COMPARISON BETWEEN DIFFERENT CORD BLOOD STEM CELL POPULATIONS IN EFFICIENCY OF TRANSDIFFERENTIATION INTO HEPATIC LINEAGE

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Abstract:
Background: Cord blood is established as a source of stem cells for hemopoeitic reconstitution. Cord blood transplants have been performed for more than 20 years now. However, cord blood stem cells as a source for regenerative medicine is still under trial. The availability of cord blood and its banking facilities make it a very useful source of hepatocytes for support of endstage liver disease. Cord blood contains a number of stem cell subsets: CD34+, CD133+, and mesenchymal stem cells (MSCs).
Objectives: This study was conducted to compare between these subsets in hepatocyte transdifferentiation efficiency. Hepatocyte lineage commitment was evaluated by alpha-fetoprotein (AFP) expression and albumin synthesis.
Methods: Cord blood is assayed for viability. Magnetic separation was done for CD34+ve, CD133+ve populations, MSCs were separated by culture on plastic flasks. Each cell fraction (CD34+ve, CD133+ve and MSCs) was cultured in liquid culture containing hepatocyte growth factor for 7 days. AFP expression was done using immunocytochemistry, albumin synthesis was measured in culture supernatant using microalbumin assay kit.
Results: All three populations showed heptocyte transdifferentiation; although with varying percentages. There was no statistically significant difference in AFP expression with MSCs showing 31% positivity, CD133+ve30% followed by CD34+ve showing 28.8%. Also, MSCs population showed the highest albumin synthesis levels, followed by CD34+ then CD133+ cells.
Conclusion: Induction of hepatocyte-like cells is possible with all three stem cell subsets of the cord blood. However, establishment of functional hepatic cells is higher in MSCs population.

Key Words: CD 34+, CD 133+, mesenchymal stem cells, umbilical cord blood, Hepatocyte differentiation

Introduction

Liver diseases have been increasing worldwide and are a considered a leading cause of death due to the prevalence of viral induced and other intractable liver diseases as primary cirrhosis and primary sclerosing cholangitis (Kim et al., 2002; Tanikawa, 1992). Most liver diseases lead to hepatocyte dysfunction with the possibility of eventual organ failure (Sellamuthu et al., 2011).

Egypt has the highest incidence of hepatitis C virus (HCV) worldwide with estimated anti-HCV antibody prevalence of 14.7% and the number of chronically infected Egyptians 9.8%, according to the most recently published Egyptian Demographic Health Survey in 2009, which was a national probability sample of the resident Egyptian population (El-Zanaty and Way 2009). HCV is a major health problem in Egypt and is the most common cause of chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, and liver transplantation in the country (Nguyen and Keeffe 2005; Abdel-Aziz et al., 2000).

The need for liver transplantation worldwide is always increasing which is hindered by the shortage of donated organs leading to critical condition. In addition, liver transplantation is associated with significant morbidity and mortality to both the donor and the recipient. Therefore, increased needs for developing alternative therapies for the treatment of liver disease especially end stage liver disease are emerging and necessary (Fox and Chowdhury 2004). Cellular therapies replacing the diseased hepatic cells by stem cells are the main approach in liver directed cell therapy (Sellamuthu et al., 2011).

Stem cells sources are the bone marrow of an adult person, the peripheral blood of an adult person and the umbilical cord blood (UCB) of a newborn baby (Sellamuthu et al., 2011). As a source of stem cells for regenerative medicine, UCB has certain advantages over the bone marrow and peripheral blood as UCB has a high concentration of highly proliferative stem cells, can be easily collected without any harm to the mother or the baby and have a low rate of infection with cytomegalovirus (McAdams et al.1996, Bromeyer 1995). In addition, UCB- derived cells are more primitive than bone marrow cells which makes it more suitable cell source for cell-based therapies, regenerative medicine and tissue engineering (Lee et al., 2010).

Several stem cell subsets are present in UCB, including; CD34+ cells, CD133+cells and mesenchymal stem cells (MSCs). CD34 has been used as a human hematopoietic stem marker and most colony forming cells are present within the CD34+ population (Holyoake and Alcorn, 1994; Sutherland and Keating, 1992). CD 133 expression defines very early subset of progenitor cells, early hematopoietic progenitor cells express CD133, which is not expressed after differentiation (Walter and Dimmeler, 2002; Yin et al., 1997). MSCs are multipotent progenitors capable of differentiating towards other cell types as adipocytes, osteocytes (Kern et al., 2006), and hepatic cells (Campard et al., 2008; Lee etal., 2004) with a low immunogenicity (Aggarwal and Pittenger, 2005).

In our study, we compared the transdifferentiation potential of each of these subsets into hepatic cells. Transdifferentiation to hepatic cells was assessed by alpha-fetoprotein (AFP) expression as AFP is one of the earliest markers for endodermal differentiation (Hammer et al., 1987), hepatic metabolic function was assessed by albumin secretion as albumin production is a specific test for the presence and metabolic activity of hepatocytes (Dunn et al., 1991).

Subjects and Methods

Ten umbilical cord blood samples were obtained with oral consent of the mother and the approval of Cairo University ethical committee. Samples were collected from the umbilical vein ex utero after spontaneous delivery of the placenta following full-term vaginal delivery. Ex utero collections are less invasive, and there is better control over technique. The cord blood was collected in a bag containing citrate phosphate dextrose anticoagulant. Mononuclear cells were isolated by centrifugation of cord blood over Ficoll-Hypaque density gradient (density 1.077, Biochrom KG, Berlin).

Isolated mononuclear cells (MNC) were assessed for viability using Trypan blue dye exclusion test. Mononuclear cells were mixed with Trypan blue dye and incubated at 37ºC for 5 minutes. Two hundred cells were counted using a light microscope at low power. Cells not taking the dye were counted as viable, whereas cells taking the dye were considered nonviable.

MNC were then divided into three parts for magnetic separation of CD133+, CD34+ cells and MSCs separation by culture on plastic flasks.

Magnetic separation was done using The MiniMACS™ Separation System (Miltenyi Biotec, Germany). 1 x 10^6 MNC were suspended in a final volume of 80 µL MACS (Miltenyi Biotech) buffer and labeled with 20 µL of microbeads with FITC (fluorescein isothiocyanate) conjugated mouse anti-human CD34/ CD133 antibodies (QBEND/10). The cells were mixed well and incubated at 4ºC for 15 min in dark. After incubation the cells were washed thrice with 500 µL of MACS buffer by spinning at 300xg for 10 min. The cells were resuspended in 500 µL of buffer and used for magnetic sorting. The column was washed with 500 µL of MACS buffer. The magnetically labeled cells were passed through the column. The cells with magnetic microbeads are retained within the column and those that are unlabelled eluted out. The eluted fraction was collected as negative fraction. The column was washed thrice with 500 µL of MACS buffer. Then the column was removed from the magnetic field. The retained cells in the column were firmly flushed out by applying pressure on the matrix of the column by a plunger supplied with the kit. These were the positive fractions which were washed twice with MACS buffer by spinning at 300xg for 5 min and resuspended in 500 µL of MACS buffer. Both fractions, magnetic and non -magnetic, were completely recovered.

Fluorescence-activated cell sorting analysis of the separated cells fractions; CD133+, CD34+ was done. Tubes were prepared for analysis; were samples were mixed with fluorescein isothiocyanate-conjugated mouse monoclonal antibody against CD133 and CD34 (Dako, Glostrup, Denmark) and with appropriate isotype-matched control monoclonal antibody. Cells were incubated with monoclonal antibody for 30 min at 4°C, washed once with phosphate-buffered saline, and resuspended in a small volume of phosphate-buffered saline for analysis by means of fluorescence-

activated cell sorting (FACScan) flow cytometer (Coulter Epics Elite, Miami, FL). Forward and side scatter gates were established to exclude cell debris and clumps before analysis for expression of CD133 and CD34.

MSCs separation was done by culture of the separated mononuclear cells into 25-cm^2 flask in complete culture media. After 24 hours of culture, non-adherent cells were washed out. Fresh medium was replaced every three days. At 70% monolayer confluence, the MSCs were enzymatically detached using 0.25% trypsin-EDTA (GIBCO BRL Grand Island, NY, USA).

Hepatocyte differentiation; $5x10^4$ cells/mL of each cell fraction (CD34+ve, CD133+ve and MSC) were cultured in Dulbecco's modified Eagle's medium (GIBCO, Sigma, St. Louis, MO) supplemented with 20% fetal calf serum (GIBCO BRL Grand Island, NY), penicillin (10,000 units/mL), streptomycin (10 mg/mL; GIBCO BRL Grand Island, NY), and 20 ng/mL hepatocyte growth factor (HGF; R&D Systems GmbH, Wiesbaden-Nordenstadt, Deutschland). Samples were incubated at 37 °C in a 5% CO^2 atmosphere for 7 days.

Cytospins and immunocytochemistry; on the7th day, cells were harvested from cultures. Cytospins were prepared by centrifugation of the cell suspension (400xg for 10 min) and cellular pellets were applied to a glass slide. For AFP immunocytochemical testing, the cells were fixed by dipping in absolute alcohol for 2 min. After slide rehydration, blockage of endogenous peroxidases was done with 3 % H_2O_2. To reduce nonspecific hydrophobic interactions between the primary antibodies and the fixed cells, the slides were incubated with a blocking solution (1:50 normal horse serum in phosphate-buffered saline). The slides were incubated with primary antibodies in incubation buffer over night at 4 °C (Biogenex, SanRamon, CA). Then incubated with secondary antibodies for 30 min (R&D Systems) followed by incubation with one to three drops of high sensitivity streptavidin-horseradish peroxidase (HRP) conjugate for 20 min (R&D Systems). One to five drops of diaminobenzidine chromogen solution (R&D Systems) were applied for 8 min (colored precipitate localizes to the sites of antigen expression as the chromogenic substrate was converted by HRP enzyme into insoluble end product). Slides were counterstained with nuclear counterstain hematoxlin (Sigma). Visualization of the stained cells was done under a microscope using a bright-field illumination. Quantitative evaluation of alpha fetoprotein-positive cells (AFP + cells) was done using Leica Qwin 500 image analyzer computer system (England). The numbers of AFP + ve cells were counted/HPF in ten fields of each culture specimen and the mean percentages were obtained.

Albumin concentration was determined in the culture supernatant using enzyme linked immunosorbent assy kit (DRG International Inc., USA) according to the manufacturer instructions.

Results

The viability of cells was estimated by using the Trypan blue dye exclusion test. The result obtained revealed that the viability of the cells was high. Mean cell viability after mononuclear cell separation was 98.8± 1.12 viable cells.

CD34+ and CD133+ cells were isolated from UCB MNC fractions by incubation with microbeads, followed by passage through Mini- MACS columns. Fluorescence-activated cell sorting analysis with anti-CD34 and CD133 antibodies was performed to determine the percentage purity of the positive fraction. Positive selection of CD133 cells yielded 52.67 ± 11.77 CD133+ cells, while positive selection of CD34 cells yielded 51.76 ± 3.04 CD34+ cells.

We examined the expression of hepatic protein markers; albumin and AFP. Albumin and AFP are liver cell functional markers used to determine and characterize hepatic cell population. The presence of albumin is a prominent feature of mature hepatocytes as the liver is the site for albumin synthesis (Sellamuthu et al., 2011).

After 7 days in culture media for hepatocyte differentiation, the cultured cells showed positive expression of AFP with varying percentages; although statistically non- significant ($p > 0.05$). MSCs showed 31% ± 1.65 positivity, CD133+ cells 30% ± 2.21 followed by CD34+ cells showing 28.8% ± 2.91.

Fig (1): Mean Alfa fetoprotein Expression after 7 day Culture

	Alfa fetoprotein (%)	P-value
MSCs	31 ± 1.65	>0.05
CD34+ cells	28.8 ± 2.91	
CD133+ cells	30 ± 2.21	

Albumin secretion was detected in the culture supernatant at the 7th day culture; the mean albumin level was 0.51 mg/L ±0.016 in MSCs culture supernatant, 0.46 mg/L ±0.049 in CD34+ cells culture and 0.4 mg/L ±0.032 in CD133+ cells culture. MSCs showed the highest albumin level in the culture supernatant compared to CD34+ cells and CD133+ cells although statistically non-significant (p >0.05).

Fig (2): Mean Microalbumin Level after 7 day Culture

	Microalbumin (mg/L)	P-value
MSCs	0.5 ±0.016	>0.05
CD34+ cells	0.46 ±0.049	
CD133+ cells	0.4 ±0.032	

Discussion

Umbilical cord blood (UCB) is a rich source of stem cells and progenitor cells, which makes it a target for extensive experimental and clinical trials. UCB derived cells being more primitive than BM- derived cells, are more suitable source for cell based therapies and regenerative medicine (Lee et al., 2010).

Several types of cells have been addressed in the UCB including; hematopoietic stem cells, mesenchymal stem cells (MSCs) (Lee et al., 2004a, Lee et al., 2004b), unrestricted somatic stem cells (Ko¨gler et al., 2004), cord blood derived embryonic stem cells (McGuckin et al., 2004), and umbilical derived mutipotent progenitor cells (Lee et al., 2007).

It has to be addressed, which cell population in the UCB is capable of differentiation into specific cell types. In our study we focused on hepatocyte differentiation, several UCB subsets were proposed by previous studies as being capable of differentiation into hepatic like cells. It has been reported that CD 45+ subpopulation of UCB cells were capable of generating hepatocytes (Ishikawa et al., 2003), CD34+ UCB cells differentiated into hepatocytes after transplantation into mouse recipient (Di Campli et al., 2006; wang et al., 2003, Danet et al., 2002). Also, MSCs isolated from UCB are capable of differentiation into hepatocyte like cells (Lee et al., 2004c). Also, other pluripotent somatic cells can be isolated from UCB and may be responsible for hepatocyte repopulation in the liver (Kögler et al., 2004).

In our work, we studied the differentiation potential of UCB stem cell subsets; CD34+ cells, CD133+cells and MSCs into hepatic cells, comparing their ability to produce albumin and AFP. Hepatic metabolic function was assessed by albumin secretion as albumin production is a specific test for the presence and metabolic activity of hepatocytes (Dunn et al., 1991). AFP is one of the earliest markers for endodermal differentiation (Hammer et., 1987), produced primarily by the visceral endoderm of the yolk sac, as well as by hepatoblasts and more differentiated fetal hepatic cells (Chen et al., 1997, Tilghman, 1985), while it is not expressed in all adult stem cells (Hong et al., 2005, Lee et al., 2004c).

Hepatocyte differentiation was induced by hepatocyte growth factor (HGF) which was considered one of the most hepatogenic-inducing functionality (Chivu et al., 2009). HGF plays an essential role in the development and regeneration of the liver (Wang et al., 2004). In previous studies, many different cytokines were studied in order to optimize conditions required for hepatic differentiation, and it was found that only fibroblast growth factor-4 (FGF-4) and HGF were able to promote hepatocyte differentiation. FGF-4 alone induced hepatocyte differentiation, however, the degree of differentiation measured by absence of immature markers such as AFP and cytokeratin-19 was higher when cells were also treated with HGF. FGF-4 is important in initial endoderm patterning and may play a role in endoderm specification (Wells and Melton, 2000) while HGF induces differentiation of hepatocytes that are not actively proliferating (Oh et al., 2000; Hamamoto et al., 1999; Yoon et al., 1999; Miyazaki et al., 1998). In a study by Tang et al., it was reported that HGF can promote the expansion of human umbilical cord blood stem cells and induce their differentiation into liver cells (Tang et al., 2006).

Albumin secretion and AFP expression were detected in the culture supernatant at the day 7 of culture; the mean albumin level was 0.51 mg/L ±0.016 in MSCs culture supernatant, 0.46 mg/L ±0.049 in CD34+ and 0.4 mg/L ±0.032 in CD133+ culture. Many other groups have studied

hepatocyte differentiation from UCB and albumin expression was detected one week after the beginning of culture (Tang et al., 2006, Teramoto et al., 2005). This showed the capability of albumin-positive cells derived from UBCs to proliferate into functional hepatocyte-like cells.

The cultured cells showed positive expression of AFP with varying percentages; MSCs showed 31% ± 1.65 positivity, CD133+ cells 30% ± 2.21 followed by CD34+ cells showing 28.8% ± 2.91. In the study by Tang et al, low level expression of AFP was detectable by day 7 and remained detectable up to day 35 (Tang et al., 2006). AFP is an early developmental marker gene of hepatoblasts (Hammer et al., 1987), indicating successful mRNA translation into specific proteins.

Based on our results, we believe that UCB-MSCs have a strong potential for differentiating into hepatic lineage cells in vitro and may be a promising source of cell therapy in intractable liver diseases as they can be easily isolated and expanded in quantities relevant to clinical application and can be cryopreserved for long periods without losing their stem cell properties in addition to having a broad differentiation potential (Kim et al., 2004, Zuk et al., 2002, Reyes et al., 2001). UCB-MSCs have a major advantage in that it does not require invasive procedures that could be harmful to the donor and can be used universally across the HLA barrier. MSCs can repair injured tissue by differentiating into damaged cell types, secreting appropriate cytokines and growth factors, and undergoing cell fusion (Prockop et al., 2003, Spees et al., 2003, Terada et al., 2002). In addition, MSCs possess the unique ability to suppress immune responses, both in vitro (Krampera et al., 2003, Tse et al., 2003) and in vivo (Polchert et al., 2008, Zappia et al., 2005, Ortiz et al., 2003).

In a study by Burra et al, UCB-MSCs were transplanted in necrotic mouse liver. Cells were recruited in the injured tissue and were able to engraft the liver and to regulate the inflammatory process (Burra et al., 2012).

Although, several studies had reported the ability of UCB-MSCs to differentiate into hepatic like cells, the application in clinical practice needs more thorough information on cell behavior in vivo. More investigations are needed to study the potential of frozen/cryopreserved cells to undergo proper differentiation into required cell type and its engraftment and cellular reconstitution ability.

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