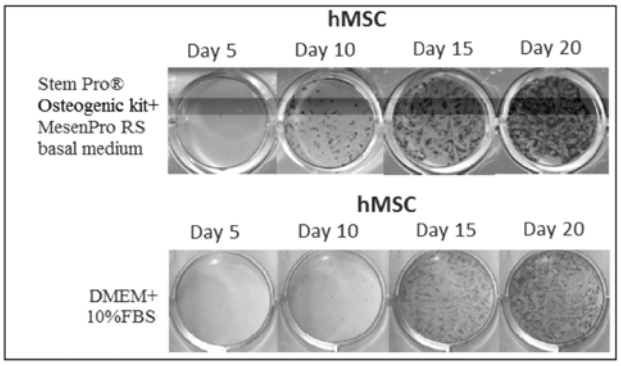

Figure 6. Alizarin red S staining of hMSCs cultured in Stem Pro® and DMEM.

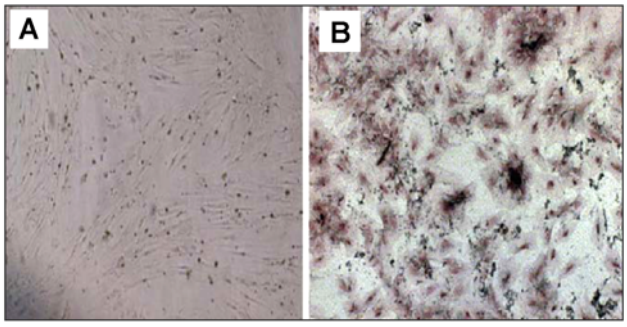

Figure 7. Von Kossa staining of hMSCs cultured in A) DMEM; B) Stem Pro®.

increase in calcium content in hMSCs grown in Stem Pro®, when compared to controls (DMEM).

3.5 Calcium Mineralization Assay

As confirmatory test for calcium deposition in the cells, we performed the *o*-cresolphthalein assay. The calcium deposition

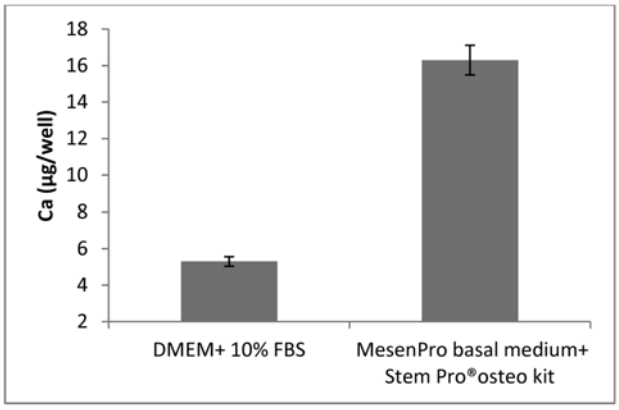

Figure 8. Calcium deposition in hMSCs. Values represent the means±SD of the results from three samples.

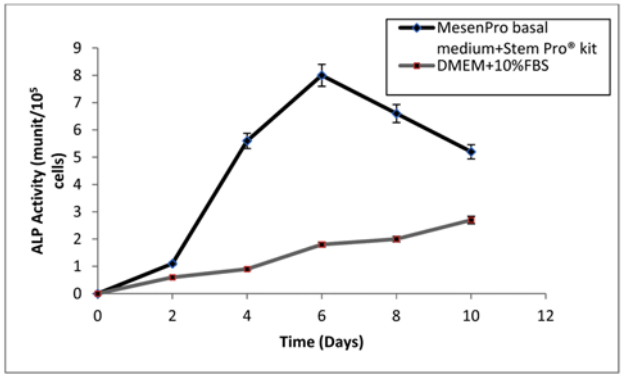

Figure 9. ALP activity of hMSCs. Values represent the means ±SD of the results from three samples.

in hMSCs cultured in osteogenic differentiation medium (Stem Pro®) is fairly high when compared to the cell grown in DMEM as control (Fig 8).

3.6 ALP Activity

There is an increased ALP activity in hMSCs cultured in osteogenic differentiation medium. The ALP activity keeps on peaking up to day6, which confirm the increased osteoblastic activity in hMSCs grown in Stem Pro® (Fig 9)

4. Discussion

We report that hMSCs from umbilical cord blood are capable of undergoing osteogenic differentiation, by presenting the bone related mineral deposition. hMSCs from cord blood shown differential response with different growth media, demonstrating better results with osteogenic differentiation media (Stem Pro®). During this study we adopted chemically defined serum free

media, which eliminate the confusing effects of serum growth factors.[11] On the other hand; hMSCs from umbilical cord blood can be regarded as osteogenic progenitor/precursor cell population that can be acquired on regular basis from caesarean section.[12] It has proven that, hMSCs from the umbilical cord blood have the skills to proliferate extensively and maintain its osteogenic differentiation *in vitro.*[13]

In our studies, results obtained from the alizarin red S, von Kossa staining and calcium assay confirms that mineralization in the hMSC layers during osteoblast differentiation when cultured in Stem Pro® osteogenic differentiation kit. This osteogenic potential of cord blood derived progenitors may be useful for autologous transplantations in near future.

For bone tissue engineering, ideal implants must mimic like native bone and enhance the osteointegration.[14] In order to achieve and enhance the osteoblast growth and to reinforce the osseous fixation, implant surface has to modify with surface coatings proteins. Various growth factors [15, 16] and extracellular matrices (ECM)[17] are currently used to surface coat the implants. Fibronectin (FN) is a prototype cell adhesion protein, widely distributed in the tissues of all vertebrates and a potential ligand for most cell types.[18] This mosaic protein is well known for its role in cell adhesion processes due to its specific amino acid sequence for the RGD motif that is recognized by specific cell membrane receptors such as integrins.[19] It is also clear from the literature that peptides with high RGD sequence are the most effective and most often employed peptide sequence for stimulated cell adhesion on to synthetic surfaces.[20] This is based upon its widespread distribution and use throughout the organism, its ability to address more than one cell adhesion receptor, and its biological impact on cell anchoring, behavior and survival.[21]

In our cell adhesion studies we noticed that hMSCs having grater affinity for fibronectin coated dishes, as compared to albumin coated dishes. This may be due to superior RGD motifs in fibronectin fallowed by CD29 and CD44surface markers on MSCs (contain β1-integrin and hyaluronic acid), well known FN receptors. [22] The mechanism of integrin mediated cell adhesion takes place in four different partly overlapping events: cell attachment, cell spreading, grouping the actin cytoskeleton and focal adhesions.[23] During the above four steps of cell adhesion, integrins are very vital in physical anchoring processes as well as in signal transduction through the cell membrane.[24]

The main drawback in tissue engineering is implant rejection due to limited bioactivity and provides minimal biological cues to guide tissue regeneration.[25] Therefore, an accelerated biomimetic process was developed by coating polymer scaffolds with a thin layer of fibronectin (FN), which is an important strategy when trying to improve the success rate for tissue regeneration.

5. Conclusion

Umbilical cord blood has proven to be an alternative source for mesenchymal stem cells and these cells are successfully differentiated into osteoblasts. The cuboidal cell morphology, phosphate and calcium deposition in the cell culture confirm them as bone forming cells. Fibronectin has shown better results for stem cell adhesion on cell culture plastic and we hope this data is further useful in 3D cell culture studies over tissue engineering implants.

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