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Isolation and characterization of hematopoietic stem cells from human umbilical cord blood for cell therapy

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Abstract: Alternatives to organ transplantation for treatment of certain liver diseases are currently under investigation. Whole liver, split liver and related living donor liver transplantation are clinically safe and well established procedures for the treatment of end-stage liver failure. However, organ donor shortage and the need of lifelong immunosuppressive treatment are still the major limitations of all these options. Therefore, the development of cell based therapeutic strategies for liver disease is under experimental evaluation. Umbilical cord blood was collected and isolated the mononucleated cell by the help of ficoll hypaque centrifugation. The collected samples were purified for CD 34+ hematopoietic stem cells using MACS. These purified hematopoietic stem cells were counted and morphologically characterized by FACS. Freshly isolated CD 34+ cells were cultured in hepatocyte differentiation media; contain growth factor of HGF and FGF-4. These growth factors induced the differentiation of hematopoietic stem cells into hepatocyte like cells. The cell density played a significant role in effecting hepatic differentiation. Optimal cell density was found to be 1.0 to 1.3X10^4 cells /cm^2. After 14 inductions, cuboidal morphology, which is characteristic of hepatocytes were observed and cells also expressed by marker genes specific for liver cells in a time depended manner.

Key words: Umbilical Cord Blood; Hematopoietic; Stem Cells; Hepatocyte; Marker Genes.

Introduction

Liver is the central metabolic organ that regulates body's energy supply, secretes several essential compounds and toxic substances by several methods, including recycling, inactivation and excretion. Loss of liver functions in acute liver failure (ALF), results in profound metabolic instability and disruption of essential functions such as acid-base balanced thermoregulation. If this is not rapidly reversed, complications such as uncontrolled bleeding occur, and dependent organs such as brain and kidneys begin to fail, reducing the chance of recovery[1]. In India, mortality rate due to liver diseases is high. The most common liver diseases are fibrosis, cirrhosis, hepatitis and cancer, occurring as a result of viral infection or alcohol abuse. Other causes of liver damage include, drug over dosage, metabolic and auto immune disorders, chemical toxins and trauma. Liver failure can progress rapidly, as in fulminant hepatic failure (FHF), or at a slower rate, as in chronic liver disease. Therefore, the liver is an organ with a tremendous capacity for self-regeneration. In most cases repair of injured liver parenchyma is taken care of by proliferation of mature adult cells, particularly hepatocytes. In severe cases of liver injury, the proliferative capacity of liver cells is not sufficient to successfully restore organ function. In such situations, hepatocyte progenitor cells and stem cells of intra hepatic and/ or extra hepatic origin may come into play in organ regeneration.

Presently, Orthotopic transplantation (OLT) is the only treatment that improves the survival rate in patients with ALF[2]. Advent of various immunosuppressive agents has improved the success rate of this procedure by preventing rejection. But shortage of donor organs, involvement of major surgery, which can be performed, only at selected advanced centers by highly skilled surgeons, and the requirement of chronic immune-suppression with its related cost and complication are the limitations of OLT. Graft rejection and sepsis are still the major causes of morbidity and mortality associated with the use of these drugs. One of the major complications after liver transplantation is severe dysfunction or primary non-function of the graft[3]. Therefore, hepatocytes transplantation has emerged as an alternative to OLT. Cell therapy has potential in treating patients with liver failure as well as metabolic deficiencies.

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Stem cells that is capable of self-renewal and is multi-potent, meaning they can differentiate into many specific cell types. Cord blood has been proposed as a source of hematopoietic stem cells alternative to bone marrow for allogeneic transplantation[4]. There is evidence for a direct positive correlation between the number of rein fused cord blood stem cells and the percentage of success of transplantation[5,6]. So that the improvement of the techniques for the in-vitro expansion of cord blood hematopoietic stem cells are at present one of the main challenges of tissue engineering. Stem cell transplantation (SCT) is increasingly used in the treatment of hematologic and non-hematologic disease of neoplastic and non-neoplastic origin[7]. Since, successful transplant depends on engraftment of pluripotent hematopoietic stem cells in the marrow microenvironment, and the transplant procedure involves infusion of stem cells into the circulation, it is assumed that stem cells are capable of homing to the marrow and docking at specific sites[8]. Therefore, Stem cell transplantation (SCT) exploits a physiological mechanism that plays a role in the regulation of normal hematopoiesis.

Recently, it has been reported that umbilical cord blood contains mesenchymal progenitor cells capable of differentiating into marrow stroma, bone, cartilage, muscle and connective tissues[9]. Furthermore, umbilical cord blood provides no ethical problems for basic studies and clinical applications. Umbilical cord blood cells can be collected without any harm to the newborn infant and umbilical cord blood hematopoietic stem cell grafts can be cryo-preserved and transplanted to a host after thawing without losing their repopulating ability[6]. For these reactions, umbilical cord blood could be a prominent source of cells for transplantation in various diseases. It remains obscure, however, whether umbilical cord blood contains stem/progenitor cells leading to endodermal cells, including hepatocytes.

The presence of non-hematopoietic progenitor cells in umbilical cord blood which can differentiate into bone and fat cells was observed[10]. Human umbilical cord blood progenitor cells differentiate into hepatocytes, *in-vitro* and *in-vivo* in fas ligand mediated liver injury model.

Materials and Methods

Isolation of mononuclear cells: Mononuclear cells were collected from human umbilical cord blood. Mononuclear cell separation was carried out within 6hrs of collection. Mononuclear cells were isolated by centrifugation, suspending the cells with ficoll hypaque in the ratio of 1:1. Cells present at interphase with the gradient medium were collected then cells were washed twice with PBS buffer. The collected cell sample was further purified for CD34+ hematopoietic stem cells using MACS and then CD34 cell counted by FACS.

Staining the cells: Stain peripheral blood samples within 24 hours of collection and all other samples within 6 hours of collection. Follow the manufacturer's recommendations for staining commercial controls. Lyses red blood cells following staining using diluted 1X BD FACS lysing solution. Use care to protect tubes from direct light. Perform the procedure at room temperature 20°-25°C.

Cell labelling: Isolated cells were labelled and sorted with anti-CD34 antibodies and eluted through mini MACS columns. The $1X10^6$ cells were suspended in a final volume of 80µl MACS buffer and are labelled with 20µl of micro beads with FITC conjugated mouse anti-human CD34 antibodies. The cells were mixed well and incubated at 4ºC for 15minutes in dark. After incubation, the cells were washed thrice with 500µl of MACS buffer by spinning at 300xg for 10 minutes. The cells are re-suspended in 500µl of buffer and are ready for magnetic sorting.

Cell culturing and hepatocyte differentiation: Mononuclear cells were suspended in DMEM supplemented with 100ml/L FBS, 100ml/L penicillin and 100ml/L streptomycin. The cells were plated at a final concentration of $1X10^6$ cells/ml, control group was cultured in DMEM supplemented with 10ml/L FBS, 100ml/L penicillin, 100ml/L streptomycin. The hepatocyte differentiation group was cultured in DMEM supplemented with 10ml/L FBS, 20ng/ml HGF, 10ng/ml FGF-4, 100U/ml penicillin and 100U/ml streptomycin and incubated for 14 days at 37ºC and 5% CO_2. Then the medium was changed every 4th day. The medium was collected at day 2,4,6,8,10,12 and 14 were stored at -20ºC for albumin, alpha-fetoprotein (AFP) assay.

Determination of viability for differentiate hepatocytes: The viability of the hepatocytes was checked by Tryphan blue dye exclusion method (TBE) and MTT (3-[4,5-dimethylthiozol-2yl]-2,5-diphenyl tetrazolium bromide) assay.

Results

Isolation of hematopoietic stem cells and characterization:

MACS: Hematopoietic and hepatic stem cells share characteristic markers such as CD34 and thy-1. Hematopoietic stem cells were subjected to magnetic cell sorting and analyzed for the presence of CD34+. CD34+ and $CD34^-$ cells were separated.

FACS: CD34+ sorted cells were counted and morphologically characterized by FACS. During sorting of umbilical cord blood cells more unwanted cells such as lymphocytes. CD34+ from hematopoietic stem cells were sorted and counted before and after sorting. The result shows the CD34+ cells were more in number after sorting (Figure.3 & Figure.4).

MTT assay: The viability of cells was estimated by using the MTT assay. The result shows the viability of cells in different incubation period (2, 4, 6, 8, 10, 12 and 14 days). It was observed that the viability rate was increased in 14th day culture compared to others (Figure.1).

Staining: Hematopoietic stem cells were isolated from human umbilical cord blood. Before culturing the mononuclear cells were stained with hematoxylin. The stained mononuclear cells were observed under microscope (Figure.2).

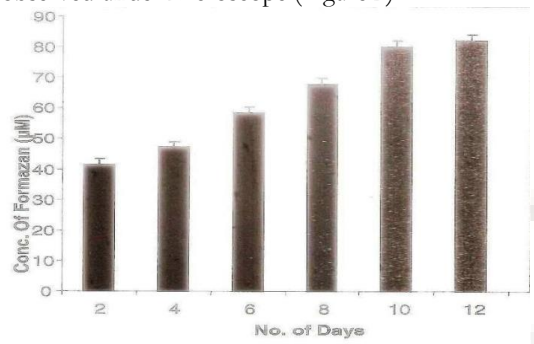

Figure 1: The formation of Formazan (µM) by MTT assay in different days.

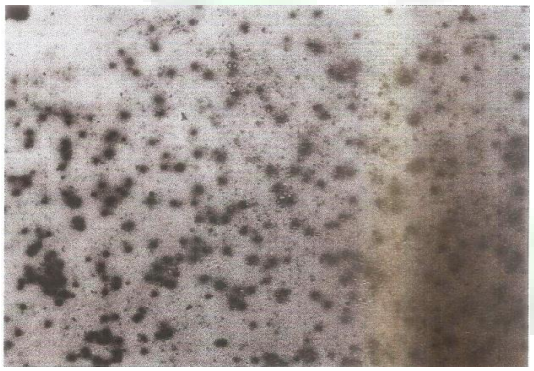

Figure 2: Mononuclear stem cells staining with hematoxylin

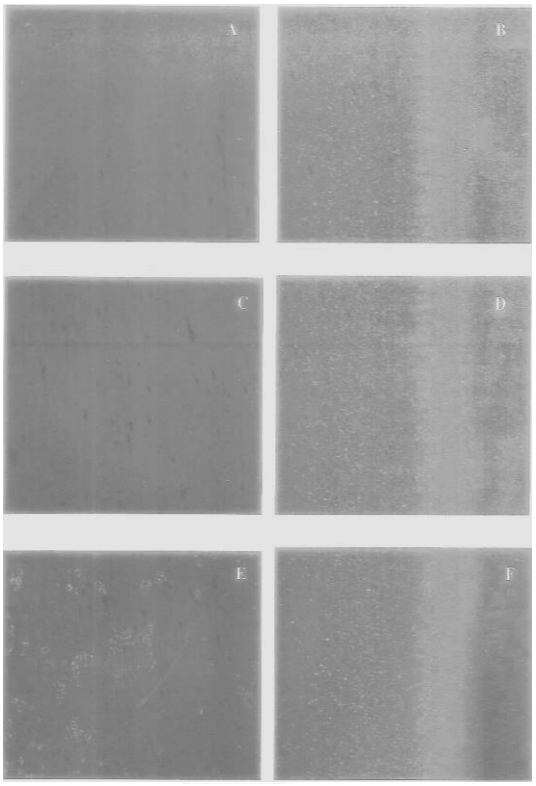

Figure 3: A-Hematopoietic stem cell culturing (Before); B-Culturing day 1; C- Culturing day 3; D-Culturing day 5; E- Culturing day 7; F- Culturing day 8.

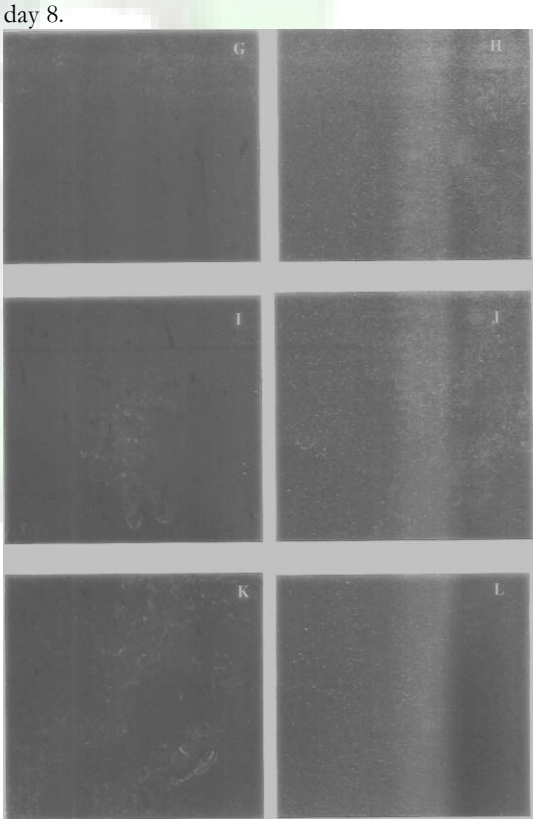

Figure 4: G-Culturing day 9; H-Culturing day 10; I- Culturing day 11; J- Culturing day 12; K-Culturing day 13; L- Culturing day 14.

Discussion

There are many hematopoietic stem cells and mononuclear stem cells in human umbilical cord blood. Many methods are used to isolate mononuclear stem cells from human umbilical cord blood[11,12]. In the present study Ficoll gradient centrifugation (1.077g/ml) was used to isolate mononuclear stem cells from human umbilical cord blood, the composition was changed in the medium many times to purify mononuclear stem cells after gradient density centrifugation. This method is relatively simple, and can easily get pure mononuclear stem cells. Mononuclear stem cells should be isolated from the sample as soon as possible. Our results showed that it was difficult to isolate mononuclear stem cells from the samples, because the activity of mononuclear stem cells is low, even loses with time prolonged. During the 14 days of differentiation, FGF-4 and HGF induced mononuclear stem cells into cells with morphological and functional characteristics of hepatocytes.

In the present study, the cells that differentiated into hepatocyte like cells could produce urea, secrete albumin, alpha fetoprotein and CK-18, and store glycogens. Urea production was characterized by hepatocyte activity, although kidney tubular epithelium also produced urea. In contrast, albumin, alpha fetoprotein and CK-18 production is a specific test for the presence and metabolic activity of hepatocytes. Only hepatocytes can generate and store glycogens.

The data suggest that human umbilical cord blood-derived mononuclear stem cells can differentiate into hepatocytes by induction of FGF-4 and HGF. HGF was first identified as blood-derived mitogens for hepatocytes. HGF and its receptor c-Met are the key factors for liver growth and function. After cultured with only HGF, adult human bone marrow mononuclear stem cells could also differentiate into hepatocytes *in-vitro*[13]. FGF-4 is mitogenic for fibroblasts and endothelial cells. Mouse embryonic stem cells grown in medium supplemented with FGF-4 could differentiate into cells expressing hepatocyte-specific genes and antigens[14]. By co-operation of HGF and FGF, the differentiation of mononuclear stem cells could be triggered and mononuclear stem cells could develop into hepatocytes.

The experimental data showed the hepatic differentiation potential of human umbilical cord blood-derived mononuclear stem cells. Human umbilical cord blood derived mononuclear stem cells have more advantages over bone marrow mononuclear stem cells. It is suggested that human umbilical cord blood-derived mononuclear stem cells are a source of cell types for cell transplantation therapy of liver diseases. The viability of the cells in each fraction is checked by TBE and MTT assay. The results thus obtained revealed that the viability of cells using TBE was significantly high. Further attempt to ascertain the functional viability of cells was studied by MTT assay; the mitochondrial dehydrogenase enzymes of the viable cells convert yellow colored tetrazolium salt to a purple colored formazon. The amount of formazon formed is directly proportional to the functional viability of the active and living cells. The data generated by MTT assay revealed that cells were functionally more active which was shown by the amount of formazon formed.

The hepatic progenitors obtained in day 13 and 14 the cells were immature as described earlier and are not fully differentiated. In absence of serum, cell proliferation will arrest, and in the presence of HGF and FGF-4, in differentiated media, differentiation will takes place. After prolonged culture, the hepatocytic morphology further matured. In conclusion human hematopoietic stem cells from different sources were able to differentiate into functional hepatocyte like cells and hence may serve as a cell source for tissue engineering and cell therapy of hepatic tissue.

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