Derivation, Expansion and Characterization of Clinical Grade Mesenchymal Stem Cells from Umbilical Cord Matrix Using Cord Blood Serum

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Background and Objectives: With increasing use of mesenchymal stem cells (MSCs) in regenerative medicine, there is greater awareness towards the need to have clinical grade products. The bovine media currently used allow good expansion to give large number of MSCs of the right quality. This report brings the significance of using cord blood serum (CBS) in the derivation of MSCs from umbilical cord matrix, to help its clinical applicability.

Methods and Results: MSCs isolated from the cord by explant cultures were expanded and characterized by flow cytometry. Cord blood serum while helping expansion, has the ability to preserve the immunophenotype and differentiation potential of the MSCs derived from the umbilical cords.

Conclusions: Our results suggest that MSCs derived and expanded in cord blood serum are better suited for clinical applications.

Keywords: Umbilical cord, Mesenchymal stem cells, Differentiation potential, Cord blood serum

Introduction

Stem cell biology has been a subject of intense investigations over the past few decades. It is expected that stem cells will play a key role in the treatment of a number of incurable diseases, although many challenges need to be overcome before routine use of these cells in clinics. One of the major technical challenges is to develop the right cell culture medium for large scale growth and expansion of the preferred cell type.

The use of bovine components in cell culture media is one of the major concerns. Currently, most researchers use

media containing fetal bovine serum (FBS) at a concentration of 10~20% for culture and expansion of various types of stem cells (1-4). In spite of its advantages, there are several concerns associated with the use of media containing animal products. One of the major concerns being transmission of diseases from the use of animal products. This has led the researchers to move away from these to other chemically defined media components, and replace it with specialized media with no serum or media supplemented with human serum (5) etc. However, obtaining human serum in large quantities is difficult and adult human serum may not be as enriched in growth factors as FBS (6). One possibility might be to use human cord blood serum as a substitute for fetal bovine serum in culturing stem cells such as MSCs. We have previously reported our success of using CBS for the culture of bone marrow derived mesenchymal stem cells (7).

Bone marrow derived mesenchymal stem cells (BMMSCs) have been an extensively studied cell type so far, for both experimental and clinical applications (8-10). Apart from the fact that bone marrow aspiration is an invasive and

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painful procedure, it has been demonstrated that both cell yield and differentiation potential of MSCs decline with age (11, 12). Umbilical cord blood is another source of mesenchymal stem cells, but the low frequency of MSCs in cord blood makes it less attractive (13). Some investigators have isolated MSCs from cord blood (14, 15) whereas others have not been so successful in culturing MSCs from all cord blood units (16, 17). There are reports of MSC isolation from other sources like adipose tissue, dental pulp, hair follicle and amniotic fluid and these cells have been used for various clinical applications (18-20). Of all the sources studied umbilical cord matrix appears to be a very good and dependable source of MSCs (21, 22). The collection of umbilical cord does not have any ethical concern and, more importantly, it is not harmful to the newborns' health. The plasticity of umbilical cord MSC (UCMSC) has been previously demonstrated by our group (23). The current study is aimed at demonstrating the usefulness and robustness of CBS to support growth and expansion of mesenchymal stem cells isolated from the umbilical cord matrix.

Materials and Methods

Preparation of cord blood serum

The cord blood is collected after delivery from pre-screened mothers after receiving an informed consent. These women were screened for infectious disease markers as per the local regulatory guidelines. No collection was made if there were any complications during delivery. The blood was collected by allowing it to flow into a sterile Schott glass bottle from the cut end after the baby is separated. Ten to 25ml of blood was collected and transported to the facility at 20~25°C within 10~12 hrs of collection.

The clotted blood was then centrifuged at 1,000 g for 30 mins at 20°C and the clear serum was collected into sterile containers. Serum samples from a minimum of three donors were pooled to eliminate donor to donor variations. The pooled sera were filter sterilized by passing through a 0.22 μ filter. Complement was inactivated by heating the serum at 56°C for 30 mins. Serum sample was labeled, aliquoted and stored at -20°C for upto 4 months for further research use.

Isolation and culture of umbilical cord mesenchymal stem cells using cord blood serum and fetal bovine serum

Umbilical cords (gestational ages 31 to 37 weeks) were obtained from the maternity hospitals after normal or cae-

sarian deliveries. The cords were collected after obtaining informed consent from the mother. Approximately 10 cms of the cord was collected into tubes containing DMEM supplemented with penicillin and streptomycin and were transported to the lab within 48 hrs of collection. Cord samples were transported at 20~22°C in a validated cold chain container. Processing was done in a GMP compliant environment. The cord was washed with phosphate buffered saline (PBS) supplemented with penicillin and streptomycin. The cord was cut open longitudinally and was cleaned to remove the blood clots. Cord explants of about 2 mm length were placed in 100 mm tissue culture dishes with culture medium. The dishes were left undisturbed in a 5% CO₂ incubator maintained at 37°C for 4~5 days after which fresh culture medium was added to the dishes. Cord samples were divided into two groups; group A samples (n=4) were cultured in DMEM/F12 (1 : 1) containing 10% FBS supplemented with 1~2 ng/ml fibroblast growth factor and group B (n=4) were cultured in DMEM/F12 (1 : 1) containing 10% CBS supplemented with 1~2 ng/ml fibroblast growth factor. Adherent cells from both groups were allowed to expand for 7~8 days by changing media at an interval of 2 days. The cells were harvested at 80~90% confluency using Tryple Select and replated into 75 cm² tissue culture flasks at a density of 5×10³ cells/cm² in appropriate cell culture media. Cells were expanded upto 8 passages.

Immunophenotyping of cultured cells

After every passage, the cells were harvested using Tryple Select and approximately 2×10⁶ cells from each passage were used for immunophenotypic analysis. Cultured cells were incubated with various mouse anti human antibodies conjugated to either FITC, PE or PerCP. Antibodies used were CD45, CD73, CD105, SSEA-4, HLA-DR, HLA-ABC, CD31, CD14, CD44, CD29 and vWF. While all antibodies were procured from BD Pharmingen, USA, only CD105 antibody was procured from Caltag Laboratories.

After incubation for 20 mins at 4°C, the cells were washed with PBS and acquired using a FACS Calibur flow cytometer (Beckton Dickinson). Cell viability was also determined by staining the cells with 7-AAD (7 Amino Actinomycin D) and analysed by using a FACS Calibur flow cytometer. Non specific fluorescence was determined by staining the cells with directly conjugated isotype matched anti-mouse monoclonal antibodies. Approximately 10,000 events were acquired and data analysis was performed using the CellQuest software.

Differentiation potential

Osteogenic Potential: For osteogenic differentiation, UCMSCs were expanded in DMEM/F12 media containing 10% FBS or 10% CBS for 24 hrs in a 37°C incubator with 5% CO₂. After 24 hrs, osteogenesis was induced by replacing DMEM/F12 medium with commercially available osteogenic induction medium. The cells were fed every other day with osteogenic induction medium for 2 weeks. Osteogenesis was confirmed by the presence of osteogenic markers such as osteopontin, osteocalcin and osteonectin by flow cytometric analyses. All osteogenic specific antibodies were procured from R&D Systems. Deposition of mineralized bone matrix was assessed by von Kossa staining after fixing the cells with ethanol (24).

Chondrogenic potential: UCMSCs cultured and expanded in DMEM/F12 containing either 10% FBS or 10% CBS were assessed for their chondrogenic potential. To induce chondrogenic differentiation, approximately 0.5×10^6 UCMSCs, were seeded in a 15 ml polypropylene tube and were maintained in commercially available chondrogenic medium supplemented with TGF β 3. The tubes were kept in 5% CO₂ incubator at 37°C. The cells grew as pellets and were fed with chondrogenic medium regularly. The pellets were collected at day 14, 21 and 29, fixed in 10% buffered formalin and paraffin embedded. Sections of 4~10 μ m thickness were made for immunohistochemical studies. Sections were stained with Safranin O and Alcian Blue. Detection of collagen type I, collagen type II and aggrecan was done using monoclonal antibodies procured from R&D systems.

Immunohistochemical studies: For the purpose of performing Alcian Blue and Safranin O staining, the sections were deparaffinised and hydrated. Pellets were then stained with 1% Alcian Blue prepared in 3% acetic acid for 30 mins. Pellets were also stained with Safranin O for 10

mins. To demonstrate chondrocyte specific markers viz collagen type I, II and aggrecan, the deparaffinised and hydrated sections were fixed with 4% paraformaldehyde. Blocking and permeabilization were done using PBS containing 1% BSA and 0.1% Triton X 100. Pellets were incubated overnight with 1 : 100 dilution of primary antibody (collagen type I, II and aggrecan). Biotinylated streptavidin horseradish peroxidase was used as the secondary antibody. 3,3'-Diaminobenzidine (DAB) was used as the substrate to produce brown colour reaction.

Cytogenetic profile: UCMSCs were incubated with growth medium containing 0.25 μ g of colcemid. After 4 hours of incubation, the cells were harvested and resuspended in 0.075M KCl and then fixed in 3 : 1 methanol/acetic acid (25). GTG banding was done on metaphase spreads obtained from cultured UCMSCs. Cells were analysed under a Olympus AX70 microscope and karyotyped using the Cytovision software.

Results

Morphology and growth kinetics of MSCs from umbilical cord matrix

MSCs were successfully isolated from all eight cords tissues processed (n=4 FBS, n=4 CBS). Approximately 4 cms of the cord was used for isolation of UCMSCs remaining 6 cms was kept at 4°C upto 7 days as a backup in case the cultures were lost to contamination. UCMSCs started to migrate from the explant after 10 to 15 days of culture (Fig. 1A). The isolated UCMSCs grew as adherent fibroblast like cells and formed a monolayer of spindle shaped cells after approximately 20~25 days of culture (Fig. 1B). The growth potential was identical in both FBS containing and CBS containing media. UCMSCs were expanded in culture by repeated harvesting and replating of

Fig. 1. Migration of UCMSC from the explants after 10~15 days (A), cell form a monolayer of adherent fibroblast like cells by day 25 (B).

Fig. 2. Expanded UCMSC showed exponential growth potential when cultured in FBS and CBS containing media (A, B). UCMSC cultured in FBS media showed a 11 fold mean expansion (C). UCMSC cultured in CBS media also showed 11 fold mean expansion (D).

cells every 7 to 8 days for upto passage 8. No change in morphology was observed in the cells that were cultured upto passage 8 in either group. On an average, $3.1 \times 10^{14} \pm 4.6 \times 10^{13}$ UCMSCs were obtained at the end of passage 8 in FBS containing medium and $1.7 \times 10^{13} \pm 1 \times 10^{12}$ in CBS containing medium. We used an automated cell counter (Coulter AcTDiff Analyser) for counting the UCMSCs. This difference in cell number was not statistically significant ($p=0.29$). UCMSCs showed an exponential growth through out the culture period (Fig. 2A, B). From explant stage i.e. passage 0 to passage 8, the UCMSCs cultured in FBS containing media showed a mean of 11 ± 4 fold expansion, while 11 ± 3 fold expansion was observed in CBS cultures (Figs. 2C, D). Fold expansion was calculated as the ratio of MSCs obtained at the nth passage to the number of MSCs obtained at passage 1. Cells cultured in FBS and CBS containing media uniformly exhibited 24 population doublings. Population

Doubling was calculated using the formula $PD = \log_2 (N/N_0)$, where N =number of cells obtained and N_0 =number of cells seeded. The viability of these cells cultured with FBS or CBS at all passage was determined to be more than 90% by 7-AAD dye exclusion method using flow cytometry.

Immunophenotyping of cultured cells

At each passage UCMSC cultured in FBS and CBS containing media were stained with monoclonal antibodies for mesenchymal stem cell markers. A phenotypically homogenous population of UCMSCs was obtained when the cells were cultured in either media as confirmed by the scatter profiles of these cells (Fig. 3A, B). Over 90% of the cells were uniformly positive for CD73, CD105, CD44, CD29 and HLA ABC antigen for both culture conditions and were negative for haematopoietic markers CD45 and CD14. It is important to note that UCMSCs also ex-

Fig. 3. (A) Undifferentiated UCMSC cultured in FBS containing medium expressed mesenchymal markers and were negative for hematopoietic and endothelial markers. (B) Undifferentiated UCMSC cultured in CBS containing medium expressed mesenchymal markers were negative for hematopoietic and endothelial markers.

pressed the stem cell marker SSEA-4. As expected the cells did not express endothelial markers CD31 and vWF. Expression of HLA-DR antigen was not observed although the cells expressed HLA-ABC antigen (Fig. 3A, B). No sig-

nificant change in the expression of these markers was observed in UCMSCs during the entire culture period (P0 to P8).

Osteogenic and chondrogenic differentiation

In order to determine if UCMSCs harvested from FBS containing medium and CBS containing medium retain similar potential for osteogenic and chondrogenic differentiation, UCMSCs were differentiated *in vitro* into osteocytes and chondrocytes in osteogenic and chondrogenic medium respectively. Flow cytometry studies of cells grown in FBS and CBS containing medium revealed that majority of the cells in osteogenic medium differentiated and expressed osteogenic markers such as osteopontin, osteonectin and osteocalcin after 14 days of culture (Fig. 4). UCMSCs expanded in FBS and CBS containing media showed the formation of mineralized matrix as assessed by von Kossa staining (Fig. 5). In addition, UCMSCs cultured in both types of media were assessed for their chondrogenic potential in micromass culture. UCMSCs formed a pellet within 24 hrs of culture in chondrogenic media in the presence of TGF β . The cell pellets were collected at 14, 21 and 29 days for histological studies. Pellet sections demonstrated the presence of cartilage specific proteoglycans by day 21 which further increased by day 29

and was confirmed by Alcian Blue and Safranin O staining (Fig. 6). Chondrogenic differentiation was also confirmed by immunohistochemical detection of collagen type I and II as well as by expression of aggrecan. As expected with chondrocytes, the intensity of collagen type I positivity was lesser compared to that of collagen type II which is suggestive of chondrocyte differentiation of UCMSCs (Fig. 7). Immunohistochemical staining of day 29 cultures did not show positivity for osteogenic markers.

Cytogenetic profile

UCMSCs cultured in FBS and CBS media exhibited normal karyology. (Fig. 8).

Discussion

MSCs are a promising cell type for regenerative medicine and tissue engineering applications. These cells have been isolated from a variety of different sources such as bone marrow, adipose tissue, umbilical cord blood, placenta and amniotic fluid (26-28). While we understand the

Fig. 4. Flow cytometric analysis of FBS and CBS expanded UCMSC showed expression of osteopontin (Panel A&D), osteonectin (Panel B&E) and osteocalcin (Panel C&F) respectively, after 2 weeks of culture in osteogenic induction medium.

Fig. 5. Formation of mineralized matrix in UCMSC cultured in FBS (A) and CBS (B) containing media.

Fig. 6. Histological analysis of pellet cultures. UCMSC expanded in FBS and CBS media were assessed for chondrogenic differentiation. Panel A shows proteoglycan staining of UCMSC expanded in FBS and Panel B shows proteoglycan staining of UCMSC expanded in CBS at various time points of differentiation.

Fig. 7. Immunohistological staining of aggrecan, collagen Type I and collagen Type II of pellets cultured for 14, 21 and 29 days. Panel A-cell pellets cultured in FBS, panel B-cell pellets cultured in CBS.

Fig. 8. Karyotypic analysis of UCMSC cultured in FBS (A) and (B) containing media.

difficulty of obtaining unlimited amount of marrow derived MSCs, other tissue sources have also been researched. Umbilical cord blood, although a good alternative source, the low frequency of MSCs has made it less attractive source for these cells. We, therefore, diverted our attention to the use of umbilical cord matrix derived MSCs which can be a source of large number of cells required for cell therapy application. In recent years several groups have successfully isolated, expanded and characterized MSCs from umbilical cord matrix (29-31). Here we have described the development of a simple protocol to derive MSCs from the umbilical cord using cord blood serum.

Clinical trials using mesenchymal stem cells for several diseases including osteogenesis imperfecta, myocardial infarction and cancer are already under progress (32, 33). Mizuno et al. (2006) and Stute et al. (2004) have used autologous serum for expansion of MSCs for clinical use (34, 35). Almost 200 ml of peripheral blood was collected for the preparation of autologous serum in one of the studies (34). Collection of such high volumes of blood is not always feasible in a clinical setting.

Morphologically, UCMSCs grown and expanded in CBS showed typical MSC - like characteristics of spindle shaped morphology and were no different than the cells that were cultured in FBS. Our data indicates that growth potential of UCMSC expanded in FBS containing media was slightly higher than those cultured in CBS containing media, although this difference was not statistically significant ($p=0.29$). About $3.1 \times 10^{14} \pm 4.6 \times 10^{13}$ UCMSC were obtained at the end of 8 passages in FBS media whereas $1.7 \times 10^{13} \pm 1 \times 10^{12}$ UCMSC were obtained at the end of 8 passages in CBS media (Fig. 1). Immunophenotyping of the cells from both the groups showed identical characteristics even at higher passages. As expected, these cells did not express any haematopoietic or endothelial cell marker. Extrapolating our observations on the yield of MSC from the umbilical cord matrix, it appears that obtaining adequate cells of the desired purity for clinical use may not be an issue.

The umbilical cord tissue contains an inexhaustible supply of MSCs that can be useful for allogenic stem cell therapy (29-31). These cells have been shown to differentiate into osteocytes, chondrocytes, neurons and other cell types (13, 28, 36). The great majority of these studies have used FBS containing medium for MSC expansion. In this report, we show that UCMSCs can be cultured and expanded in CBS as efficiently as in FBS containing medium. Previously, our group has also demonstrated that CBS containing medium could be used to expand

BMMSC and these cells exhibited efficacy in a preclinical model of Parkinson's disease (37). Since the production process of CBS is relatively simple, CBS would be a cheaper alternative to FBS. It can be manufactured in house, by most hospital based institutions with a proper certification of analysis.

We believe that this is an important milestone towards fulfilling the goal of using xenofree medium to grow MSCs for regenerative medicine applications.

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Potential conflict of interest

The authors have no conflicting financial interest

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